Effects of urea and trimethylamine N-oxide on fluidity of liposomes and membranes of an elasmobranch

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Barton, Kimby N., Mary M. Buhr, and James S. Ballantyne. Effects of urea and trimethylamine N-oxide on fluidity of liposomes and membranes of an elasmobranch. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R397–R406, 1999.—The effects on membrane fluidity of two solutes of biological importance in elasmobranch fishes, urea and trimethylamine oxide (TMAO), were determined using elasmobranch red blood cell plasma membranes and artificial liposomes. Fluorescence polarizations of three probes with differing sites of insertion (1,6-diphenylhexatriene, diprimeric acid, and trans-parinaric acid) were used to study the effects of physiological levels of urea (400 mM) and TMAO (200 mM) separately and together in a 2:1 urea:TMAO ratio (400 mM:200 mM). In the elasmobranch erythrocyte membrane, there was a trend toward an increase in the order of the gel-phase domains when treated with urea, although this was not statistically significant. This effect was counteracted by the presence of TMAO. To determine if the organic solutes were acting directly on the membrane lipids or on the integral proteins, phase-transition profiles of protein-free dipalmitoyl phosphatidylcholine liposomes were determined. These profiles showed that urea again increased the order of the gel-phase domains of the bilayer; however, this effect was not counteracted by the presence of TMAO. We suggest that the increased order in the gel-phase domains may be an indirect effect of a decrease in the order of the fluid-phase domains. This increase in fluidity may be due either to a disruptive effect of urea on the hydrophobic core of the membrane or to indirect effects mediated by changes in the integral membrane proteins. This study is the first to demonstrate that urea and TMAO may act as counteracting solutes in the elasmobranch erythrocyte membrane and that the counteraction appears to be at the level of the integral proteins rather than the membrane lipids.

Effects of urea and trimethylamine N-oxide on fluidity of liposomes and membranes of an elasmobranch

Previous studies have demonstrated an effect of some organic solutes on membrane structure (11, 12, 30). Several species that survive dehydration and/or freezing produce and accumulate cytoplasmic organic solutes, especially sugars (11, 30). Dimethyl sulfoxide, glycerol, sucrose, trehalose, and urea have all been shown to minimize freezing and dehydration damage to cells and cellular components (12).

There is some evidence from model membrane systems that urea may disrupt hydrophobic interactions (27, 43, 51). This effect of urea on hydrophobic bonding, if exerted in vivo, could result in membranes that are too fluid and unstable. Membrane composition is altered in response to a variety of environmental factors to maintain important membrane properties such as fluidity (see Ref. 18 for review). This modification in response to physical challenge is termed “homeoviscous adaptation” (35). The homeoviscous response can include alterations in relative proportions of phospholipid head group class, changes in degree of unsaturation and chain length of the phospholipid fatty acyl chains, and changes in cholesterol levels (7, 18, 35). Although several studies have focused on the homeoviscous response to such factors as temperature, pressure, ionic strength, and diet (see Ref. 18 for review), no studies have investigated the effects of physiological concentrations of a chaotropic agent such as urea. If the effects of urea on membranes are not counteracted by TMAO, one would predict that animals with high levels of urea in their tissues would have membranes adapted to its presence. This is supported by a study of the basolateral plasma membrane of the liver of the little skate (Raja erinacea) that showed that the fluidity of these membranes, when measured at body temperature and in the absence of urea, was very low compared with published values for other organisms (9, 38). This low fluidity in the skate membrane may result from the fluidity measurements being made in a solution that did not contain either urea or TMAO. Another study comparing the liver mitochondrial membranes of a urea-retaining elasmobranch to those of two other, non-urea-retaining fishes also supports the contention that urea affects membrane structure. The fatty acyl chains of the mitochondrial membrane phospholipids in the skate contained much higher levels of saturated fatty acids and lower polyunsaturated fatty acid levels, and the chain lengths were shorter than in the other non-urea-retaining fishes (15). This highly saturated membrane composition is unusual and is consistent with that needed to have highly ordered membranes to maintain membrane integrity in the presence of physiological amounts of urea, possibly indicating membrane adaptation to urea.

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The purpose of the present study was to ascertain whether urea has a disruptive effect on membrane molecular order in an elasmobranch membrane and to determine if this effect is counteracted by the presence of a counteracting solute such as TMAO. In addition, we wished to determine if the effects of the solute in the biological membranes resulted from a disruption of the membrane protein structure or from an effect of the solutes directly on the membrane lipid. To accomplish this, plasma membranes were isolated from the red blood cells of an elasmobranch, R. erinacea, and the fluidity of the membranes was assessed in the presence of urea and TMAO separately or together in a 2:1 urea:TMAO ratio. To assess whether the solutes were having a direct effect on the membrane lipids, the fluidity of artificial membranes composed entirely of phospholipid [dipalmitoyl phosphatidylcholine (DPPC)] liposomes was measured. All fluidity measurements were made using fluorescence polarization of three fluorescent probes: 1,6-diphenylhexatriene (DPH), cis-parinaric acid (cPNA), and trans-parinaric acid (tPNA). These probes were used because they insert into different areas in the membrane. DPH and cPNA are associated with lipid in both the gel and fluid phases. tPNA strongly partitions into the gel phase of the membrane. Thus using cPNA and tPNA in tandem allows for better evaluation of the heterogeneity in the organization of lipid and lipid-protein systems (see Ref. 36 for review).

MATERIALS AND METHODS

Elasmobranch Membranes

Experimental animals. R. erinacea were obtained by otter trawling in Passamaquoddy Bay, New Brunswick, in late August and transported to Guelph. Animals were maintained at the Marine Biology Laboratory at the University of Guelph in salt water (1,000 mOsm) at 10°C (±1.0°C) for 1–12 mo before use. Fish were fed a diet of krill (Murex Aqua Foods, Langley, British Columbia, Canada) and chopped herring to satiety daily.

Erythrocyte membrane preparation. Blood samples were taken from four male and six female skates (approximate weights 350–800 g, n = 10) by cardiac puncture using a heparinized syringe (500 U heparin/ml 0.9% NaCl). Membranes were obtained by a combination of chemical and physical processes based on the method of Jackson (19). Blood was diluted in 10 vol 0.1% NaCl (pH 7.6) and centrifuged for 5 min at 1,000 g at 4°C in a Sorvall RC5C centrifuge (DuPont). The supernatant was decanted, and the pellet was resuspended in 10 vol of NaCl solution and centrifuged again. At the end of this spin, the supernatant was decanted and a small sample of pellet was frozen at −80°C for marker enzyme assays. The remaining pellet was resuspended and centrifuged in a series of dilute phosphate buffers (5, 2.5, and 1.25 mM NaH₂PO₄, pH 7.6). All suspensions were centrifuged for 5 min at 1,000 g. Samples were rinsed and resuspended in each buffer two times. Between successive dilutions, the pellets were squeezed through a heparinized syringe with a 25-gauge needle. Then pellets were resuspended in 5 vol suspension solution [0.1 mM MgCl₂, 1 mM sodium tetrathionate, and 0.1 mM phenyl methane sulfonate (PMSF)] and vortexed vigorously for 1 min. Five volumes of a digestion buffer [0.1 mM MgCl₂, 10 mM NaH₂PO₄, 1 mM sodium tetrathionate, and 0.1 mM PMSF, pH 7.6; after Jackson (19)] were then added to the samples, followed by incubation for 15 min at room temperature (21°C). Finally, the samples were centrifuged at 12,000 g and 4°C for 20 min. The supernatant was decanted, and the final membrane preparation was stored at −80°C for further analysis.

Approximately 2 h before fluorescence measurements, the membrane preparations were thawed at room temperature, suspended in 2 vol of a saline solution (10 mM Tris, 150 mM NaCl, pH 7.4), and homogenized using three passes of a Potter-Elvehjem homogenizer equipped with a Teflon pestle. Each replicate of the elasmobranch membrane preparation consisted of membranes pooled from two animals. Protein in each sample was determined by the method of Bradford (3), using BSA as a standard.

Artificial Membranes

Liposome preparation. For liposome preparation, stock solutions of DPPC in 2:1 chloroform:methanol (1 µmol lipid in 100 µl chloroform:methanol) were placed in Kimax tubes and the solvent was evaporated to dryness under a steady stream of N₂ gas. The lipids were then hydrated in one of four different buffers: a control buffer (10 mM Tris, 150 mM NaCl, pH 7.4), a urea buffer (400 mM urea), a buffer containing TMAO (200 mM TMAO), or a buffer containing both urea and TMAO in a 2:1 ratio (400 mM urea:200 mM TMAO). All treatment buffers were prepared in the control medium. The working concentrations for the liposomes were 0.3 µmol DPPC in 1.5 ml treatment buffer. After buffer was added to the liposomes, they were vortexed vigorously for 30 s and then bubbled with N₂ gas for 10 min. Liposomes were heated above their transition temperature to 65°C in a Fisher Versa Bath (Fisher Scientific, Mississauga, Ontario, Canada) and used for fluorescence measurements.

Fluorescence measurements. Fluorescence intensity was measured using an AlphaScan spectrofluorometer (Photon Technology International, South Brunswick, NJ) equipped with excitation and dual-emission monochromators and a four-position sample turret equipped with a water jacket. Temperature was controlled using a BioCool II portable controlled-rate freezer (model BC-11-4; FTS Systems, Stone City, NY) that was programmed to achieve the indicated temperature in the cuvette. A Teflon-coated magnetic stirring bar was placed in each cuvette to continuously mix the contents.

Before collecting the polarization data, we determined a G-factor value (37) for each cuvette at the appropriate wavelengths. Samples were excited with vertically polarized light at 358 nm for DPH and 322 nm for tPNA and cPNA. Vertically and horizontally polarized emitted light was read at 428 nm for DPH and 410 nm for tPNA and cPNA. Slit widths were 5 nm for the excitation and 8 nm for the emissions.

The membrane probes cPNA, tPNA, and DPH were prepared as previously described (4).

Red blood cell membranes were analyzed in 1-ml quartz cuvettes using 50 µg/ml plasma membrane protein and 1 µM tPNA, 1 µM cPNA, or 1 µM DPH. Solutions of 4 M urea, 2 M TMAO, and 4 M 2 M urea:TMAO were added to cuvettes to give a final concentration of 400 mM urea, 200 mM TMAO, and 400 mM:200 mM urea:TMAO. Control cuvettes contained samples mixed with control buffer (10 mM Tris, 150 mM NaCl, pH 7.4). Blank samples that contained membrane and buffer with no probe were run for every replicate. Cuvettes were shaken vigorously to ensure complete mixing of solution and membrane and incubated at room temperature for 10 min. Samples were then incubated for another 10 min at the assay temperature (10 ± 1°C). The assay tempera-
ture chosen was the temperature at which the animals had been maintained. Readings were taken every 2 min for total of 40 min (giving 20 measurements/sample) at a data collection rate of 20 points/s.

All fluorescence measurements on liposomes were performed in 3-ml quartz cuvettes. One of three different fluorescent probes was then added to the liposome preparations. Samples were then incubated with the probe for 10 min at room temperature, followed by another 15 min at 50°C to ensure that they had reached the appropriate temperature before the beginning of the experiment. Fluorescence polarization readings were taken over a temperature range from 20 to 50°C that contained the phase-transition zone for DPPC liposomes. Temperatures were cooled from 50 to 20°C at a rate of 1°C/min (referred to as "cooling" stage) and then warmed back up from 20 to 50°C at the same rate (referred to as "warming" stage). This allowed for the determination of any thermal hysteresis associated with the effects of the solutes. For all fluorescence readings, there was always a blank containing buffer and liposome but no probe subtracted from each sample.

Fluorescence emission intensities were measured using the T format for the liposomes and L format for the membrane studies; the latter was necessitated by a mechanical problem with one emission monochromator. Fluorescence intensities were transformed into polarization values using the Perrin equation (34). All agents used during fluorescence measurements were tested for quenching of fluorescence of DPH, tPNA, and cPNA and were found to be without effect (data not shown).

Chemicals

cPNA and tPNA were purchased from Molecular Probes (Eugene, OR). DPH, DPPC, and chemicals for marker enzyme assays were purchased from Sigma (St. Louis, MO). Chemicals for treatment solutions were purchased from Fisher Chemical (Mississauga, Ontario, Canada).

Statistical Analysis

Polarization values of native membrane samples were shown to remain constant over time, and therefore mean values of 20 readings were calculated and used for subsequent analyses. Polarization values of the native membranes were analyzed using one-way ANOVA (Proc ANOVA; Statistical Analysis Software (SAS); P < 0.05) (39). Assumptions of normality were tested using the Shapiro-Wilk statistic available in Proc univariate (SAS), and each probe was then tested separately. Power analyses were performed to determine the power of the test for each separate probe (52).

Data from the phase-transition experiments with the liposomes were analyzed using nonlinear regression. To reduce variation among replicates within an experiment, initial polarization values were subtracted from subsequent values within each replicate, and these adjusted polarization values were pooled within each experiment for analysis (5). How-ever, absolute polarization values for parameters a and b were included for purposes of comparison with literature values (Table 3). The form of this relationship is displayed in Fig. 1. Data from the cooling stage were fitted to a Lorentzian cumulative distribution (adjusted $r^2 = 0.9979$, F$_{crit} = 2.649.8771$) using table curve 2D software (Jandel Scientific). The equation for the curve is described by

\[ y = a + \frac{1}{2} \left( \frac{\arctan \frac{x - c}{d} + \frac{\pi}{2}}{\pi} \right) \]

(1)

where $y$ is a measurement of the dependent variable, $a$ is the parameter used to estimate the polarization value in the fluid phase (lower range of graph), $b$ is the $y$-intercept that estimates polarization value in gel-phase domain; $c$ is the phase-transition temperature; $d$ is the average width around inflection point.

Fig. 1. Statistical parameters described in MATERIALS AND METHODS. a, estimation of polarization value in fluid-phase domain; b, $y$-intercept that estimates polarization value in gel-phase domain; c, phase-transition temperature; d, average width around inflection point.

where $y$ is a measurement of the dependent variable, $a$ is the parameter used to estimate the polarization value in the fluid phase (lower range of graph), $b$ is the $y$-intercept, which estimates the polarization value in the gel phase (higher range of the graph), $c$ is the inflection or break point, and $d$ is the average width around the inflection point. The graph of the phase transitions for the warming stage had a different shape from the cooling data; thus the $a$ parameter was dropped for testing these data. This gave Eq. 2

\[ y = b \left[ \frac{\arctan \frac{x - c}{d} + \frac{\pi}{2}}{\pi} \right] \]

(2)

where the $b$, $c$, and $d$ parameters are the same as in Eq. 1. Estimations of the parameters for Eqs. 1 and 2 were made using the nonlinear least squares estimates given by Proc NLIN (SAS). The iterative method used was the multivariate secant or false position method. Parameters were estimated, and ANOVA was performed using a general linear model (Proc GLM) procedure to determine if there were differences between treatments and probes for the various parameters. The assumption of normality was tested using the Shapiro-Wilk statistic available in Proc univariate (SAS). Because of problems normalizing data and failure of nonparametric tests, each probe was analyzed separately. Data for the warming and cooling stages of the phase transitions were analyzed separately, and then comparisons were made between the $b$, $c$, and $d$ parameters between the two groups. Log, square root, or inverse transformations were performed on the parameters as required to normalize these data. The same analysis was performed on the warming data, and the parameters were also transformed as required. Differences between treatment means were determined using Tukey’s multiple comparison method (SAS; $P < 0.05$) (39).

The $d$ parameter is included for the sake of completeness but is not discussed further because it did not appear to have any physiological significance.

An ANOVA was performed on parameters from the cooling and warming transitions (Proc GLM, SAS) to determine if the
different treatments altered the transition temperature of the gel to fluid-phase transition (P < 0.05). Differences between treatment means were determined using Tukey’s multiple comparison method (SAS; P < 0.05) (39).

RESULTS

Elasmobranch Membranes

Marker enzyme and phospholipid compositional studies of membrane purity (data not shown) indicate that although the preparation consists mostly of erythrocyte plasma membrane, there was some contamination with mitochondria and other organelles. The mitochondrial membrane was enriched 13.7%, the endoplasmic reticulum 6.0%, and the plasma membrane 2.6% based on marker enzyme data, and there were no significant differences between replicates (P < 0.05). For the purposes of our study, this contamination would not affect any of the conclusions.

Figure 2 illustrates the effects of urea and TMAO on membrane fluidity (as measured by polarization value of 1,6-diphenylhexatriene (DPH), cis-parinaric acid (cPNA), and trans-parinaric acid (tPNA)] of elasmobranch erythrocyte membranes. Values are means ± SE (n = 5).

Liposome Studies

The effects of urea (400 mM), TMAO (200 mM), and urea and TMAO together (400 mM:200 mM) on the polarization values of the probe DPH in the DPPC liposomes are presented in Fig. 3, A and B, and the individual parameter values are summarized in Tables 1 and 2. The polarization values of both the gel and fluid phases were not altered in the presence of either urea or TMAO alone or in combination (P < 0.05). However, the transition temperature of the fluid to gel-phase transition in the cooling curve (i.e., c in Fig. 1) shows that urea significantly increased the transition temperature (P < 0.05) relative to the other treatments. This shift in the transition temperature did not occur in the warming curve.

Figure 4 illustrates the effects of the treatments on the polarization value of the probe tPNA. In the cooling curve (Fig. 4A), there were no significant differences in parameters a, b, or c. Again, the individual parameter values and significant differences between treatment means are summarized in Tables 1 and 2. In the warming curve, urea, separately and in combination with TMAO, decreased (P < 0.05) the fluidity of the liposomes in the gel phase (P < 0.05) relative to the control. This stiffening effect was not counteracted by the presence of TMAO, and TMAO alone had no effect on the fluidity of the liposomes. Neither of the organic solutes had any effect on the transition temperature in either the cooling or the warming curves.

Figure 5 illustrates the effects of the three treatments on the polarization values of cPNA. The organic solutes had no effect on the fluidity of the gel or fluid phases or on the transition temperature of the liposomes in either the cooling (Fig. 5A) or warming (Fig. 5B) curves. Individual parameter means and significant differences are summarized in Tables 1 and 2.

Thermal Hysteresis

The transition temperatures and the fluidity of the lipids in the gel phase were compared between the warming and cooling curves for each combination of treatment and probe. For all the probes and treatments studied, it was found that the transition temperatures were significantly higher in the warming curves than in the cooling curves (P < 0.001). There were no differences in the polarization values in the gel phase between the warming and cooling curves.

DISCUSSION

It has been well documented that the organic solute urea has a disruptive effect on both protein and lipid structure and that this effect on protein is counteracted by the presence of methylamine solutes, such as TMAO (27, 33, 43, 49, 50). This study is the first to demonstrate an effect of physiological levels of urea on the fluidity of both artificial and native membrane structures and a counteracting effect of TMAO on this effect.
in protein-containing elasmobranch membranes but not in an exclusively lipid system.

Assessment of the effects of urea and TMAO on the membrane fluidity using three different fluorescent probes allowed for a better description of the activities of urea and TMAO in a complex, heterogeneous system. The lack of a probe that inserts exclusively into the fluid phase is problematic, although effects on this phase can be inferred by examining the effects of all three probes.

Elasmobranch Membrane Studies

Urea is known to be highly permeant, crossing both artificial and native membranes (8, 21, 22, 25, 33). Previous studies of membrane fluidity and composition have suggested that elasmobranchs have unusually ordered membranes in the absence of urea and TMAO (14, 38). Thus it was expected that the presence of these organic solutes would increase the fluidity of the elasmobranch membrane. Fluorescence polarization values of the probes DPH and cPNA showed no differences between the fluidity of membranes treated with these solutes. This may result from the partitioning behavior of the probes within the bilayer. Both DPH and cPNA partition equally into gel and fluid-phase lipids (34, 36), and, therefore, it is difficult to make conclusive measurements on a complex system using these probes. In a situation in which a membrane is composed of two areas, one of which is highly viscous and the other highly fluid, the polarization value obtained experimentally would be a weighted average of these two values (23). Thus a region of extremely high fluidity could be masked by a region of extremely low fluidity.

In contrast to DPH and cPNA, tPNA preferentially partitions into more ordered and compact gel-phase lipid domains (36). The increase in the polarization of tPNA in the presence of urea alone suggests that there is an increase either in the order of the densely packed gel phase lipids or an increase in the number of gel-phase microdomains present in the bilayer. The fact that this increase in the order or number of gel-phase domains was not detected by DPH or cPNA suggests that it may have been masked by a decrease in the order of a more fluid region. This is supported by the results of a similar study on the effects of several chaotropic agents on phase transitions of dipalmitoyl lecithin liposome bilayers that found that some of these compounds caused a change in the packing within the gel-phase lipids but did not necessarily change the size or number of the gel-phase domains (20).

A comparison of calcium effects on lipids concluded that calcium was having an ordering effect on membrane structure based on an increase in the polarization values of both tPNA and cPNA in the gel phase of various liposomes and native membranes (37). Thus urea may be having a direct effect on regions of more compact lipid or may be acting indirectly to alter the fluidity of lipids surrounding these regions.

It is well established that the structural and compositional properties of the bilayer can determine the catalytic properties of membrane transport proteins (6,
Solutes Effects on Membranes

Table 1. Individual parameter values for cooling phase of DPPC liposomes using probes DPH, tPNA, and cPNA

<table>
<thead>
<tr>
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<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
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<tbody>
<tr>
<td>DPH</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>-0.004 ± 0.001</td>
<td>0.27 ± 0.00</td>
<td>30.64 ± 0.17†</td>
<td>-0.95 ± 0.07</td>
</tr>
<tr>
<td>Urea</td>
<td>-0.003 ± 0.001</td>
<td>0.27 ± 0.00</td>
<td>32.64 ± 0.17†</td>
<td>-0.95 ± 0.07</td>
</tr>
<tr>
<td>TMAO</td>
<td>-0.005 ± 0.001</td>
<td>0.27 ± 0.00</td>
<td>30.88 ± 0.19†</td>
<td>-0.91 ± 0.08</td>
</tr>
<tr>
<td>Urea + TMAO</td>
<td>-0.002 ± 0.001</td>
<td>0.27 ± 0.00</td>
<td>30.58 ± 0.18†</td>
<td>-0.87 ± 0.08</td>
</tr>
<tr>
<td>tPNA</td>
<td></td>
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<tr>
<td>Control</td>
<td>-0.003 ± 0.001</td>
<td>0.21 ± 0.02</td>
<td>31.30 ± 0.28</td>
<td>-0.47 ± 0.06†</td>
</tr>
<tr>
<td>Urea</td>
<td>0.002 ± 0.004</td>
<td>0.27 ± 0.04</td>
<td>31.33 ± 0.31</td>
<td>-0.76 ± 0.06*</td>
</tr>
<tr>
<td>TMAO</td>
<td>-0.005 ± 0.001</td>
<td>0.22 ± 0.02</td>
<td>31.86 ± 0.27</td>
<td>-0.75 ± 0.05*</td>
</tr>
<tr>
<td>Urea + TMAO</td>
<td>-0.006 ± 0.004</td>
<td>0.25 ± 0.02</td>
<td>31.70 ± 0.28</td>
<td>-0.63 ± 0.06</td>
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<tr>
<td>cPNA</td>
<td></td>
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<tr>
<td>Control</td>
<td>0.004 ± 0.002</td>
<td>0.25 ± 0.01</td>
<td>30.67 ± 0.15</td>
<td>-0.67 ± 0.05</td>
</tr>
<tr>
<td>Urea</td>
<td>0.000 ± 0.002</td>
<td>0.25 ± 0.01</td>
<td>30.63 ± 0.16</td>
<td>-0.59 ± 0.05</td>
</tr>
<tr>
<td>TMAO</td>
<td>-0.001 ± 0.002</td>
<td>0.24 ± 0.01</td>
<td>30.77 ± 0.15</td>
<td>-0.66 ± 0.05</td>
</tr>
<tr>
<td>Urea + TMAO</td>
<td>0.000 ± 0.002</td>
<td>0.25 ± 0.01</td>
<td>30.72 ± 0.16</td>
<td>-0.69 ± 0.05</td>
</tr>
</tbody>
</table>

Values are pretransformation means ± SE. Number of determinations are as described in Figs. 3–5. DPH, 1,6-diphenylhexatriene; TMAO, trimethylamine oxide; tPNA, trans-parinaric acid; cPNA, cis-parinaric acid; DPPC, dipalmitoyl phosphatidylcholine. a, Estimation of polarization value in fluid-phase domain; b, y-intercept that estimates polarization value in gel-phase domain; c, phase-transition temperature; d, average width around inflection point. *Significantly different from control; †significantly different from Urea; ‡significantly different from TMAO; P < 0.05.

16, 17, 32). Conversely, structural changes in membrane proteins may alter the properties of the surrounding lipid. It is possible that the fluidity changes caused by urea result from an effect of urea on proteins embedded in the bilayer.

The ordering effect of urea on the gel-phase lipids of the elasmobranch membrane was counteracted by the presence of TMAO. This counteraction may be a result of the protection of protein by TMAO in the native membrane or a direct effect of TMAO on the membrane lipids.

The polarization values for DPH in the elasmobranch membrane are similar to those found for the brush-border membranes of 20°C-acclimated rainbow trout (see Ref. 18 for review) and in isolated trout hepatocytes (48), indicating fluidities similar to those of teleost fishes and demonstrating that the fluidity values determined by our system are comparable to published values.

Liposome Studies

The effect of urea and TMAO on the erythrocyte membrane may result from an indirect effect of these solutes on the membrane proteins or a direct effect on the membrane lipids. To help clarify the possible site of action of these solutes, we examined the fluidity of a protein-free liposome system composed of the major phospholipid of the elasmobranch membrane (~32% phosphatidylcholine). Similar to the results from the elasmobranch membranes, fluorescence polarization of the probes DPH and cPNA in the liposomes showed no differences between the treatments above or below the transition temperature. Both of these probes partition equally between straight-chain, compact lipids and regions of higher fluidity, and therefore an increase in the order of one phase may not be detected if there is a decrease in the order of the other phase. Urea significantly decreased the fluidity of the gel-phase lipids; however, unlike the effect in the elasmobranch membrane, this decrease in fluidity was not counteracted by the presence of TMAO.

The increase in the order of the gel-phase lipids in the presence of urea and urea in combination with TMAO may be caused by a combination of the effect of urea on the acyl chains in the hydrophobic core and its effect on the phospholipid head groups. The solubility diffusion model of membrane theory predicts that lower membrane fluidity reduces the ability of permeant molecules to diffuse through the lipid bilayer (14, 22, 40, 41). It is possible that in the gel phase the liposomes may have been too rigid to allow permeation of the urea alone or in combination with TMAO.
Phospholipids are hydrated with, in the case of phosphatidylcholines, 10–12 water molecules hydrogen bonded around the polar head groups (reviewed in Ref. 11). When the hydration layer is removed, the lateral spacing of head groups decreases, leading to increased van der Waals interactions between hydrocarbon chains (10). Urea may have a head group effect on membrane phospholipids, and this effect may be different for gel- and fluid-phase domains.

The increase in the order of the gel-phase lipids in the presence of urea was not counteracted by the presence of TMAO in the tPNA-monitored domain of the DPPC liposomes. The inability of TMAO to counteract the effect of urea could be caused by its inability to penetrate past the positively charged DPPC head groups in both the gel- and fluid-phase domains. In the native membrane, several different phospholipid species of differing charges are present that may have enabled TMAO to diffuse into the bilayer core. Alternately, it is plausible that TMAO does not counteract the effect of urea on artificial membranes because it does not have the same counteracting effect on lipids as it does on proteins.

Further insight into the effects of urea on model membranes is evident from the studies using DPH. Urea increased the transition temperature of the gel- to liquid crystal-phase transition in the cooling profile of DPH. In contrast, tPNA and cPNA showed no difference in the phase transition temperature between treatments in both the warming and cooling profiles. DPH differs from cPNA in that it partitions into the hydrophobic domains of the bilayer and is distributed between at least two sites, one parallel to the hydrocarbon chains and the other between the two bilayers (9, 33). Thus the polarization values obtained from the DPH are a weighted average of the different sites of insertion. The increased melting temperature observed with DPH may result from an effect of urea on the probe that is inserted between the two bilayers.

A comparison of the unadjusted polarization values (see Table 3) and thermal transition temperatures obtained in this study are similar to those previously published (1, 2, 46).

**Thermal Hysteresis**

Thermal hysteresis refers to the phenomenon whereby the phase-transition temperature of lipids during cooling differs from that measured during heating (45). A study on the effects of alcohols on thermodynamic reversibility of phase transitions found that for phosphatidylcholine these transitions were not thermodynamically reversible at high alcohol concentrations (31).

Heating curves reveal the existence of marked endothermic transitions that have been shown, using a range of physical techniques, to be associated with the melting of lipid chains (7). Phase-transition data have indicated that cooling and heating curves may differ and that previous thermal history may affect phase transitions (7, 31, 42). The large difference between the transition temperatures in the warming and cooling...
phases in the present study was due to a lag in the ability of the circulator to reheat the samples. This will not influence the difference found between the treatments within the particular phases; however, it will prevent further discussion of the differences in the transition temperatures between the cooling and warming phases. Examination of the thermal hysteresis in the present study shows that treatment effects on the polarization value in the liquid crystal and gel phases are independent of previous thermal history because the treatment effects are similar in both the warming and cooling phases.

In conclusion, elasmobranchs use a counteracting solute strategy, maintaining high levels of urea and TMAO both intra- and extracellularly (14). Thus native elasmobranch erythrocyte membranes may be adapted to the presence of these solutes on both sides of the membrane. The results of the present study demonstrate that urea and TMAO may act as counteracting solutes in elasmobranch membranes, although the counteraction appears to be at the level of the integral protein rather than on the membrane phospholipids. Although the effect of urea on the fluidity of the elasmobranch membranes was not statistically different from the controls, it demonstrated a trend toward a counteractive effect that was supported by the results from the liposome studies. The effects of these solutes are complex, with interactions with both membrane lipids and proteins, and they may vary in membranes of different phospholipid and protein composition. Further studies should be conducted on a variety of membranes to determine if this effect is observed in other biological systems. Such effects may be important determinants of membrane properties and function, especially during periods of changing urea levels. The effects of urea and the methylamine TMAO on the native and model membranes we have observed may also be acting in regions of the mammalian kidney, where urea and methylamine levels are high and variable.
REFERENCES


