Fieser, L. F., & Fieser, M. (1959) *Steroids*, pp 27–35, Reinhold, New York.
Glauert, A. M., Dingle, J. T., & Lucy, J. A. (1962) *Nature (London) 196*, 953.
Holz, R., & Finkelstein, A. (1970) *J. Gen. Physiol. 56*, 125.
Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkool, G. (1973a) *Proc. Natl. Acad. Sci. U.S.A. 70*, 480.
Jost, P. C., Griffith, O. H., Capaldi, R. A., & Vanderkool, G. (1973b) *Biochim. Biophys. Acta 311*, 141.
Jost, P. C., Nadakavukaren, K. K., & Griffith, O. H. (1977) *Biochemistry 16*, 3110.
Kang, S. Y., Gutowsky, H. S., & Oldfield, E. (1979a) *Biochemistry 18*, 3286.
Kang, S. Y., Gutowsky, H. S., Hsung, J. C., Jacobs, R., King, T. E., Rice, D., & Oldfield, E. (1979b) *Biochemistry 18*, 3257.
Kinsky, S. C. (1970) *Annu. Rev. Pharmacol. 10*, 119.
Knowles, P. F., Watts, A., & Marsh, D. (1979) *Biochemistry 18*, 4480.
Longmuir, K. L., Capaldi, R. A., & Dahlquist, F. W. (1977) *Biochemistry 16*, 5746.
Mantsch, H. H., Saitô, H., & Smith, I. C. P. (1977) *Prog. Nucl. Magn. Reson. Spectrosc. 11*, 211.
Marsh, D., Watts, A., Maschke, W., & Knowles, P. F. (1978) *Biochem. Biophys. Res. Commun. 81*, 397.
Nakamura, T., Inoue, K., Nojima, S., Sankawa, U., Shoji, T., Kawasaki, T., & Shibata, S. (1979) *J. Pharmacobio-Dyn. 2*, 374.
Ogihara, Y., Inoue, O., Otsuka, H., Kawai, K., Tanimura, T., & Shibata, S. (1976) *J. Chromatogr. 128*, 218.
Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978a) *Biochemistry 17*, 2727.
Oldfield, E., Gilmore, R., Glaser, M., Gutowsky, H. S., Hsung, J. C., Kang, S. Y., King, T. E., Meadows, M., & Rice, D. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4657.
Rice, D., & Oldfield, E. (1979) *Biochemistry 18*, 3272.
Rouser, G., Nelson, G. J., & Fleisher, S. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 5–64, Academic Press, London and New York.
Seelig, A., & Seelig, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1747.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353.
Shany, S., Bernheimer, A. W., Grushoff, P. S., & Kim, K.-S. (1974) *Mol. Cell. Biochem. 3*, 179.
Smith, I. C. P., Bennett, L. G., Blackwell, B., Bloom, M., Butler, K. W., Davis, J. H., Jennings, H. J., Johnson, K. G., Martin, A., Moyna, P., Stockton, G. W., & Tulloch, A. P. (1978) in *Biomolecular Structure and Function* (Agris, P. F., Ed.) pp 3–24, Academic Press, New York.
Steiner, M., & Holtzem, H. (1955) in *Modern Methods in Plant Analysis* (Peach, K., & Tracey, M. V., Eds.) Vol. 3, pp 67–68, Springer-Verlag, West Berlin.
Stockton, G. W., & Smith, I. C. P. (1976) *Chem. Phys. Lipids 17*, 251.
Stockton, G. W., Polnaszek, C. F., Leitch, L. C., Tulloch, A. P., & Smith, I. C. P. (1974) *Biochem. Biophys. Res. Commun. 60*, 844.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954.
Stockton, G. W., Johnson, K. G., Butler, K. W., Tulloch, A. P., Boulanger, Y., Smith, I. C. P., Davis, J. H., & Bloom, M. (1977) *Nature (London) 269*, 267.
Tschesche, R., & Wulff, G. (1973) *Fortschr. Chem. Org. Naturst. 30*, 461.

Mechanism of Exchange of Cytochrome b_5 between Phosphatidylcholine Vesicles†

Thomas L. Leto, Mark A. Roseman,‡ and Peter W. Holloway*

ABSTRACT: The intervesicle exchange of cytochrome b_5 has been studied by fluorescence quenching. The binding of cytochrome b_5 to 1,2-bis(9,10-dibromostearoyl)-*sn*-glycerol-3-phosphorylcholine vesicles results in a quenching of cytochrome b_5 fluorescence whereas the fluorescence is enhanced upon binding to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine vesicles. This difference in cytochrome b_5 fluorescence upon binding was used to study the kinetics of cytochrome b_5 intervesicle exchange between the "quenching" and "enhancing" vesicles. Separation of the two cytochrome b_5–vesicle complexes by density gradient centrifugation provided direct evidence for cytochrome b_5 intervesicle exchange. Both the fluorescence assay and the density gradient assay yield the same value for the extent of cytochrome b_5 exchange, obtained after equilibration, between the two types of vesicles. Both experiments also indicate that cytochrome b_5 binds in a reversible fashion and has an equal affinity for the two types of vesicles. The kinetics of the exchange process are consistent with a mechanism involving the transfer of cytochrome b_5 through the aqueous phase and rule out a mechanism involving vesicle collision.

Considerable recent interest has been focused on the phenomenon of intermembrane transfer or exchange of membrane components. Such processes may prove to be of importance in membrane biosynthesis and may, in part, account for the observed heterogeneity in turnover rates of membrane lipids and proteins (Omura et al., 1967). Intermembrane exchange of lipids has been described in a number of model systems, occurring both spontaneously (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Doody et al., 1978; Roseman & Thompson, 1979) or in the presence of exchange proteins (Wirtz, 1974). Spontaneous intermembrane transfer of some membrane proteins has also been demonstrated recently in vitro. The exchange of cytochrome b_5 (Roseman et al., 1977) and cytochrome b_5 reductase (Enoch et al., 1977) between

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908. *Received November 30, 1979.* This work was supported by Grants GM 23858 and 5T32 CA 09109 from the National Institutes of Health.
‡ Present address: Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, MD 20014.

0006-2960/80/0419-1911$01.00/0

phospholipid vesicles represents the two best documented cases of integral membrane proteins undergoing exchange.

Cytochrome b_5 has been localized on the membranes of several subcellular organelles by immunochemical labeling studies (Fowler et al., 1976; Jarasch et al., 1979), although the specificity of the antibodies used in these experiments is uncertain [see Fukushima & Sato (1973)]. In view of the possible broad subcellular distribution of cytochrome b_5, we have been interested in the mechanism by which cytochrome b_5 transfers from membranes and in the possibility of such a process occurring in vivo. The in vivo occurrence of intermembrane exchange of cytochrome b_5 could explain the movement of cytochrome b_5 from its site of synthesis on the rough endoplasmic reticulum (Harano & Omura, 1977) to the other cell membranes. This process would represent an alternative to the membrane flow hypothesis [see Morre' et al. (1979)].

In this paper we describe a novel method for following the kinetics of cytochrome b_5 exchange between phospholipid vesicles. Based on the data presented here, we suggest that the protein binds in a reversible fashion to these vesicles and that the exchange of cytochrome b_5 involves a transfer of the membrane-dissociated protein through the aqueous phase.

Materials and Methods

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (POPC)[1] was obtained from R. Berchtold Biochemicals (Bern, Switzerland) and was used without further purification. The preparation of a homogeneous population of small unilamellar vesicles by sonication is described elsewhere (Barenholz et al., 1977). These vesicles are very similar to egg phosphatidylcholine preparations in size, density, and sedimentation value (Roseman et al., 1978).

1,2-Bis(9,10-dibromostearoyl)-*sn*-glycerol-3-phosphorylcholine (BrPC) was prepared by bromination of dioleoylphosphatidylcholine (Sigma, St. Louis, MO) according to the method described by Dawidowitz & Rothman (1976). The product was purified by silicic acid chromatography (Litman, 1973). Vesicles from the bromolipid were made by sonication under nitrogen to clarity as previously described (Barenholz et al., 1977). Following sonication the solution was centrifuged at 60000*g* for 20 min and the upper two-thirds of the supernatant was retained for the experiments described below. This preparation, when subjected to molecular sieve chromatography on Sepharose 4B, eluted as a single peak in the included volume. The buffer used in all experiments was 10 mM Tris–acetate and 1 mM EDTA, pH 8.1.

Rabbit liver cytochrome b_5 was prepared by the method described by Ozols (1974).

Fluorescence measurements were made with a Perkin-Elmer MPF-44A spectrofluorometer used in the ratio mode. Solutions were irradiated at 295 nm with a 310-nm cutoff filter in the emission beam path, and the fluorescence emission was monitored at 338 nm. Excitation and emission slits were set at 3 and 6 nm, respectively. All kinetic experiments were done at 25 °C.

Separation of cytochrome b_5–lipid complexes according to density was achieved on 0–2 M sucrose gradients in Tris buffer centrifuged for 16 h at 200000*g*. Gradients were composed of steps of 2 mL of 2 M sucrose, 7 mL of 1 M sucrose, and 2 mL of buffer containing the protein–vesicle mixtures. Following centrifugation the gradients were separated into 12 equal fractions. Each fraction was analyzed for cytochrome b_5 content from the reduced minus oxidized difference spectra (Omura & Sato, 1964) and for lipid by phosphate analysis (Bartlett, 1959). Further lipid analysis of each gradient fraction was as follows. Each fraction was extracted with chloroform–methanol, according to the method of Bligh & Dyer (1959), and evaporated to dryness. Approximately 50 mg of potassium *tert*-butoxide in 2 mL of dimethyl sulfoxide (Gunstone & Hornby, 1969) was then added, and the mixture was incubated for 16 h at 37 °C. Fatty acids were recovered by acidification with HCl and extraction with pentane. The dried pentane extracts were methylated with 14% BF_3 in methanol (Morrison & Smith, 1964) for 15 min at 100 °C. The methylated samples were then extracted with pentane, and the extract was analyzed by gas–liquid chromatography, utilizing a 3-m glass column packed with 3% OV-17, 100–200 mesh, Gas Chrom Q support (Applied Science Laboratories, State College, PA). Gas–liquid chromatography was performed with a Shimadzu GC-4B chromatograph with an FID-4C flame ionization detector programmed from 160 to 210 °C (program rate 5 °C/min). Peaks were quantitated with a Shimadzu Chromatopac E1A integrator.

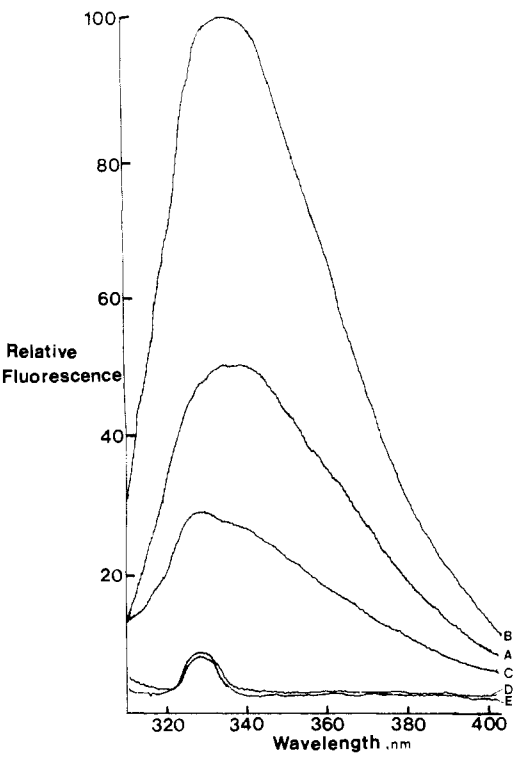

FIGURE 1: Fluorescence emission spectra of cytochrome b_5 (1.4 μM) solutions at 25 °C, uncorrected for photomultiplier response: (A) cytochrome b_5 alone; (B) in the presence of 0.5 mM POPC vesicles; (C) in the presence of 0.5 mM BrPC vesicles; (D) light scattering by 0.5 mM POPC vesicles; (E) light scattering by 0.5 mM BrPC vesicles.

Results

The binding of cytochrome b_5 to the two types of vesicles used in this study results in opposite effects on cytochrome b_5 fluorescence. The fluorescence emission spectra presented in Figure 1 indicate the changes in cytochrome b_5 fluorescence observed after a 30-min incubation of cytochrome b_5 with lipid vesicles made from either POPC or BrPC. The fluorescence increase upon binding of cytochrome b_5 to POPC vesicles results in a 1.85-fold enhancement of protein fluorescence. An enhancement of cytochrome b_5 tryptophan fluorescence upon binding to phosphatidylcholine vesicles has previously been

[1] Abbreviations used: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine; BrPC, 1,2-bis(9,10-dibromostearoyl)-*sn*-glycero-3-phosphorylcholine.

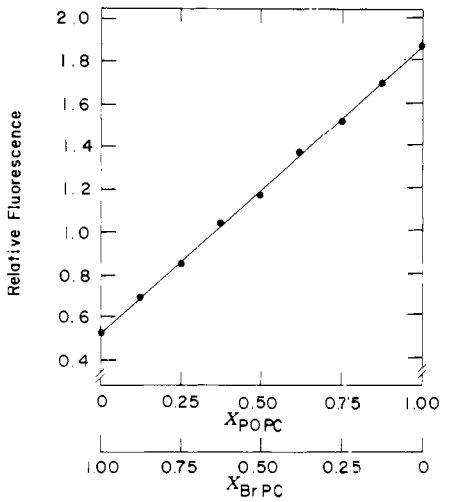

FIGURE 2: Relationship between the observed cytochrome b_5 fluorescence and the mole fraction of POPC (X_{POPC}) and BrPC (X_{BrPC}) vesicles. 1.4 μM cytochrome b_5 was incubated for 30 min at 25 °C with 0.5 mM BrPC or POPC vesicles. These two solutions were then mixed in varying proportions, and measurements were made relative to the fluorescence intensity observed for cytochrome b_5 in the absence of lipid.

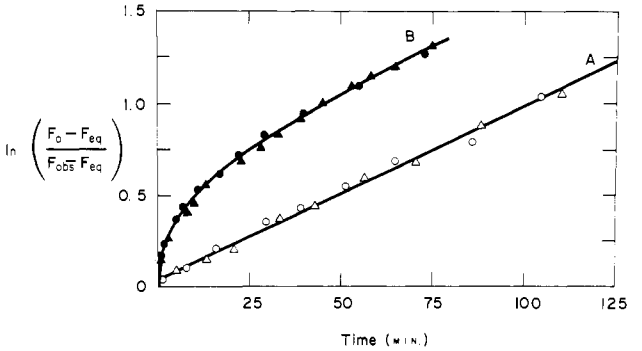

FIGURE 3: Kinetics of cytochrome b_5 exchange between POPC and BrPC vesicles as detected by changes in cytochrome b_5 tryptophan fluorescence. After a 60-min incubation of cytochrome b_5 with donor vesicles at 25 °C, an equivalent amount of acceptor vesicles was added. F_{obsd}, fluorescence observed during the reaction time course; F_0, fluorescence at zero time, upon addition of acceptor vesicles; F_{eq}, equilibrium fluorescence, fluorescence observed after 7 h. (A) Donor vesicles were BrPC and acceptor vesicles were POPC: (△) [BrPC] = 2.0 mM, [POPC] = 2.0 mM, [cytochrome b_5] = 5.1 μM; (○) [BrPC] = 0.2 mM, [POPC] = 0.2 mM, [cytochrome b_5] = 0.51 μM. (B) Donor vesicles were POPC and acceptor vesicles were BrPC: (▲) [POPC] = 2.0 mM, [BrPC] = 2.0 mM, [cytochrome b_5] = 5.0 μM; (●) [POPC] = 0.2 mM, [BrPC] = 0.2 mM, [cytochrome b_5] = 0.51 μM.

reported (Leto & Holloway, 1979; Dufourcq et al., 1975; Vaz et al., 1978). The fluorescence quenching observed upon binding to the BrPC vesicles results in a reduction of cytochrome b_5 fluorescence by a factor of 0.54. Tryptophan fluorescence quenching seen with vesicles which have bromine substituted on the number 9 and 10 positions of the phospholipid hydrocarbon chains seems to indicate that the tryptophans are fairly well buried in the hydrophobic portion of the bilayer. These results are in agreement with our previous experiments which showed that binding of cytochrome b_5 to egg phosphatidylcholine vesicles dramatically decreases tryptophan quenching by acrylamide, an aqueous collisional probe (Leto & Holloway, 1979). Fluorescence titration experiments, similar to those previously described with egg phosphatidylcholine (Leto & Holloway, 1979), result in a constant fluorescence intensity above 80 phosphatidylcholine molecules per molecule of cytochrome b_5 for both the quenching and enhancing vesicles, suggesting that both vesicles have a similar binding capacity (data not shown).

On the basis of the observed difference in cytochrome b_5 fluorescence illustrated in Figure 1, experiments were conducted to establish whether cytochrome b_5 fluorescence could be used quantitatively to determine the distribution of cytochrome b_5 between two vesicle populations. In these experiments cytochrome b_5 is bound separately to the quenching or the enhancing vesicles and the two types of protein–vesicle complexes are mixed to give the same final lipid and protein concentrations but varying ratios of the two lipid vesicles. The relationship between the observed protein fluorescence and the fraction of either vesicle type in solution is illustrated in Figure 2. The observed protein fluorescence exhibits a linear dependence on the fraction of each vesicle species. Incubation times of up to 7 h did not alter this relationship. On the basis of these observations, the following expression is applicable for predicting the cytochrome b_5 fluorescence intensity in these vesicle mixtures:

$$F_{obsd} = F_{en}X_{POPC} + F_qX_{BrPC} \quad (1)$$

where at a given concentration of protein and lipid, F_{en} is the fluorescence observed for cytochrome b_5 bound to POPC vesicles, F_q is the fluorescence observed for cytochrome b_5 bound to BrPC vesicles, F_{obsd} is the observed fluorescence for any vesicle mixture, and X_{POPC} and X_{BrPC} are the mole fractions of POPC and BrPC, respectively.

The ability to quantitatively differentiate between cytochrome b_5 bound to fluorescence quenching and enhancing vesicles prompted a series of experiments to investigate the intervesicle transfer of cytochrome b_5. The design of the fluorescence experiments made it possible to monitor the initial binding to the first (donor) vesicle population and, after addition of the second (acceptor) vesicle population, the kinetics of the exchange process. In addition, the final stable fluorescence could be used to calculate the distribution between the two types of vesicles. When this experiment was preformed, it was found that an initially enhanced spectrum of POPC-bound cytochrome b_5 decreased in intensity upon addition of BrPC vesicles and an initially quenched spectrum of BrPC-bound cytochrome b_5 became enhanced with time upon addition of POPC vesicles. These observations suggest that intervesicle cytochrome b_5 exchange occurs from both the enhancing (POPC) and quenching (BrPC) vesicles. Although these results are consistent with the occurrence of intervesicle exchange of cytochrome b_5, it is also possible that intervesicle transfer of lipid or vesicle fusion could explain the observed fluorescence changes. The density gradient experiments, which are discussed below, demonstrated that the two types of vesicles remained intact in this experiment and that no mixing or transfer of lipids occurred; thus, these fluorescence changes are attributed to cytochrome b_5 intervesicle transfer. The magnitude of the fluorescence change occurring with cytochrome b_5 exchange was affected by the ratio of the donor and acceptor vesicles. For example, in three separate experiments with donor/acceptor vesicle ratios of 1:1, 3:7, and 1:9 the equilibrium fluorescence value, which was determined several hours after addition of the acceptor vesicles, agreed with the F_{obsd} value predicted from eq 1. These results, taken in conjunction with the data in Figure 2, suggest that cytochrome b_5 has an equal affinity for both types of vesicles.

The kinetics of cytochrome b_5 exchange, as detected by fluorescence changes, are presented in Figure 3. In these experiments cytochrome b_5 is incubated with donor vesicles for 60 min prior to addition of the acceptor vesicles. Complete binding to the donor vesicles was indicated by maximal

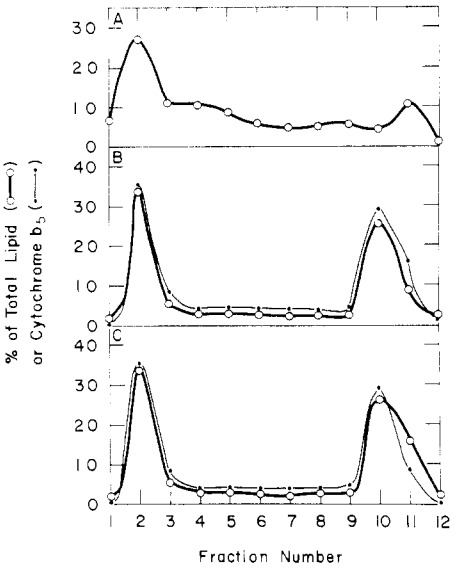

FIGURE 4: Exchange of cytochrome b_5 between phospholipid vesicles, as detected by density gradient centrifugation. Samples were placed on the tops of gradients, and fractions were collected from the bottom (gradient bottom on left). (A) 2.2-μmol amounts of POPC and BrPC vesicles were incubated together at 25 °C for 7 h prior to centrifugation. (B) 5.1 nmol of cytochrome b_5 was incubated with 2.0 μmol of BrPC (donor) vesicles for 60 min at 25 °C followed by the addition of 2.0 μmol of POPC (acceptor) vesicles and incubation for 7 h at 25 °C prior to centrifugation. (C) Same as (B) except POPC was the donor vesicle and BrPC was the acceptor.

fluorescence enhancement (or quenching) within this first incubation period. The fluorescence changes are expressed relative to the equilibrium fluorescence value, F_{eq}, which is evaluated 7 h after the addition of the second (acceptor) vesicle population. It was found that F_{eq} had the same value in both experiments of Figure 3, at a given concentration of protein, regardless of which vesicle type was used as donor and acceptor. In addition, F_{eq} agreed with the predicted fluorescence value calculated from eq 1 for a 1:1 mixture of the two types of vesicles. This shows that the same equilibrium distribution of cytochrome b_5 is attained from either direction. When BrPC vesicles are used as the donor, exchange with respect to cytochrome b_5 concentration can be described by a simple first-order rate expression over the first 65% of the reaction. A more complicated expression is needed to describe the initial exchange process from the POPC donors. These points will be discussed later. The important observation is that the fractional rates of exchange are unaffected by a 10-fold dilution of vesicles, showing that exchange is first order with respect to the donor–acceptor vesicle concentration.

The assumption made in relating the fluorescence data to cytochrome b_5 exchange is that the donor and acceptor vesicles remain as two separate populations over the time course of the analysis. The validity of this assumption was verified by the density gradient experiment described below. Density gradient separation of brominated and nonbrominated vesicles was first demonstrated in the lipid exchange studies of Dawidowitz & Rothman (1976). In this previous study separation was achieved by sedimentation velocity, whereas our experiments achieved an isopycnic separation of the two types of vesicles. The density gradient profile presented in Figure 4A illustrates a separation of an equimolar mixture of POPC and BrPC vesicles which have been incubated for 7 h at 25 °C prior to centrifugation. The profiles presented in parts B and C of Figure 4 represent a separation of cytochrome b_5–vesicle mixtures used in the fluorescence exchange experiment described in Figure 3. Since the fluorescence experiment indicated that exchange equilibrium had been achieved after 7 h at 25 °C, both samples used in parts B and C of Figure 4 were centrifuged for 7 h after the addition of the acceptor vesicles. The exchange experiment in Figure 4B used BrPC vesicles as the donor and POPC vesicles as the acceptor, while that in Figure 4C used POPC vesicles as the donor vesicles. As can be seen in parts B and C of Figure 4, the ratio of cytochrome b_5 to lipid is relatively constant throughout the gradient and cytochrome b_5 appears to partition equally between the two vesicle populations. These results are consistent with the fluorescence data and indicate again that the same equilibrium distribution of cytochrome b_5 is obtained from both directions and that cytochrome b_5 has an equal affinity for the two populations of vesicles.

The density gradients were analyzed to determine if intervesicle lipid transfer or vesicle fusion had occurred in the presence or absence of cytochrome b_5 during the 7-h incubation period. Dawidowitz & Rothman (1976) have presented evidence suggesting fusion of dioleoylphosphatidylcholine and bromolipid vesicles in a similar experiment. The presence of appreciable amounts of lipid in the middle fractions of the gradient in Figure 4A is suggestive of vesicles of hybrid composition. The lipids in the density gradients in Figure 4 were extracted and analyzed by the procedure outlined under Materials and Methods. Since direct gas–liquid chromatographic analysis of the fatty acid methyl esters of the bromolipid did not give suitable quantitative results, the dehydrobromination reaction with potassium *tert*-butoxide in dimethyl sulfoxide was used. This reaction gave two major peaks which were resolvable from the methyl esters of palmitate and oleate in the gas–liquid chromatographic system described above. Analysis of gradient A, which did not contain cytochrome b_5, revealed that 27% of the phospholipid in fraction 3 and 32% of the lipid in fraction 4 were POPC. Since the samples were placed on the top of the gradient, the presence of light lipid in the dense fractions must have arisen from a transfer or fusion process. In contrast to these results, the presence of cytochrome b_5 (parts B and C of Figure 4) appeared to inhibit mixing of lipid between vesicles. Analysis of gradient B revealed that greater than 90% of the lipid in fraction 2 was BrPC and greater than 95% of the lipid in fractions 10 and 11 was POPC. Thus, it appears that cytochrome b_5 may inhibit fusion events by preventing collisional contact between vesicles, possibly by electrostatic repulsion of the hydrophilic domain of the protein.

Discussion

Previous studies in this laboratory have shown that cytochrome b_5 exchanges between phospholipid vesicles (Roseman et al., 1977). We now present a method for following the kinetics of this process by monitoring cytochrome b_5 fluorescence as the protein transfers between two vesicle populations which have different effects on cytochrome b_5 fluorescence. Binding to POPC vesicles causes an enhancement of cytochrome b_5 fluorescence while binding to BrPC vesicles causes a quenching of fluorescence. In addition, the ability to separate the two vesicle populations used in the exchange experiments provides a direct method for correlating the observed fluorescence changes with an actual transfer of protein from one vesicle type to the other.

Intervesicle exchange of cytochrome b_5 can be achieved by two possible mechanisms. These two routes are presented schematically in Figure 5. According to the first scheme, which we call the aqueous transfer mechanism, the exchange of cytochrome b_5 is accomplished as a result of a reversible binding equilibrium. Since the equilibrium of cytochrome b_5

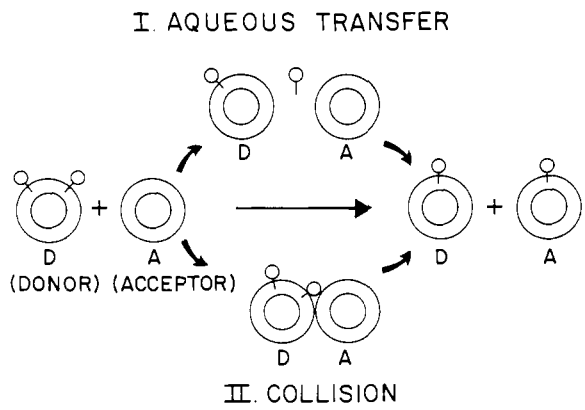

FIGURE 5: Two possible mechanisms for cytochrome b_5 intervesicle exchange.

lies well in favor of the membrane-associated form (i.e., free aqueous cytochrome b_5 is undectable), the rate of exchange by the aqueous transfer model is limited by the rate of dissociation of cytochrome b_5 from the donor vesicle. By this mechanism the fractional rate of equilibration should be independent of vesicle concentration because acceptor vesicles do not affect the rate-limiting step of the reaction.

The second mechanism for cytochrome b_5 exchange (collisional model, scheme II) requires the collision of donor and acceptor vesicles. The transient collisional complex could conceivably provide some type of hydrophobic channel for protein transfer, thus enabling exchange to occur without dissociation of the protein from lipid. This exchange mechanism is kinetically distinguishable from the aqueous mechanism since the fractional rate of equilibration would show a linear dependence on the concentration of donor and acceptor vesicles.

The kinetic data in Figure 3 rule out the collisional model and are consistent with an aqueous transfer mechanism. Although the transfer of cytochrome b_5 from POPC vesicles to BrPC vesicles does not appear to be a simple first-order process with respect to cytochrome b_5 concentration, the absence of any dependence on vesicle concentration excludes the collisional model in this case. According to our proposed mechanism, the slope of these plots in Figure 3 should equal the rate constant for the equilibration of cytochrome b_5 between the two vesicle populations. This slope should also equal the sum of the rate constants for the limiting steps of the forward and backward reactions, which we interpret as the dissociation of protein from either type of vesicle. Consistent with this interpretation, we expect the same limiting slopes regardless of which vesicles are used as donor and acceptor. As can be seen, the limiting slopes in Figure 3 do appear to be parallel, although the initial rate of exchange from the POPC vesicle appears to be faster. This could be attributed to a lower affinity of the POPC vesicle for cytochrome b_5 at higher protein/vesicle ratios (i.e., negative cooperativity).

Our results which support an aqueous transfer mechanism can be compared to some of the recent studies on spontaneous transfer of other soluble amphiphiles between phospholipid vesicles, which also appears to involve the same mechanism. These studies include the transfer of phospholipids (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977), fluorescent phospholipid analogues (Roseman & Thompson, 1979), and fatty acids (Doddy et al., 1978). Cytochrome b_5 has a two-domain structure with a sequence of ~30 nonpolar amino acids on the carboxyl end of the peptide, which serves to anchor the protein to the membrane. Though the nature of this binding involves an intimate hydrophobic association with the nonpolar portion of the lipid bilayer, as these studies confirm, we propose that the protein's mobility between membrane structures arises from the existence of a free aqueous form, which is in equilibrium with the membrane-bound protein.

The above discussion assumes that reversible binding is occurring and this was directly shown in the exchange experiment performed at different ratios of POPC and BrPC vesicles. In these studies the same equilibrium distribution of protein was achieved irrespective of which vesicles were donor and acceptor; hence, all protein must bind in a reversible (exchangeable) form to both types of vesicles. This reversible binding model which we describe for cytochrome b_5–vesicle interactions allows for an alternative explanation of the data recently presented by Enoch et al. (1979) concerning the orientation of cytochrome b_5 in membranes and its ability to undergo exchange. Based on cytochrome b_5 susceptibility to carboxypeptidase digestion and the ability of cytochrome b_5 to undergo exchange in different systems, these authors suggest that the hydrophobic domain of cytochrome b_5 can assume at least two different orientations in the membrane. We suggest that what Enoch et al. (1979) have distinguished as "loose" and "tight" binding, implying different orientations of cytochrome b_5 in the membrane, could alternatively involve different affinities of cytochrome b_5 for different membrane preparations. The inability to observe cytochrome b_5 exchange from microsomal membranes onto phospholipid vesicle may be indicative of a higher affinity of cytochrome b_5 for the microsomal membrane. According to our reversible binding model, a higher affinity could reflect a slower rate of dissociation of cytochrome b_5 from the microsomal membrane. Considering the exchange kinetics which we observed with phospholipid vesicles, it is conceivable that exchange of microsomal bound cytochrome b_5 would require several hours. Even at this rate the exchange process may still be of physiological importance, considering that the estimated half-life of cytochrome b_5 in the cell is 120 h (Omura et al., 1967). Exchange experiments with natural membrane preparations performed over longer time periods, using acceptor membranes of comparable affinity, need to be done to adquately address the question of cytochrome b_5 intermembrane exchange in vivo.

Cytochrome b_5 has shown some membrane binding specificity in model systems. Lipid charge (Faucon et al., 1976), cholesterol content (Tajima & Sato, 1979), the presence of other membrane proteins, and membrane curvature (Enoch et al., 1979) all appear to influence cytochrome b_5 membrane binding. The methodology presented here will hopefully be applicable to an examination of some of these factors affecting cytochrome b_5 membrane interactions.

Acknowledgments

The authors thank Kirk Tanner for the preparations of cytochrome b_5, Leslie Shelton for assistance in the lipid analysis, and Caroline Holloway for her critical reading of this manuscript.

References

Barenholz, Y., Gibbs, D., Litman, B. J., Goll, J., Thompson, T. E., & Carlson, F. D. (1977) *Biochemistry 16*, 2806.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.

Dawidowitz, E. A., & Rothman, J. E. (1976) *Biochim. Biophys. Acta 455*, 621.

Doody, M. C., Pownall, H. J., & Smith, L. C. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1832.

Duckwitz-Peterlein, G., Eilenberger, G., & Overath, P. (1977) *Biochim. Biophys. Acta 469*, 311.
Dufourcq, J., Faucon, J. F., Lussan, C., & Bernon, R. (1975) *FEBS Lett. 57*, 112.
Enoch, H. G., Fleming, P. J., & Strittmatter, P. (1977) *J. Biol. Chem. 252*, 5656.
Enoch, H. G., Fleming, P. J., & Strittmatter, P. (1979) *J. Biol. Chem. 254*, 6483.
Faucon, J. F., Dufourcq, F., Lussan, C., & Bernon, R. (1976) *Biochim. Biophys. Acta 436*, 283.
Fowler, S., Remacle, J., Trouet, A., Beaufay, H., Berthet, J., Wibo, M., & Hausen, P. (1976) *J. Cell Biol. 71*, 535.
Fukushima, K., & Sato, R. (1973) *J. Biochem. (Tokyo) 74*, 161.
Gunstone, F. D., & Hornby, G. M. (1969) *Chem. Phys. Lipids 3*, 91.
Harano, T., & Omura, T. (1977) *J. Biochem. (Tokyo) 82*, 1541.
Jarasch, E., Kartenbeck, J., Bruder, G., Fink, A., Morre', D. J., & Franke, W. W. (1979) *J. Cell Biol. 80*, 37.
Leto, T. L., & Holloway, P. W. (1979) *J. Biol. Chem. 254*, 5015.
Litman, B. J. (1973) *Biochemistry 12*, 2545.
Martin, F. J., & MacDonald, R. C. (1976) *Biochemistry 15*, 321.
Morre', D. J., Kartenbeck, J., & Franke, W. W. (1979) *Biochim. Biophys. Acta 559*, 71.
Morrison, W. R., & Smith, L. M. (1964) *J. Lipid Res. 5*, 600.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370.
Omura, T., Siekevitz, P., & Palade, G. E. (1967) *J. Biol. Chem. 242*, 2389.
Ozols, J. (1974) *Biochemistry 13*, 426.
Roseman, M. A., & Thompson, T. E. (1979) *Biophys. J. 25*, 172a.
Roseman, M. A., Holloway, P. W., Calabro, M. A., & Thompson, T. E. (1977) *J. Biol. Chem. 252*, 4842.
Roseman, M. A., Lentz, B. R., Sears, B., Gibbs, D., & Thompson, T. E. (1978) *Chem. Phys. Lipids 21*, 205.
Tajima, S., & Sato, R. (1979) *Biochim. Biophys. Acta 550*, 357.
Vaz, W. L. C., Jähnig, F., Austin, R. H., & Schoellman, G. (1978) *FEBS Lett. 87*, 269–272.
Wirtz, K. W. A. (1974) *Biochim. Biophys. Acta 344*, 95–117.

Action of Detergents on Membranes: Differences between Lipid Extracted from Red Cell Ghosts and from Red Cell Lipid Vesicles by Triton X-100†

Ruby I. MacDonald

ABSTRACT: A comparative study was undertaken of the solubilization of red cell ghosts and red cell lipid vesicles by Triton X-100 (Triton) to assess the influence of ghost membrane proteins on the amounts and on head-group and fatty acyl compositions of phospholipids removed from both types of membranes. Similar amounts of phospholipid and of cholesterol were solubilized from ghosts and liposomes at a Triton/lipid mole ratio of 1:1, but ~1.5–2 times as much phospholipid and cholesterol was released from ghosts as from liposomes at higher Triton/lipid ratios. Both ghosts and liposomes were less readily solubilized than vesicles of red cell lipid from which cholesterol had been removed. No differences between phospholipid compositions of Triton-extracted lipid from ghosts and liposomes were demonstrable at a Triton/lipid ratio of 1:1, which releases almost no protein from ghosts. At higher Triton/lipid ratios, which release nearly all intrinsic membrane protein from ghosts, however, more phosphatidylserine was removed from ghosts than from liposomes. The latter difference was apparent, regardless of whether ghosts and liposomes were subjected to the same level of Triton or whether ghosts and liposomes were subjected to different levels so as to release the same amounts of lipid from both types of membranes. The selective cosolubilization of phosphatidylserine and certain membrane proteins from ghosts but not from liposomes indicates that phosphatidylserine and at least one of those membrane proteins are associated in untreated ghost membranes. This protein-dependent, selective solubilization of ghost lipid by Triton is distinguishable from a protein-independent, selective solubilization of both ghost and liposome lipids by Triton, the soluble lipid from both membranes having a lower content of sphingomyelin relative to the total amount of lipid extracted.

The mechanism by which detergents solubilize membranes is of interest because of their usefulness not only for the isolation and purification of membrane proteins but also for the resolution of membrane structure. On exposure to various mild detergents, some proteins are released from their membrane sites in association with specific phospholipid(s) not representative of the total membrane lipid, e.g., glycophorin (Armitage et al., 1977; van Zoelen et al., 1977; Buckley, 1978), the erythrocyte anion channel or band III (Ross & McConnell, 1978), and Na^+,K^+-ATPase (Hokin & Hexum, 1972). In addition to demonstrations of coisolation of individual proteins with specific phospholipid(s), intrinsic membrane proteins are also released from erythrocyte ghosts along with a mixture of phospholipids, the composition of which differs from that of total red cell lipid (Yu et al., 1973; Kirkpatrick et al., 1974; Coleman et al., 1976). These findings could signify interactions in the intact membrane between the extracted protein(s) and lipid(s) and/or between the nonextracted protein(s) and lipid(s), which would have survived exposure to detergent. Alternatively, a cosolubilization of specific lipids with certain proteins could reflect a similar affinity of detergent for those proteins and lipids, which would enter the same micelle independently of each other.

† From the Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201. *Received October 2, 1979.* This research was supported by National Institutes of Health Grant R01 GM23153.

0006-2960/80/0419-1916$01.00/0