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Structural Changes in Membranes Produced by the Binding of Small Amphipathic Molecules†

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ABSTRACT: In their interactions with membranes, amphipathic small molecules exhibit detergent-like properties. At sufficiently high concentrations (above their critical micelle concentrations, if they form micelles), they substantially dissolve membranes. At lower concentrations, between maximally antihemolytic and lytic, we show here that the amphipaths significantly perturb membrane structure. Each of six small-molecule amphipaths was shown by hygroscopic desorption filtration to induce the extraction of small but significant amounts of membrane components, partly in the form of vesicular fragments, from red blood cell membranes. These extracts were enriched in the lipid to protein ratio as compared to the intact membrane, and the protein composition was highly unrepresentative. A similar set of extractions from sarcoplasmic reticulum membranes was induced by the six amphipaths. We conclude that small-molecule amphipaths, at concentrations lower than lytic, promote gross redistributions of components in the plane of a membrane that result in the observed extractions.

The interactions of amphipathic small molecules with membranes are of great interest in physiology and biochemistry. Such amphipaths include the bile salts, many anesthetics and tranquilizers, mitochondrial uncouplers, and many other drugs and metabolites. In addition, many of the molecular probes used to study membrane fluidity and other properties are amphipaths. These molecules have two distinct domains, one hydrophobic and the other hydrophilic. It is widely believed that over a broad range of concentrations most amphipaths dissolve in the fluid lipid regions of a membrane, intercalating their hydrophobic domains into the hydrophobic interior of the lipid bilayer, while their hydrophilic domains are positioned in the region of the lipid polar head groups. This is thought to be responsible for the fluidity changes that are generally

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observed when amphipaths bind to membranes. However, it is becoming increasingly clear that the situation is more complex than this simple picture suggests. It is likely that there are several different modes of association of amphipaths with membranes, depending on the structure of the particular amphipath and its concentration, as well as on the composition of the membrane under study. It is also likely that the membrane association of amphipaths results in significant changes in the structure of the membranes, which is the major thesis of this paper.

Important to an understanding of this complex behavior is an appreciation of the fact that all amphipaths exhibit detergent-like properties. The detergent properties of amphipaths such as sodium dodecyl sulfate or sodium deoxycholate are of course well recognized, but amphipaths such as chlorpromazine (Scholtan, 1955; Conrad & Singer, 1981) or tetracaine (Fernandez, 1980) also behave as detergents: at concentrations exceeding their critical micelle concentrations (cmc), they form micelles in aqueous solution similar to those formed by conventional detergents. At such large concentrations, amphipaths often disrupt membranes and cause at least their partial dissolution [cf. Leterrier et al. (1974)], presumably by the formation of mixed micelles containing the amphipath along with particular membrane components. At concentrations below their cmc, amphipaths generally interact with membranes without dissolving them. At these lower concentrations, however, the effects of amphipaths on membranes may still reflect the detergent-like affinities for membrane components that the amphipaths exhibit in mixed-micelle formation at higher concentrations. These lower concentrations are in the range where certain of these amphipaths exhibit important pharmacological and physiological effects.

In the present paper, we demonstrate that a wide range of small-molecule amphipaths at concentrations below their cmc induce significant structural changes in membranes. We have studied in detail the effects of six common amphipathic compounds (chlorpromazine, methochlorpromazine, tetracaine, indomethacin, 2,4-dinitrophenol, and decanol) on human red blood cell (RBC) membranes and on rabbit muscle sarcoplasmic reticulum (SR) membrane vesicles. Each amphipath was used mainly at a concentration corresponding to its maximum retardation of the rate of hypotonic hemolysis of intact RBC, i.e., at a concentration corresponding to its presumed maximum stabilization of the RBC membrane (Seeman, 1972). The amphipath-treated membranes were subjected to hygroscopic desorption filtration (Conrad & Singer, 1981) because we suspected that the amphipaths were extracting components from the membrane. In this technique, an equilibrated suspension of membranes and amphipaths is filtered through a porous hydrophobic filter, which completely retains the intact membranes in a nearly dry state and removes all of the fluid phase containing the amphipath and any solubilized membrane components or small membrane fragments through the filter to be collected for analysis.

We have found that under these conditions each of the amphipaths induces a small but significant extraction of membrane components into the filtrate. At higher concentrations of an amphipath, increased extraction is induced. The extraction is highly selective in both lipid and protein components and differs for different amphipaths acting on the same membrane. The data suggest that the amphipaths induce specific structural rearrangements in the membrane which result in the observed extractions.

Materials and Methods

Cells and Membranes. Human RBC were prepared from serum drawn from normal donors into ACD (0.038 M citric acid, 0.095 M sodium citrate, and 0.136 M glucose, pH 7.4) and were washed 3 times with PBS (0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4), carefully removing the buffy coat by aspiration. A few experiments were carried out with sheep and rat RBC collected in the same way as with the human cells. In order to examine an entirely different membrane system, we also prepared sarcoplasmic reticulum (SR) vesicles, starting with the back and leg muscles of a rabbit, by the procedure of Martonosi (1968).

Labeling of Cells and Membranes with ^{125}I. Most of the experiments with human RBC were carried out by first labeling *intact* cells with ^{125}I by the method of Hubbard & Cohn (1972). One milliliter of a mixture containing $(1-2) \times 10^9$ cells in PBS, 20 μg of lactoperoxidase (powder, Sigma), 5 μmol of glucose, 0.4 μg of glucose oxidase (type V, Sigma), and 200 μCi of carrier-free $Na^{125}I$ (Amersham) was incubated for 10 min at 22 °C with occasional mixing; the reaction was terminated by the addition of PBS containing NaI instead of NaCl, followed immediately by centrifugation of the cells. ^{125}I-Labeled RBC were then washed 4 times with the NaI-containing PBS, 3 times with PBS, and 2 times with isotonic Tris–saline [0.146 mM NaCl and 0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4]. The labeled cells were mixed with a 10-fold excess of fresh unlabeled RBC, and ghosts were then prepared by a slight modification of the method of Dodge et al. (1963) that avoided the use of phosphate in the hypotonic lysis buffer by substituting Tris. The faintly pink ghosts were then placed in isotonic Tris–saline containing 10^{-5} M $CaCl_2$ and were incubated for 60 min at 37 °C to induce resealing (Schwoch & Passow, 1973). The ghosts were then collected in isotonic Tris–saline, and aliquots were stored frozen at −20 °C. Each aliquot, once thawed, was used for a set of filtration experiments, and any remainder was discarded. Such single freezing and thawing allowed the ghosts to remain sealed.

Ghosts that were ^{125}I labeled and treated in this manner appeared as intact spheres when viewed by Nomarski optics in the light microscope and were smooth-surfaced when observed by transmission electron microscopy after negative staining with 1% phosphotungstic acid.

For comparison, a few experiments were carried out by lysing ^{125}I-labeled human RBC by the more gentle method of Ting-Beall et al. (1981), which gives sealed pink ghosts. For the experiments involving sheep and rat RBC, the intact cells were ^{125}I labeled, and the labeled ghosts were prepared by the same procedures used with the human cells.

^{125}I-Labeled SR vesicles were prepared by using the light vesicle fraction in 0.1 M KCl and 5 mM histidine, pH 7.3, as described for the labeling of the RBC.

Hygroscopic Desorption. The hygroscopic desorption experiments were carried out by using a modification of the apparatus described by Conrad & Singer (1981), so that the filtrate could be collected under vacuum for analysis. The top hydrophobic filter was a 10-μm-thick polycarbonate film[1] of 0.4-μm maximum pore size (Nuclepore) that was used without any preconditioning treatment. The middle and bottom filters of the original apparatus were replaced by a sintered glass disk,

[1] In our previous experiments (Conrad & Singer, 1979, 1981), polycarbonate films of 2-μm thickness obtained from Mobay Chemical Corp. were irradiated for us by Nuclepore to produce the porous hydrophobic top films. This special procedure was discontinued by Nuclepore, and we have since had to resort to the 10-μm-thick films provided by Nuclepore which are less satisfactory for the purposes of hygroscopic desorption.

and the filtrate was collected into a glass scintillation vial. Most of the desorption experiments were carried out as follows. ^{125}I-Labeled ghosts, at a concentration between 30 and 75 nmol of phosphate/mL, and a specific radioactivity of (4–5) × 10^3 cpm/nmol of phosphate, were added either to isotonic Tris–saline or to the same buffer containing an amphipath at the concentration indicated in the second column of Tables I and VI. The mixtures were incubated at 37 °C, generally for 10–60 min. A 1.5-mL aliquot was then applied to a polycarbonate top filter, and filtration was carried out under vacuum. (With these thick and unconditioned films, the flow rate in the absence of a vacuum was negligible.) The filter was rinsed twice with 0.5 mL of buffer for the saline control, or buffer plus amphipath for the treated sample, and the filter was then removed for radioactive counting of the membrane retained on it. The filter washings, plus the washings of the glass disk, were combined with the filtrate. The filtrate mixture was then processed in one of two ways: for subsequent protein analysis, it was dialyzed extensively (without protein loss) against distilled water and then lyophilized; for subsequent lipid analyses, the mixture was lyophilized directly.

In some cases, the collected filtrates were applied directly to polycarbonate top filters of smaller (0.08 μm) maximum pore size in order to retain any vesiculated membrane fragments that might have arisen during the first filtration. These small pore filters were then counted to determine the label retained or were processed for examination by scanning electron microscopy.

In other experiments, the ^{125}I-labeled human RBC ghosts were subjected to hygroscopic desorption by using the original three-part filter assembly of Conrad & Singer (1979, 1981), and without the application of a vacuum. After the usual incubation of the ghosts with chlorpromazine, the sample was applied, and filtration was carried out until no perceptible liquid remained on the top filter. The three filters were then disassembled and counted separately. However, it was not feasible to recover the filtrate for further analyses in these experiments, and only the membrane retained on the top filter could be counted as a check on the vacuum filtration procedure that was generally used.

For the hygroscopic desorption of ^{125}I-labeled SR vesicles, the same procedures were used as for the RBC, except that polycarbonate top filters of 0.1-μm diameter maximum pore size were employed (instead of 0.4 μm as used for the RBC) in order to retain the smaller vesicles.

Centrifugal Separation of Amphipath-Treated ^{125}I-Labeled RBC Ghosts. A few experiments were carried out in which, after the usual incubation of the labeled ghosts with the amphipath solution or the buffer control, the ghosts, instead of being filtered, were centrifuged at 27000*g* for 10 min, and the supernatant was removed for counting to determine if labeled membrane components were released.

Analysis of the Filtrates. (A) Protein Analyses. Lyophilized filtrate samples or ^{125}I-labeled RBC ghosts or ^{125}I-labeled SR were dissolved in gel sample buffer [0.015 M Tris-HCl, pH 8.0, 5% sodium dodecyl sulfate (SDS), 20% sucrose, 2% β-mercaptoethanol, and 0.002% bromphenol blue] and heated at 56 °C for 5 min. Discontinuous SDS–polyacrylamide gel electrophoresis (PAGE) was performed by using slab gels and the system of Laemmli (1970). The gels were stained with Coomassie blue, dried under vacuum, and autoradiographed.

In such experiments with RBC preparations, the proteins visualized by autoradiography were only those that were labeled by ^{125}I-lactoperoxidase treatment of intact RBC. In order to determine whether additional proteins were extracted in the filtration experiments, the lyophilized amphipath filtrates of *unlabeled* ghosts were dissolved in 1% SDS, and the solution was then ^{125}I labeled with the Bolton–Hunter reagent (Shing & Ruoho, 1981). The samples, after appropriate dilution, were then electrophoresed on SDS–polyacrylamide gel slabs and autoradiographed as described above.

(B) Lipid Analyses. Lipid components of membranes also became iodinated by the ^{125}I-lactoperoxidase procedure [cf. Mersel et al. (1976) and Schlager (1980)]. In order to determine what types of labeled lipids were extracted from the membranes by the amphipaths, some simple fractionations based on the polarity of the lipids, followed by thin-layer chromatography (TLC) separations of the fractions and autoradiography, were carried out on the amphipath extracts of the ^{125}I-labeled ghosts. The aim of these analytical procedures was not to identify and quantitate every compound extracted from the ghosts, but rather to determine qualitative features of the extractions, and in particular whether different amphipaths promoted the extraction of different types of lipids.

The procedure outlined in Figure 1 was employed, combining the methods of Irwin & Irwin (1979) and Jungalwala et al. (1977). This resulted in a reproducible separation of four somewhat overlapping lipid fractions, A1, A2, A3, and B. Fraction A1 contained mainly the least polar lipids (cholesterol, free fatty acids), A2 mainly the neutral glycolipids (Vance & Sweeley, 1967), A3 mainly the phospholipids, and B mainly the gangliosides (Irwin & Irwin, 1979) present in the RBC membrane. (Gangliosides are not present in SR membranes.) Aliquots of these fractions were counted for their ^{125}I-label content; the remainder was dried under N_2. Fraction B was then resuspended in 1 mL of distilled water, dialyzed extensively against distilled water to remove salts, and then lyophilized. [Gangliosides remain inside the dialysis bag under these conditions (Formisano et al., 1979).] Fraction A2 was then subjected to alkaline methanolysis (Vance & Sweeley, 1967) to remove phospholipids before further analysis.

All four lipid fractions were then analyzed by thin-layer chromatography on silica gel plates (Schleicher & Schuell, 250 μm). The following developing solvents were used: A1, hexane–diethyl ether–acetic acid (80:20:1) (Blank & Snyder, 1975); A2 and A3, chloroform–methanol–water (65:25:4) (Kuksis, 1977); and B, chloroform–methanol–0.25% $CaCl_2$ (60:35:8) (Irwin & Irwin, 1979). After evaporation of the solvents, the plates were autoradiographed. Control experiments showed that under these conditions the R_f values for the major identifiable radioactive bands in the RBC ghost extract were the same as those for the equivalent nonradioactive compounds, confirming the findings of Schlager (1980).

Results

Characterization of Labeled Membranes. The significance of the extraction results presented below depends on the assumption that the membranes studied were not structurally altered by the ^{125}I labeling. To test this point, we found that the labeled intact RBC showed the same hemolysis protection curves (Seeman, 1972) as unlabeled RBC with all of the amphipaths used in the extraction studies (not shown). Therefore, we conclude that the ^{125}I-lactoperoxidase labeling did not of itself induce any significant structural change in the RBC membranes. Furthermore, in some of the experiments described below, unlabeled membranes were used and showed the amphipath-induced extraction.

Whole labeled RBC membranes, prepared as described under Materials and Methods, had about 92% of the total label

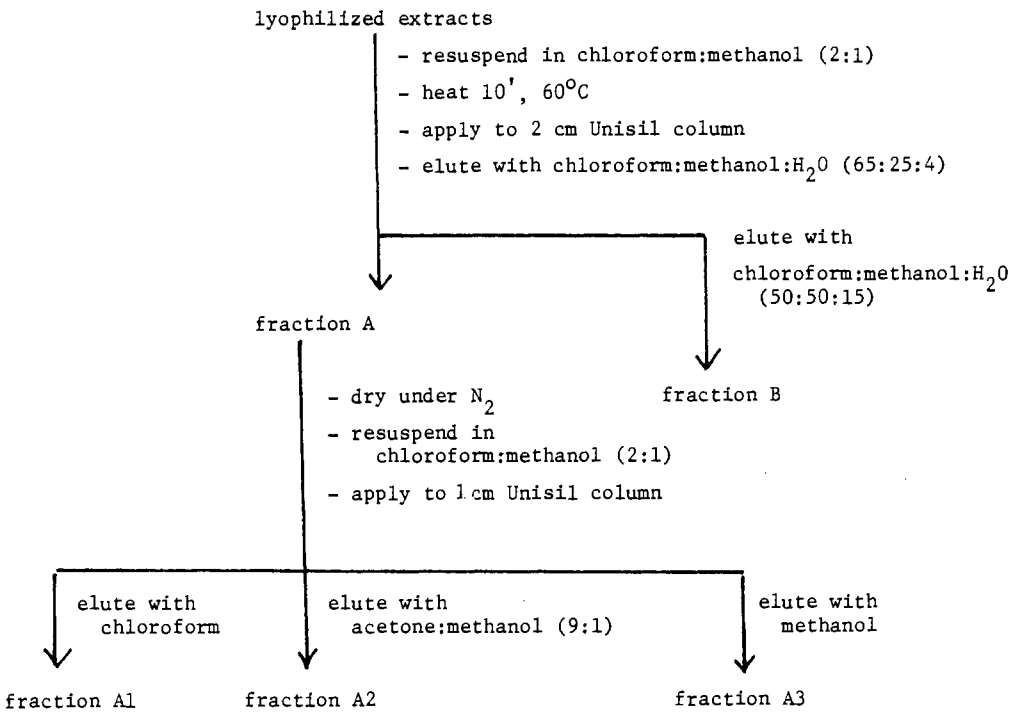

FIGURE 1: Flow chart for the procedures used in the fractionation of the lipids extracted from membranes by amphipaths.

Table I: Amphipath-Induced ^{125}I-Label Extraction from RBC Ghosts

agent	concn (mM)	(cpm of test extract)/(cpm of control extract)		% extracted cpm in lipid	% extracted cpm in protein
		desorption	centrifugation		
buffer control		1.00	1.00	13.7	86.3
chlorpromazine	0.06	1.27	1.05	14.9	85.1
methochlorpromazine	0.30	1.91	1.42	16.7	83.3
tetracaine	0.50	1.43	0.95	13.5	86.5
decanol	0.01	1.37	0.97	14.0	86.0
dinitrophenol	10	1.43	1.00	16.6	83.4
indomethacin	0.60	1.61	1.00	15.6	84.4
intact ghosts				7.45[a]	92.6[a]

[a] Percent total cpm in this fraction.

in protein and 8% in lipids (i.e., extractable into organic solvents). With whole SR membranes, the values were 83% and 17%, respectively. (In the absence of the enzymes lactoperoxidase and glucose oxidase, the total labeling was less than 1% of these values.) Enzyme-catalyzed iodination of the lipids of lysosomes and mammalian cells has been observed before (Mersel et al., 1976; Schlager, 1980).

Characterization of the Hygroscopic Desorption Filtration Experiments. (A) Retention of Membranes by Filters. The polycarbonate top filters of 0.4-μm pore diameter retained 95 ± 2% of the counts applied as ^{125}I-labeled RBC membranes in buffer (without added amphipath). Filters with 0.2 μm pore size gave similar results, while with still smaller pore-size filters the flow rate with the RBC membranes was too low to be useful. With the SR membranes, 0.1-μm pore filters were optimal. These retained 98 ± 2% of the applied counts with buffered suspensions of ^{125}I-labeled SR membranes.

(B) Conditions of Extraction. The concentration of each of the six amphipaths used for extraction was generally that which we determined afforded maximum retardation of the rate of hypotonic lysis of intact RBC (Seeman, 1972). These single concentrations were used throughout most of these studies, and are listed in Table I, column 2. The time of incubation of the amphipath with the membrane prior to filtration was 10 min, because it was shown that longer incubation had no effect, except with methochlorpromazine. The latter required 60 min of incubation to produce maximum extraction of counts. Only a narrow range of membrane concentrations could be used, the lower limit defined by the sensitivity of the assays and the upper limit by the flow rate through the filter. For RBC membranes, most experiments were carried out with suspensions containing 75 nmol of membrane phospholipid P per mL.

To determine if the amphipath-induced extraction of membrane components depended on the condition of the RBC membrane, some hygroscopic desorption experiments were carried out with intact labeled RBC or with pink ghosts whose preparation minimized membrane perturbations (Ting-Beall et al., 1981). Upon incubation with amphipaths as in the experiments with the standard RBC membranes, the percent of the ^{125}I label extracted into the filtrate was about the same in each case (not shown). Further analysis of the composition of the filtrates was rendered difficult by the large amounts of hemoglobin that were also present in these samples. The results were, however, consistent with the conclusion that the amphipath-induced extraction of the standard RBC membranes was not simply the result of structural alterations in the membranes produced during their preparation.

Analysis of the Extracts of RBC Membranes. (A) Protein:Lipid Ratios. In all of the experiments to be presented, the amphipath-induced extracts are compared with the filtrates obtained in the absence of amphipaths (buffer controls). These

Table II: Concentration Dependence of Amphipath-Induced Extraction of RBC Ghosts during Hygroscopic Desorption Filtration

agent	concn (mM)	(cpm of test extract)/(cpm of control extract) ratio
chlorpromazine	0.001	1.01
	0.06	1.27
	0.10	2.11
	0.60	lysis
decanol	0.001	0.99
	0.010	1.37
	0.10	1.63
	10	lysis

controls were not negligible. In the case of the ^{125}I-labeled RBC membranes, the control filtrates contained about 5% of the original counts, and of these counts, 14% was in the lipid fraction and 86% in protein (Table I) compared to 8% and 92%, respectively, for the whole ghosts. In other words, the buffer filtrates did not simply have the composition of the original labeled RBC membrane but were enriched in lipid. Further characterization of the control filtrates is given in subsequent sections.

With each of the amphipaths at the concentration of their maximum stabilization of the RBC membrane, there was a significant increase (27–91%) in the total counts extracted from the labeled RBC membranes into the filtrate above the buffer control (column 3, Table I). (The values given represent the average of 8–10 experiments, each done in triplicate. The experimental error for the values in column 3 of Table I is about ±0.1.) The increase in extracted counts was observed in both total lipid and protein fractions (columns 5 and 6, Table I). For most amphipaths, the ratio of lipid to protein in the extract was significantly larger than for the whole membrane (bottom row, Table I).

A few experiments were carried out to determine the effect of amphipath concentration on the total ^{125}I label extracted, without further analysis into protein and lipid fractions. As shown in Table II, the amount extracted increased with increasing concentration of either chlorpromazine or decanol, until a concentration of either amphipath was reached which substantially dissolved the RBC membranes.

(B) Protein Composition. The autoradiographic profiles in SDS–PAGE of the proteins of whole RBC membranes prepared from ^{125}I-labeled intact RBC (Figure 2, lane a) were dominated by the overlap region between band 3 and PAS-1. In addition, less intensely labeled bands of lower molecular weight components were observed (Mueller & Morrison, 1977; Thompson et al., 1980). The amphipath-induced extracts generally showed the same labeled proteins but in different proportions than in the whole RBC membrane (Figure 2, lanes b–h).

In these experiments, however, only those proteins of the membranes of the intact RBC that were labeled by the ^{125}I-lactoperoxidase procedure could be observed. More interesting results were obtained when the filtrates from unlabeled membranes were analyzed for their protein composition by dissolving and labeling them by the Bolton–Hunter procedure. Whole RBC membranes dissolved and so labeled gave an autoradiographic profile on SDS–PAGE that corresponded fairly well to the standard Coomassie blue staining pattern (Figure 3, lane a). The labeled protein profiles of the amphipath-induced extracts, however, were remarkably different from those of the whole membranes. The extracts were not only devoid of the major peripheral proteins (such as spectrin

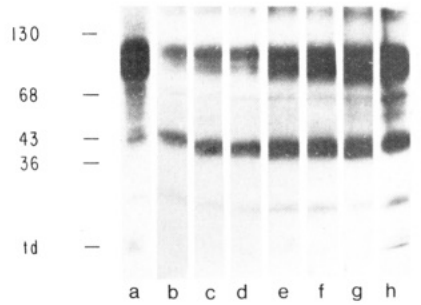

FIGURE 2: Autoradiogram of electrophoretic separations on 9% SDS–polyacrylamide gels of extracts of ghosts from ^{125}I-lactoperoxidase-prelabeled RBC. Extracts were lyophilized, dialyzed against distilled H_2O, lyophilized, and dissolved in 200 μL of sample buffer (0.015 M Tris-HCl, pH 8.0, 5% SDS, 20% sucrose, and 2% β-mercaptoethanol), and 50-μL aliquots were applied to each well. The gels were stained with Coomassie blue, dried, and autoradiographed on Kodak XAR-5 film at −70 °C with intensifying screens for 2–5 days. Lane a shows whole ghosts before extraction; the remaining lanes were of the following extracts: (b) buffer control; (c) chlorpromazine; (d) decanol; (e) tetracaine; (f) indomethacin; (g) methochlorpromazine; and (h) dinitrophenol.

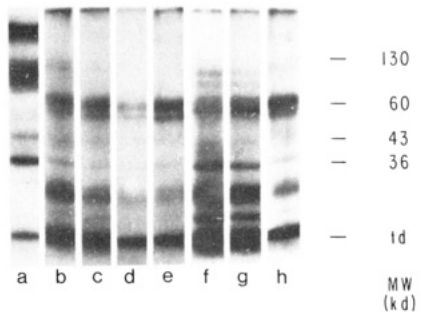

FIGURE 3: Autoradiogram of electrophoretic separations on a 9% SDS–polyacrylamide gel of extracts of ghosts from unlabeled RBC postlabeled with ^{125}I by using the Bolton–Hunter reagent. Extracts were lyophilized, dialyzed against distilled H_2O, lyophilized, resuspended in 100 μL of 0.1 M $NaHCO_3$, pH 8.7, and 1% SDS, and labeled with the Bolton–Hunter reagent as described (Shing & Ruoho, 1981). Each sample was diluted with an equal volume of 2× sample buffer, and 50-μL aliquots were applied to each well. The gels were further treated as described in Figure 1. Whole ghosts were treated in the same way as the extracts. Lane a, whole ghosts before extraction; lane b, buffer-control extract. The remaining lanes were for the same amphipaths as in Figure 2.

bands 1 and 2 and actin band 5) but also showed a marked enrichment of several minor membrane proteins (such as proteins in the region of band 4.5) over band 3 and PAS-1. Of further interest, the extracted protein profiles differed significantly for different amphipaths. For example, the profiles for methochlorpromazine (lane g) and tetracaine (lane e) were remarkably different from one another, although both amphipaths are positively charged, whereas the profile for indomethacin (lane f) was very similar to that for methochlorpromazine, although the two amphipaths are oppositely charged. The proteins extracted by the amphipaths, therefore, are clearly a special subset of the proteins of the RBC membrane, excluding the major peripheral proteins, and differ with different amphipaths, showing no simple correlation of their overall composition to the charge of the amphipath molecule.

(C) Lipid Composition. The total labeled lipid was resolved into four fractions of different polarity according to the scheme outlined in Figure 1, and the percent of the extracted lipid recovered in each fraction was determined (Table III). The most striking feature of these results was the enhanced proportion of counts in fraction B (consisting mainly of gangliosides) compared to whole RBC membranes (first row). Although the buffer-control filtrates already showed an en-

Table III: Distribution of ^{125}I Label among Different Lipid Fractions in RBC Ghost Extracts[a]

agent	% recovery of total lipid cpm	lipid fraction A1	A2	A3	B
intact ghosts	68.6	0.19	0.27	0.36	0.18
buffer control	84.4	0.09	0.20	0.43	0.28
chlorpromazine	84.3	0.08	0.19	0.37	0.36
methochlorpromazine	73.8	0.04	0.21	0.40	0.35
tetracaine	91.2	0.06	0.12	0.31	0.51
decanol	92.1	0.07	0.15	0.39	0.39
dinitrophenol	75.9	0.11	0.19	0.39	0.31
indomethacin	70.4	0.07	0.12	0.23	0.58

[a] Expressed as the ratio of cpm in fraction/cpm in total lipid extracted.

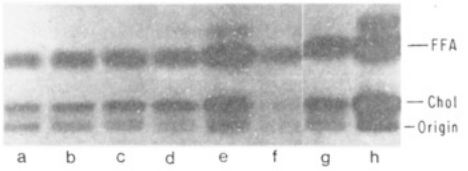

FIGURE 4: Autoradiogram of thin-layer chromatogram of lipid fraction A1 from RBC ghosts and extracts. After preparation of fraction A1 as described in Figure 1, equal volumes of each sample were dried under N_2 and resuspended in 50 μL of 2:1 chloroform:methanol, and 25-μL aliquots were applied to silica gel G plates in streaks. The chromatograms were developed with hexane:diethyl ether:acetic acid (80:20:1), dried, and autoradiographed as described in Figure 2. Bands were identified by comigration with authentic standards run at the same time. The lanes were of the following extracts: (a) buffer control; (b) chlorpromazine; (c) decanol; (d) tetracaine; (e) indomethacin; (f) methochlorpromazine; and (g) dinitrophenol. Lane h was of fraction A1 obtained from whole ghosts.

richment in fraction B, the amphipath-induced extracts were still more highly enriched in this fraction, but to a variable extent depending on the amphipath. Accompanying this enrichment in fraction B was a relative depletion in fraction A1 (consisting mainly of nonpolar lipids and free fatty acids). Examination of Table III shows that there were significant differences in the proportions of the different lipid fractions extracted by different amphipaths, although no simple correlation with amphipath charge could be discerned.

Each of the four fractions from each amphipath-induced extract was resolved into several bands by TLC and autoradiography. Generally, the same components that characterized a given lipid fraction from whole RBC membranes also appeared in that fraction from an amphipath-induced extract, more or less independent of the amphipath. Typical chromatograms for lipid fractions A1 and B are shown in Figures 4 and 5, respectively. Some subtle differences between different amphipaths are apparent in these figures, however; for example, the ratio of free fatty acids to cholesterol extracted by methochlorpromazine (Figure 4, lane f) is much larger than for the other amphipaths.

(*D*) *Other Properties of the Extracts.* One question raised by these extraction results is the following: What is the state of the extracted material; e.g., is it vesicular or micellar? To examine this question, filtrates from the RBC membranes extracted on 0.4-μm pore-size filters were applied to a 0.08-μm pore-size filter under vacuum. From 20% to 50% of the applied counts were retained on the latter filter, depending on the amphipath used in the extraction (Table IV). No significant additional counts were retained if 0.03-μm pore-size filters were used instead of 0.08 μm. The material collected on the 0.08-μm filters was examined by scanning electron microscopy (SEM) (Figure 6) and appeared to consist mainly of irregular

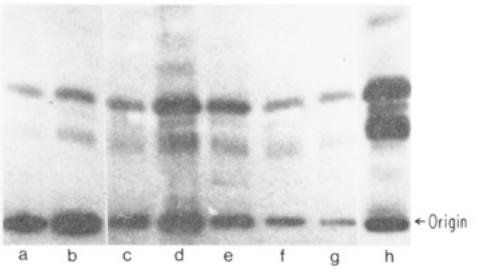

FIGURE 5: Autoradiogram of thin-layer chromatogram of lipid fraction B from RBC ghosts and extracts. After preparation of fraction B as described in Figure 1, the samples were dialyzed against distilled water, lyophilized, and resuspended in 50 μL of chloroform:methanol (2:1), and 25-μL aliquots were applied to silica gel G plates in streaks. The chromatograms were developed with chloroform:methanol:0.25% $CaCl_2$ (60:35:8), dried, and autoradiographed as described in Figure 2. The lane designations are the same as those in Figure 4.

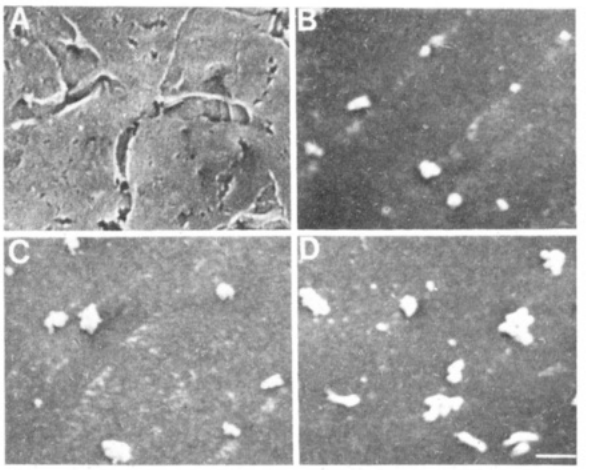

FIGURE 6: Scanning electron micrographs of (A) the whole RBC ghosts left on a 0.4-μm pore-size filter after desorption with buffer and (B) the filtrate from the buffer desorption, (C) from a chlorpromazine desorption, and (D) from a dinitrophenol desorption of the RBC ghosts refiltered on a 0.08-μm pore-size filter. The filters were processed for scanning electron microscopy as described (Conrad & Singer, 1979). The bar represents 0.1 μm.

Table IV: Retention of Label on Small-Pore Filters of Amphipath-Induced Extracts from RBC Ghosts[a]

extract	% retention
buffer control	23.1
chlorpromazine	24.5
methochlorpromazine	18.4
tetracaine	39.7
decanol	25.9
dinitrophenol	49.7
indomethacin	49.2

[a] 0.08-μm pore-size filters were used; see the text.

small vesicles. For amphipaths such as dinitrophenol for which a larger fraction (~50%) of the original counts in the extracts was retained on the 0.08-μm filter, a larger amount of vesicular material was observed (Figure 6D). It is to be noted that vesicular material was also observed in the buffer-control extracts (Table IV and Figure 6B). We conclude from these experiments that a substantial fraction, but possibly not all, of the components extracted by the amphipaths from the RBC membranes was in the form of small vesicles.

These observations raised the next question: Were these vesicles and other materials removed from the RBC membranes directly upon addition of the amphipaths to the membrane, or were they removed only after subjecting the amphipath-treated membranes to filtration by hygroscopic de-

Table V: Extraction of ^{125}I-Labeled Material from Ghosts of Different Species[a]

agent[b]	RBC source		
	human	sheep	rat
chlorpromazine	1.27	1.05	1.57
methochlorpromazine	1.91	1.10	1.52
tetracaine	1.43	1.38	1.50
decanol	1.37	1.00	1.11
dinitrophenol	1.43	2.83	1.45
indomethacin	1.61	1.93	1.35

[a] Values are expressed as the ratio of cpm of test extract to cpm of control extract. [b] Agent concentrations are as in Table I.

sorption? To explore this question, the ^{125}I-labeled RBC membranes were incubated with the amphipaths as usual, but instead of subjection of the suspensions to hygroscopic desorption, they were centifuged at 27000*g* for 10 min, and the supernatants were counted. The results in Table I, column 4, were obtained. Under these conditions, [i.e., those of the centrifugal method used to measure the apparent binding of the amphipath to the membrane (Seeman, 1972)], the buffer control contained 3.5% of the total counts in the supernatant. None of the amphipath-treated samples, with the exception of methochlorpromazine, showed any increase in supernatant counts above the control. Furthermore, SEM examination of unfiltered amphipath-treated RBC membranes did not reveal any indication of vesicular debris associated with the membranes. We infer that the physical extraction of at least the vesicular material derived from the amphipath-treated membranes largely occurred only upon subjecting the membranes to filtration by hygroscopic desorption, and not before.

Extracts of Sheep and Rat RBC Membranes. A few similar experiments were carried out with ^{125}I-labeled RBC membranes from sheep and rat. The interest in performing these experiments derives from the finding (Coleman et al., 1980; Lowe & Coleman, 1981) that sheep RBC are significantly more resistant to glycocholate-induced lysis than are human or rat RBC, perhaps because of their larger sphingomyelin content. Upon measuring the total counts extracted by the same concentrations of different amphipaths, the comparative results in Table V were obtained. While chlorpromazine, methochlorpromazine, and decanol did indeed extract fewer counts above saline controls from sheep RBC membranes than from either the human or the rat membranes, the reverse was true for dinitrophenol and indomethacin. No simple correlations were evident for the amphipath-extraction results of the different species of RBC; on the contrary, a specificity of the extraction for a given amphipath and a particular membrane was indicated.

Analysis of the Extracts of SR Membranes. The same types of experiments described for the amphipath-induced extraction of RBC membranes were carried out with ^{125}I-labeled SR membrane vesicles. The filtrates from hygroscopic desorption experiments of amphipath-treated SR membranes showed significant increases in total counts extracted over buffer controls (column 3, Table VI) and in both total lipid and total protein extracted (columns 4 and 5, Table VI) for all six amphipaths. The data in Table VI were obtained by using SR membrane suspensions containing 135 nmol of membrane phospholipid P per mL; closely similar values were obtained at 75 nmol/mL, the membrane concentration used in the RBC experiments. The original SR membranes contained 83% of the ^{125}I label in protein and 17% in lipid, but the extracts were markedly enriched in lipid. The buffer-control extract was itself enriched in lipid compared to the original membranes, but each of the amphipaths induced extracts which were still further enriched in lipid. All of these results are strikingly parallel to those observed with the RBC membranes.

Table VI: Amphipath-Induced ^{125}I-Label Extraction from SR Vesicles

agent	concn (mM)	(cpm of test extract)/(cpm of saline extract) ratio	% extracted cpm in lipid	% extracted cpm in protein
buffer control		1.00	36.2	63.8
chlorpromazine	0.06	1.39	43.0	57.0
metho-chlorpromazine	0.30	1.95	48.3	51.7
tetracaine	0.50	1.44	44.6	55.4
decanol	0.01	1.19	43.0	57.0
dinitrophenol	10	1.78	43.7	56.3
indomethacin	0.60	1.42	41.1	58.9
intact vesicles			17.3[a]	82.7[a]

[a] Percent total cpm in this fraction.

Table VII: Distribution of ^{125}I Label among Different Lipid Fractions in SR Vesicle Extracts[a]

agent	concn (mM)	% recovery of lipid cpm	lipid fraction			
			A1	A2	A3	B
intact vesicles		77.8	0.02	0.19	0.63	0.12
buffer control		87.7	0.06	0.32	0.15	0.47
chlorpromazine	0.06	83.7	0.09	0.35	0.09	0.47
metho-chlorpromazine	0.30	73.9	0.06	0.69	0.04	0.21
tetracaine	0.50	94.8	0.04	0.41	0.26	0.29
decanol	0.01	64.1	0.13	0.27	0.17	0.43
dinitrophenol	10	83.4	0.04	0.12	0.57	0.27
indomethacin	0.60	96.5	0.13	0.23	0.34	0.30

[a] Expressed as the ratio of cpm in the fraction to cpm in total lipid extracted.

The protein composition of intact SR membranes is dominated by the Ca^{2+}-ATPase (MacLennan, 1970), and this was the major protein observed upon carrying out SDS–PAGE on the amphipath-induced extracts (not shown).

The lipids in the SR extracts were separated into four fractions as with the RBC membranes, and the distribution of label among these fractions is shown in Table VII. In whole SR membranes (first row), fraction A3 contains the largest fraction of label, with lesser amounts in A2 and B and very little in A1. These proportions are markedly altered in all of the extracts, including the buffer control. The proportion of label in fraction B was elevated in most of the amphipath-induced extracts as was the case with the RBC membranes; however, the label in this SR lipid fraction was not of ganglioside origin, since the counts were not retained after dialysis of the fraction as with the RBC. The lipid components in these several fractions were not further characterized. The results indicate that the extracts of SR membranes induced by different amphipaths show differences in the label distribution among the four fractions.

Discussion

Previous studies from this laboratory (Conrad & Singer, 1979, 1981) on the anomalous binding characteristics of small-molecule amphipaths to membranes led us to suspect that amphipaths, at lower than lytic concentrations, might

seriously perturb membrane structures and even extract components from membranes. The present study was undertaken to examine these possibilities. Six small-molecule amphipaths, three (chlorpromazine, methochlorpromazine, and tetracaine) positively charged at pH 7.4, two (dinitrophenol and indomethacin) negatively charged, and one (decanol) electrically neutral, were chosen for study, along with two quite different membrane preparations, human RBC ghosts and rabbit skeletal muscle SR membranes. Each amphipath was used at a concentration at which it maximally stabilizes intact RBC against hypotonic hemolysis, under which conditions it might be least expected to extract membrane components. Nevertheless, our experiments indicate that at those concentrations, each amphipath induces membrane structural changes and promotes the extraction of membrane components.

This study was carried out by subjecting the amphipath-equilibrated membranes to hygroscopic desorption (Conrad & Singer, 1979, 1981). In this procedure, the intact membranes are essentially completely retained on a hydrophobic filter while almost all of the aqueous phase is removed from the filtrate. In most of the experiments, intact RBC or SR vesicles were prelabeled by the ^{125}I-lactoperoxidase technique; the labeled ghosts were prepared from the RBC, and these ghosts and the labeled SR were subjected to amphipath treatment and hygroscopic desorption; the filtrates were collected and analyzed for their labeled components. With all amphipaths, the filtrates contained significantly more counts than for buffer controls, from both RBC and SR membranes.

When the composition of the filtrates of labeled RBC membranes was examined, both protein and lipid components were found to be increased over buffer controls, but the overall lipid:protein ratios were considerably larger in the filtrates than in the original membranes. The amphipath-induced filtrates had a remarkably different protein distribution compared to the intact RBC membranes. The major peripheral proteins (spectrin and actin included) were absent, and a subset of minor proteins of the membrane predominated (Figure 3). These proteins may correspond to a minor set of integral glycoproteins of the RBC membrane migrating between bands 4.1 and 5 which are especially highly labeled by treatment with galactose oxidase followed by [^{3}H]borohydride (Steck & Dawson, 1974), indicating that their oligosaccharide chains terminate in galactose or N-acetylgalactosamine. The functional characteristics of these integral proteins are not known. Among the lipids in the filtrates, the gangliosides were particularly enriched (Table III). Superimposed on these similarities, however, different amphipaths induced the extraction of distinctive patterns of protein and lipid components. The extraction process could therefore not be ascribed simply to prior membrane damage and the subsequent excision of different amounts of the same damaged regions by the action of the different amphipaths.

The amphipaths induced extractions from the two membranes that showed striking similarities, considering the very different molecular characteristics of SR and RBC membranes. In both cases, for example, while both protein and lipid extraction was enhanced, the lipid:protein ratio in the filtrates was markedly larger than that for the original membranes. The very important conclusion is that the effects of the amphipaths in promoting the extraction of membrane components are not confined to RBC membranes but are also observed with SR membranes of quite different composition.

In the interpretation of the structural significance of these results, account must first be taken of two facts. One is that no significant release of ^{125}I-labeled components into the supernatant was produced upon incubation of the RBC membranes with amphipaths and then sedimentation of the membranes (Table I, column 4). This suggests that the extractions observed in the main body of our experiments were induced during the filtration step in hygroscopic desorption. Second, the extractions with buffer controls were not negligible. In the absence of any amphipaths, about 5% and 2% of the total counts of the labeled RBC and labeled SR, respectively, were found in the filtrates. These buffer-control filtrates themselves exhibited enhanced lipid:protein ratios and specialized protein and lipid compositions as compared to the original membranes. About 23% of the counts in the buffer-control filtrates from RBC membranes could be recovered as small vesicular fragments (Figure 6B); the nature of the other 77% is not known.[2] Similar amounts of label were found in buffer-control filtrates of intact RBC or of RBC membranes prepared by the method of Ting-Beall et al. (1981), so it does not appear that the buffer-control extractions were due to damage in the course of RBC membrane preparation. The nature of the extraction results is also much more consistent with the idea that we are dealing with a small alteration affecting essentially all of the membranes in a preparation, rather than a large alteration affecting only a small fraction of the membranes.

There are two possible nontrivial explanations of the buffer-control results: either (a) domains of the size of the vesicular fragments in Figure 6B and containing the special protein and lipid compositions observed in the control filtrates *preexist* in the RBC membrane and are susceptible to excision upon hygroscopic desorption filtration or (b) the composition is originally uniform in the plane of the membrane on a scale corresponding to the areas of the vesicular fragments in Figure 6B, but nonuniformity on this scale is rapidly *induced* by the mechanical stress of filtration, and this results in the excision of membrane fragments of special composition.

The mechanism of action of the amphipaths on the membrane would be different depending on which of these two possibilities was correct. Either (a) the amphipaths restructure the preexisting domains in the membrane, changing the lipid and protein composition and the amount of membrane subsequently excised upon filtration, or (b) the nonuniformity that is induced only in response to the stress of filtration is changed in the presence of the amphipath, and the amounts and the composition of membrane fragments (and perhaps micelles[2]) that are excised are accordingly altered.

We have no experimental way of discriminating between these two possibilities at the present time. RBC and SR membranes do not exhibit in the electron microscope morphologically distinct domains as do the plasma membranes of polarized cells in tissues. There is therefore no direct evidence for preexisting domains in these membranes. Using fatty acids and fluorescent probes, Klausner et al. (1980) have presented indirect evidence that lymphocytes contain distinctive lipid domains in their membranes, but these experiments encounter similar problems to ours: do these domains preexist in the

[2] The remaining material may be in the form of very small vesicles not retained on the 0.03-μm pore-size filters, or perhaps in micellar form, in the buffer-control extracts and in the amphipath-induced extracts. Evidence for the formation of mixed micelles between amphipaths and membrane components, upon binding of amphipaths to intact membranes, has been provided (Conrad & Singer, 1981). The marked enrichment of gangliosides in the buffer-control filtrates and particularly in the amphipath-induced extracts is of particular interest in this connection because gangliosides are relatively unstable in bilayer structures (Barenholz et al., 1980). Their enrichment in the extracts may therefore reflect in part the entry of gangliosides into mixed micelles containing other specific membrane components and amphipathic molecules.

membrane, or are they induced by the probes used to detect them?

Despite the uncertainty of whether domains preexist or are induced in RBC and SR membranes, it is important to appreciate that the conclusion derived from the amphipath-induced extraction experiments is the same: the amphipaths must alter the structure and composition of these domains and do so in a manner that is distinctive for different amphipaths. Whether they do this solely by intercalating in the membrane, or by binding to the membrane more superficially in some other mode (Conrad & Singer, 1981; Bondy & Remien, 1981), is not clear. It should also be appreciated that for most of the extraction experiments each of the amphipaths was employed at the concentration at which it maximally *stabilizes* intact RBC against hypotonic hemolysis, and the total counts extracted were correspondingly small. At larger concentrations, however, larger extraction effects are observed (Table II), and at still larger concentrations, lysis is produced. This is not surprising, since at the larger concentrations many of these amphipaths form detergent-like micelles (Scholtan, 1955; Fernandez, 1980). These detergent-like effects on membranes of small-molecule amphipaths at high concentrations have been reported previously [cf. Leterrier et al. (1974), Boivin & Galand (1975); and Fernandez (1981)], including the case of the bile salt glycocholate (Billington & Coleman, 1978).

The conclusion that small-molecule amphipaths, at concentrations that are well below lytic, can induce profound rearrangements of protein and lipid components in the plane of the membrane has important implications for the various physiological and biochemical effects exerted by different amphipaths on membranes. These effects include anesthesia [or the blocking of conductance in nerve cell membranes (e.g., by tetracaine)], uncoupling of oxidative phosphorylation by mitochondrial membranes (for example, by dinitrophenol or indomethacin), changes in hormone-mediated adenylate cyclase activity (Salesse & Garnier, 1979; Salesse et al., 1982), and changes in the shape of intact RBC (Deuticke, 1968). In the past, these effects have variously been ascribed to the intercalation of amphipaths into the lipid bilayer [cf. Seeman (1972)], to differential intercalation of specific amphipaths into the two halves of the asymmetric lipid bilayer (Sheetz & Singer, 1974), to binding of the amphipaths to the lipid–protein interfaces in the membrane interior (Metcalfe et al., 1974), or to binding of the amphipaths directly to membrane proteins. To this list of possible mechanisms, which all assume that no significant rearrangement of membrane components is induced by amphipaths, must be added the possibility that gross redistributions of the lipids and proteins in the plane of the membrane may contribute to the effects of amphipaths on various membrane functions.

Acknowledgments

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Registry No. 2,4-Dinitrophenol, 51-28-5; methochlorpromazine, 19077-31-7; tetracaine, 94-24-6; 1-decanol, 112-30-1; chlorpromazine, 50-53-3; indomethacin, 53-86-1.

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