Probing the Ligand Binding Sites of Fatty Acid and Sterol Carrier Proteins: Effects of Ethanol†

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ABSTRACT: Direct effects of ethanol on the interaction of cytosolic lipid transfer proteins with ligands are not known. In this study, recombinant liver fatty acid binding protein (L-FABP) and sterol carrier protein-2 (SCP-2) were used in conjunction with a series of fluorescent fatty acid probe molecules to compare the relative dielectric properties of the ligand binding sites and to examine the effects of ethanol *in vitro* on ligand interaction with these proteins. L-FABP and SCP-2 exhibited broad but distinct ligand specificities. Although NBD-stearic acid bound with high affinity to both proteins, emission spectra showed that the relative dielectric constant of the ligand binding site in SCP-2 was significantly lower than in L-FABP, 2 vs 24. Furthermore, affinities of L-FABP for NBD-fatty acid probes were NBD-stearic acid > NBD-lauric acid >>> NBD-hexanoic acid, NBD-acetic acid. In contrast, SCP-2 bound only NBD-stearic acid with a K_d of 0.23 μM and B_{max} of 0.98 mol/mol. This observation of SCP-2 specifically binding the fluorescent NBD-stearic acid was confirmed with RdB-stearic acid and the naturally fluorescent *cis*-parinaric acid, both of which had similar affinities and stoichiometries. Ethanol *in vitro* had no effect on L-FABP−NBD-stearic acid binding. However, ethanol at physiological concentrations (25 mM) dramatically inhibited NBD-stearic acid binding to SCP-2. In conclusion, the data show that both L-FABP and SCP-2 specifically bind fluorescent fatty acids. However, the ligand binding sites of L-FABP and SCP-2 differed dramatically in their dielectric properties and their sensitivity to ethanol.

Cellular lipids are involved not only in energy production/storage but also in regulating membrane structure and function. These roles are disrupted by chronic or acute ethanol administration (Pignon et al., 1987; Wood et al., 1990; Rao et al., 1993; Schroeder & Wood, 1992; Schroeder, 1988). Ethanol may directly alter the structure of membrane lipids and their interaction with proteins either by altering membrane parameters such as fluidity of specific domains (Schroeder & Wood, 1992; Wood et al., 1995) or by modifying the dielectric properties of lipid/protein interfaces (Wood et al., 1995; Schroeder et al., 1994, 1995; Colles et al., 1995; Rottenberg, 1992). Alternately, ethanol may indirectly alter membrane lipid/protein interactions by disrupting the cellular machinery for intracellular lipid trafficking and targeting. It has been reported that ethanol *in vitro* modified the lipid composition of intracellular transport vesicles (Slomiany et al., 1992) and that cytosolic proteins are necessary for mediation of sterol transport between such vesicles (Lange et al., 1991). Fatty acid binding proteins and sterol carrier proteins may be important in the cytosolic trafficking and targeting of fatty acids and cholesterol (Schroeder et al., 1990a,b, 1991, 1993; Billheimer & Reinhart, 1990; Moncecchi et al., 1991; Butko et al., 1992; Hapala et al., 1994; Woodford et al., 1994; Jefferson et al., 1991; Peeters et al., 1989; Spener et al., 1989). Chronic ethanol consumption altered the expression of L-FABP[1] in liver (Pignon et al., 1987) and SCP-2 in brain (Myers-Payne et al., 1995). However, nothing is known regarding the effects of ethanol *in vitro* on the interaction of these proteins with lipids.

In the present investigation, the interaction of liver fatty acid binding protein (L-FABP) and sterol carrier protein-2 (SCP-2) was investigated with a series of fluorescent fatty acid probe molecules in the presence and absence of ethanol *in vitro*. It was observed that ethanol at clinically relevant concentrations dramatically specifically reduced the interaction of NBD-stearic acid with SCP-2, but not with L-FABP.

MATERIALS AND METHODS

Materials. Rat liver recombinant L-FABP and intestinal fatty acid binding protein, I-FABP, were isolated as described earlier (Lowe et al., 1987). Human liver recombinant SCP-2 was isolated as described earlier (Matsuura et al., 1993). The

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[1] Abbreviations: liver sterol carrier protein-2, SCP-2; liver fatty acid binding protein, L-FABP, (9*Z*,11*E*,13*E*,15*Z*)-octatetraenoic acid, *cis*-parinaric acid; 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]octadecanoic acid, NBD-stearate; 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoic acid, NBD-lauric acid; 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid, NBD-hexanoic acid; 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]acetic acid, NBD-acetic acid; *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino chloride, NBD-chloride; and octadecyl rhodamine B, chloride salt, RdB-stearic acid.

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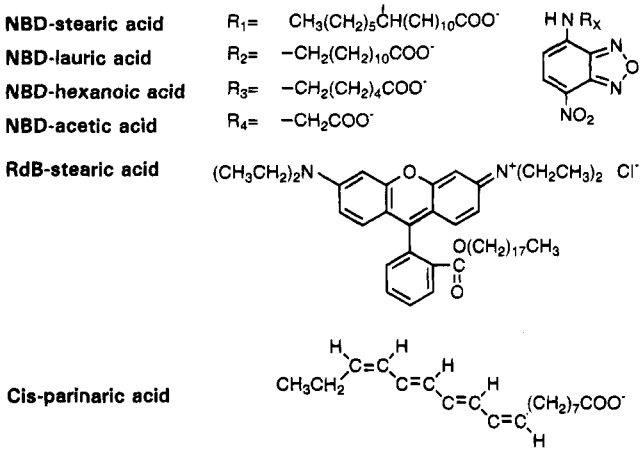

FIGURE 1: Structures of fluorescent NBD-labeled lipids and other fluorescent ligands.

recombinant proteins offered the advantage of being readily isolated from bacteria in pure form in the absence of any other fatty acid or sterol binding protein contaminants observed in native tissues (e.g. SCP, albumin, ligandin, etc.). The following fluorescent ligands were obtained from Molecular Probes, Eugene, OR: (9*Z*,11*E*,13*E*,15*Z*)-octatetraenoic acid (*cis*-parinaric acid), 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]octadecanoic acid (NBD-stearic acid), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino chloride (NBD-chloride), and octadecyl rhodamine B, chloride salt (RdB-stearic acid). The following NBD-acyl probes were synthesized by reacting NBD-chloride with either 12-aminododecanoic acid, 6-aminohexanoic acid, or 2-aminoacetic acid and purified by thin layer chromatography as described earlier (Fager et al., 1973) to produce 12-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoic acid (NBD-lauric acid), 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoic acid (NBD-hexanoic acid), and 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]acetic acid (NBD-acetic acid), respectively. Structures for these probe molecules are shown in Figure 1. Dioxane was from Fluka Chemical Co. (Ronkonkoma, NY). Ethyl alcohol-200 proof dehydrated alcohol, United States Pharmaceutical, puctilious (containing no denaturants and no fluorescent residues), was obtained from Quantum Chemical Co. (Cincinnati, OH).

Steady State Fluorescence Instrumentation and Methods. Steady state fluorescence measurements were obtained using an ISS Photon Counting Fluorometer (ISS, Champaign, IL) equipped with a 300 W Xe-arc lamp. Constant temperature was maintained using a Neslab Instruments cooling system (Plymouth, NH). Intensity measurements were performed in L-format. For experiments in which the emission monochromator was not in line, Janos GG-375 filters (Janos Technology, Townshend, VT) were used in the emission paths to reduce contributions from light scattering. The inner filter effect was avoided through use of samples with total absorbance at the excitation wavelength below 0.12. Data were automatically collected using ISS software.

Interaction of respective ligands with L-FABP or SCP-2 was determined at 22 °C by comparison of fluorescent ligand intensity in the absence and presence of protein. Concentrated stock solutions of fluorescent ligands in 25 mM phosphate buffer, pH 7.4, were prepared in the absence of ethanol or other solvent, unless otherwise stated. Fluorescence intensity was measured in the L-format and averaged over 9 s. Lipid concentration was as indicated in 25 mM PBS, pH 7.4 buffer. Protein concentration was as indicated. Relative fluorescence intensity, corrected for incident light, was measured in the presence of protein after a 5 min incubation to assure maximal interaction. Excitation wavelengths were as follows: NBD probes, λ_{ex} = 466 nm; *cis*-parinaric acid, λ_{ex} = 313 nm; and rhodamine, λ_{ex} = 556 nm. Emission wavelengths were as indicated in the legends to the figures.

For all solvent studies, 0.1 μg of probe was added per milliliter of solvent and vortexed, and fluorescence parameters were determined at 24 °C. The dielectric constants of dioxane/water and ethanol/water mixtures were taken from previously published sources (Turner & Brand, 1968; Zannoni et al., 1983).

Ligand Binding Analysis. A modified version of the fluorescence assay of Nemecz et al. (1991) based on the method of Lohman and Bujalowski (1991) was used to construct ligand binding curves and analyze the data for K_d and B_{max}. In order to obtain the maximum fluorescence intensity of fully bound fluorescent probe, the ligand concentration was held constant at 0.025 μM and L-FABP or SCP-2 concentration was varied from 0.01 to 1.28 μM. Binding assays were then performed by holding the protein concentration at 0.025 μM (SCP-2) or 0.05 μM (L-FABP) and increasing the ligand concentration. Alternately, ligand concentration was held constant and protein concentration was varied. The data were analyzed by fitting the calculated bound and free ligand data to the binding isotherm, $Y = AX/(B + X)$. In this equation, A is equal to the maximum bound ligand and B is equal to the K_d. The number of binding sites was obtained by dividing the fluorescence signal of the maximum bound ligand by the fluorescence signal of bound ligand equimolar to the protein used in the assay. This constant was determined as described above.

RESULTS

Effect of Solvent Dielectric on Spectral Properties of NBD-Stearic Acid. Ethanol is a relatively polar solvent that may disrupt the interactions of NBD ligands with SCP-2 or L-FABP within the respective ligand binding pockets. Therefore, it is necessary to investigate the dielectric properties of the SCP-2 and L-FABP protein probe binding sites. The structure of NBD-stearic acid is shown in Figure 1. Two NBD-stearic acid fluorescence parameters were sensitive to solvent dielectric: fluorescence intensity and maximal emission wavelength. First, the fluorescence intensity of NBD-stearic acid (0.3 μM) was highly dependent on solvent dielectric constant and showed the following relative fluorescence intensities: 6031 in dioxane (dielectric of 2), 1170 in ethanol (dielectric of 24), and 149 in water (dielectric of 80) (Figure 2). Second, the emission maximum of NBD-stearic acid shifted from 531 nm in dioxane (dielectric of 2) to 551 nm in water (dielectric of 80) (Figure 3). This represented a 20 nm red shift in emission maximum. The emission maxima of equal concentrations of NBD-stearic acid, NBD-lauric acid, NBD-hexanoic acid, or NBD-acetic acid at the same solvent dielectric, e.g. 0.3 μM in ethanol, did not significantly differ from each other. Therefore, the dielectric properties sensed by the NBD fluorophore were not related to the acyl chain length of the fatty acid to which each was linked.

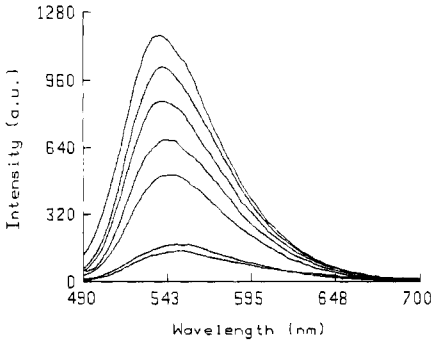

FIGURE 2: NBD-stearic acid emission spectra in a series of ethanol/water mixtures. NBD-stearic acid (0.3 μM) was dissolved in solvent and excited at 466 nm. Emission spectra were obtained as described in Materials and Methods. Curves from top to bottom represent NBD-stearic acid in 100% ethanol, 70% ethanol, 60% ethanol, 40% ethanol, 30% ethanol, 10% ethanol, and water.

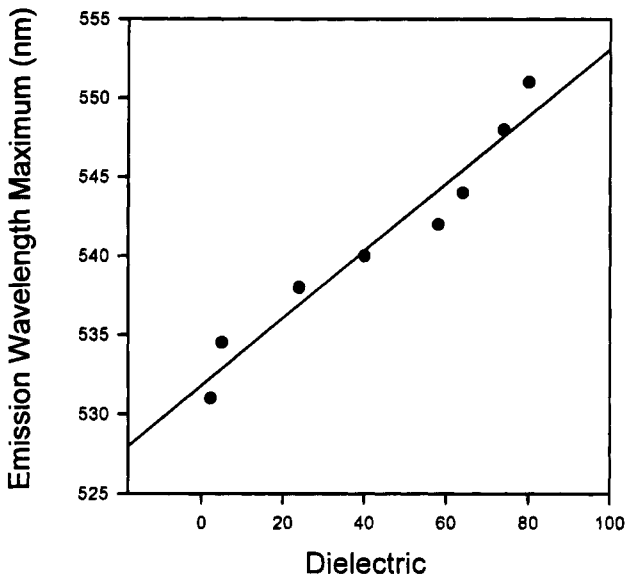

FIGURE 3: Effect of solvent dielectric constant on NBD-stearic acid emission spectra. NBD-stearic acid (0.3 μM) was dissolved in solvent and excited at 466 nm. Emission spectra were obtained as described in Materials and Methods. Data were from NBD-stearic acid in ethanol/water mixtures, except for dielectric constants below 20 which were obtained with dioxane/water mixtures as described in Materials and Methods.

Although the NBD-stearic acid microenvironment in the SCP-2 and L-FABP binding sites may not be precisely correlated with observations in organic solvent mixtures, relative differences in apparent dielectric constant or range of constants may be inferred. The data in Figure 3 provide a relative calibration of the dielectric properties of the NBD ligand binding sites in SCP-2 and L-FABP.

Fluorescence Emission Spectral Properties of NBD-Fatty Acids Interacting with SCP-2 and L-FABP. The interaction of several NBD-fatty acid probes with SCP-2 and L-FABP was examined (Figure 4). The NBD-stearic acid (0.3 μM) poorly fluoresced in aqueous buffer. However, in the presence of SCP-2, the fluorescence intensity of NBD-stearic acid increased 26-fold (Figure 4). At the same time, the emission maximum shifted 25.5 nm from 551 to 525.5 nm. When compared to the calibration curve of NBD-stearic acid in solvents with differing dielectric constants (Figure 3), the emission wavelength of NBD-stearic acid bound to SCP-2 was correlated with NBD-stearic acid localized in a microenvironment with a relative dielectric near 2. In contrast,

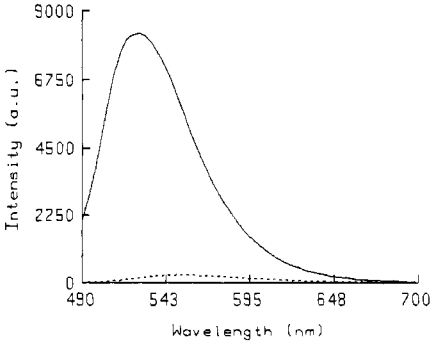

FIGURE 4: Interaction of NBD-stearic acid with SCP-2. NBD-stearic acid and SCP-2 concentrations were 0.3 and 0.27 μM, respectively.

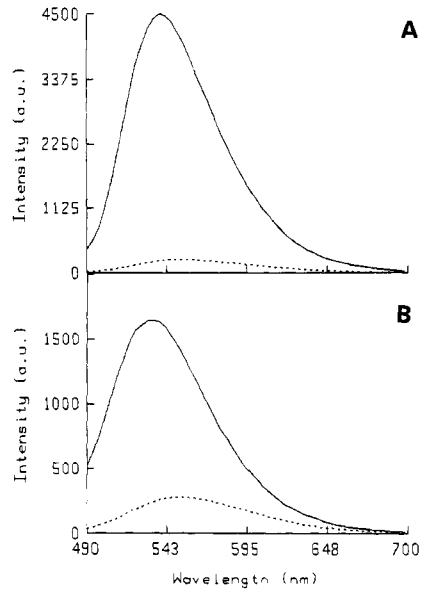

FIGURE 5: Interaction of NBD-fatty acid probes with SCP-2. NBD-fatty acid and SCP-2 concentrations were 0.3 and 0.8 μM, respectively. Panel A: NBD-stearic acid. Panel B: NBD-lauric acid.

SCP-2 did not significantly enhance the fluorescence intensity or shift the emission maxima of shorter chain NBD-fatty acid probes: NBD-lauric acid, NBD-hexanoic acid, or NBD-acetic acid (not shown). In summary, SCP-2 spectral shifts indicated that SCP-2 interacted best with the 18-carbon NBD-stearic acid compared to 12-carbon or shorter NBD-fatty acids tested. Thus, the NBD-stearic acid binding to SCP-2 was not a property attributable to the NBD group by itself.

In addition to interacting with SCP-2, the NBD-stearic acid also interacted with L-FABP (Figure 5). In the presence of L-FABP, the fluorescence intensity increased 15.3-fold (Figure 5A). Concomitantly, the NBD-stearic acid emission maximum shifted 12.7 nm from 551 to 538.3 nm. When compared to the calibration curve of NBD-stearic acid in solvents with differing dielectric constants (Figure 3), the emission wavelength maximum correlated to NBD-stearic acid being localized in the L-FABP binding site in an environment with a relative dielectric constant near 24. This was very similar to the dielectric constant of NBD-stearic acid in pure ethanol. Unlike SCP-2, L-FABP also bound NBD-lauric acid (Figure 5B). Under the same conditions as for NBD-stearic acid binding to L-FABP, L-FABP increased the fluorescence intensity of NBD-lauric acid 7-fold

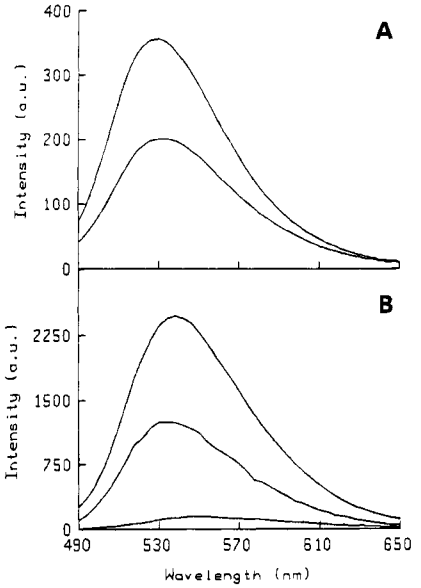

FIGURE 6: Displacement of L-FABP- and SCP-2-bound NBD-stearic acid with oleic acid. Panel A: top curve, 0.1 μM NBD-stearic acid, 0.1 μM SCP-2; bottom curve, 0.1 μM NBD-stearic acid, 0.1 μM SCP-2, and 2.5 μM oleic acid. Panel B: top curve, 0.3 μM NBD-stearic acid, 0.8 μM L-FABP; middle curve, 0.3 μM NBD-stearic acid, 0.8 μM L-FABP, and 5 μM oleic acid; bottom curve, 0.3 μM NBD-stearic acid.

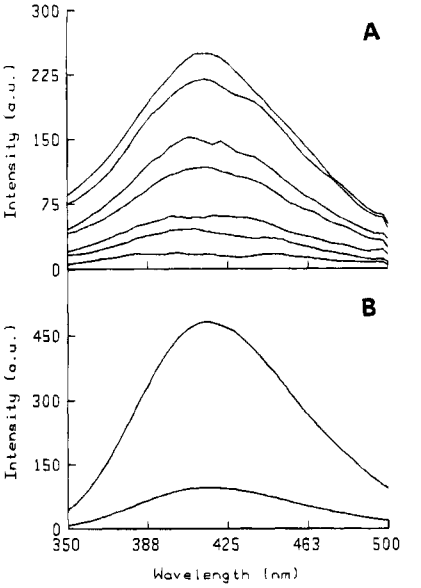

FIGURE 7: Emission spectra of *cis*-parinaric acid interacting with SCP-2 and L-FABP. Interaction of *cis*-parinaric acid with SCP-2 and L-FABP was measured as described in Materials and Methods. Panel A: bottom curve, 0.5 μM *cis*-parinaric acid; increasing from bottom to top, samples contained 0.2, 0.5, 0.8, 1.2, and 1.4 μM SCP-2. Panel B: bottom curve, 0.25 μM *cis*-parinaric acid; top curve, 0.25 μM *cis*-parinaric acid, 0.5 μM L-FABP.

and shifted the emission maximum 15 nm from 551 and 536 nm. This emission maximum wavelength correlated with a relative dielectric constant near 18. Although, the emission maximum wavelength indicated that the NBD group of the shorter chain NBD-lauric acid was localized in a slightly more hydrophobic microenvironment in L-FABP than the longer chain NBD-stearic acid, the nearly 2-fold smaller increase in fluorescence intensity indicated that the L-FABP had a lower affinity for the NBD-lauric acid than for NBD-stearic acid. L-FABP did not increase the fluorescence intensity or shift the emission emission maximum of NBD-hexanoic acid or NBD-acetic acid (not shown).

NBD-Stearic Acid as a Fatty Acid Binding Site Probe. Although it is well-accepted that L-FABP has broad ligand specificity with respect to fatty acids and other hydrophobic lipids (Schroeder et al., 1993; Nemecz et al., 1991; Nemecz & Schroeder, 1991; Paulussen & Veerkamp, 1990), specific binding of a fatty acid to SCP-2 has hitherto not been reported. Since the aforementioned data clearly indicated that NBD-stearic acid interacted with both SCP-2 and L-FABP, it was important to demonstrate that the interaction with SCP-2 was not a unique property of this fatty acid analogue.

First, the specificity of NBD-stearic acid binding was examined by competition with oleic acid. Oleic acid competed for the same SCP-2 ligand binding site as NBD-stearic acid, as indicated by 48% decrease in NBD-stearic acid fluorescence intensity (Figure 6A). The fact that a 25-fold excess of oleic acid only decreased SCP-2-bound NBD-stearic acid fluorescence intensity by 48% was consistent with a higher affinity of SCP-2 for NBD-stearic acid than oleic acid. Oleic acid also competed for the same L-FABP ligand binding site as NBD-stearic acid, also as indicated by 47% decreased fluorescence intensity of NBD-stearic acid bound to L-FABP (Figure 6B). Again, the observation that a 17-fold excess of oleic acid only decreased L-FABP-bound NBD-stearic acid fluorescence intensity by 47% was consistent with L-FABP having a higher affinity for NBD-stearic acid than oleic acid.

Second, NBD-stearic acid binding to SCP-2 and L-FABP was compared to that of another fatty acid binding protein, I-FABP, whose ligand specificity is exclusively for fatty acids (Nemecz et al., 1991a,b; Paulussen & Veerkamp, 1990). L-FABP has two ligand binding sites while I-FABP has only one ligand acid binding site (Nemecz et al., 1991a,b). Over the same concentration range used to examine interaction of NBD-stearic acid with SCP-2 and L-FABP, I-FABP increased the intensity of NBD-stearic acid 8-fold (data not shown). This was about half that observed with L-FABP (Figure 5A), consistent with a single binding site in I-FABP. However, the I-FABP-bound NBD-stearic acid emission maximum shifted to 544 nm, which corresponded to a relative dielectric of 56 when compared to the calibration curve in Figure 3. These results indicated that the microenvironment wherein the NBD fluorophore of NBD-stearic acid resided in I-FABP differed significantly from that of SCP-2 or L-FABP. The more polar microenvironment of the NBD fluorophore when NBD-stearic acid was bound to I-FABP was due to the fact that, in contrast to L-FABP, the fatty acid in I-FABP is oriented with the fatty acid carboxyl located deep in the binding pocket of the protein and this pocket has considerable polar character [review in Schroeder et al. (1993)].

Third, *cis*-parinaric acid, a conjugated polyene without an extrinsic reporter group (Figure 1), was used to determine whether the addition of the extrinsic reporter group (NBD) to stearic acid (Figure 1) conferred SCP-2 binding ability to this probe. However, this was not the case. *cis*-Parinaric acid also bound to SCP-2 (Figure 7A) and L-FABP (Figure 7B) as indicated by ligand concentration dependent increased fluorescence intensity. When *cis*-parinaric acid was bound to SCP-2 and L-FABP, the ratio of the amplitude of the

Table 1: Affinities of SCP-2 and L-FABP for NBD-Fatty Acids and Other Fluorescent Ligands[a]

	SCP-2		L-FABP	
ligand	K_d (μM)	B_{max} (mol/mol)	K_d (μM)	B_{max} (mol/mol)
NBD-stearic acid	0.234 ± 0.047	0.983 ± 0.075	0.022 ± 0.002	1.570 ± 0.023
NBD-lauric acid	none	none	1.05	0.88
NBD-hexanoic acid	none	none	none	none
NBD-acetic acid	none	none	none	none
cis-parinaric acid	0.400 ± 0.019	0.67 ± 0.30	0.412 ± 0.033	1.65 ± 0.44
RdB-stearic acid	0.259 ± 0.056	1.20 ± 0.08	0.201 ± 0.004	3.30 ± 0.08

[a] Ligand binding was determined as described in the Materials and Methods. Values represent the average ± standard error (n = 3–6).

excitation peaks at 322/294 nm was 1.14 and 1.32, respectively. These ratios of the amplitude of the excitation peaks at 322/294 nm for *cis*-parinaric acid bound to the respective proteins were compared to a calibration curve of *cis*-parinaric acid in organic solvents (Nemecz et al., 1991b). This yielded relative dielectric constants of 2 and 27 for *cis*-parinaric acid bound to SCP-2 and L-FABP, respectively.

Fourth, rhodamine B-stearate (RdB-stearate), a stearic acid probe with an extrinsic reporter group rhodamine B esterified at the carboxyl group of stearic acid (Figure 1), was used to determine if replacement of the fatty acyl carboxyl group with a relatively polar fluorophore (RdB) modified binding of the acyl probe to SCP-2 and L-FABP. Both proteins bound the RdB-stearate (see Table 1, below) with roughly similar affinities (see below).

In summary, both SCP-2 and L-FABP bound the 18-carbon chain length fluorescent fatty acid probes whether an intrinsic (conjugated tetraene) or extrinsic reporter group (NBD, RdB) was present or absent. Likewise, both proteins bound these fatty acid probes whether the carboxyl terminal was or was not replaced by a larger polar group. The fluorescent NBD-stearic acid binding competed with oleic acid in both proteins, indicating that the fluorescent fatty acid bound at the same site as native fatty acids. The relative affinities of SCP-2 and L-FABP for these fatty acids are detailed in the following section.

Relative Affinities of SCP-2 and L-FABP for Fluorescent Ligands. The affinities of L-FABP for the NBD-fatty acid and other probes was determined as described in Materials and Methods. Typical data showing the effect of increasing L-FABP in the presence of constant NBD-stearic acid are indicated in Figure 8. NBD-stearic acid interaction with L-FABP displayed a concentration dependent fluorescence intensity increase (Figure 8A). L-FABP displayed saturation binding of NBD-stearic acid (Figure 8B). When the data were transformed into a Lineweaver–Burke plot, straight lines indicative of a single class of binding sites were obtained (not shown). For NBD-stearic acid binding of L-FABP, Lineweaver–Burke analysis yielded a K_d of 0.022 ± 0.002 μM and basically two binding sites per mole of protein (B_{max} = 1.57 ± 0.02 mol/mole of L-FABP) as shown in Table 1. In addition, L-FABP similarly bound other 18-carbon fatty acids, but with lower affinity: *cis*-parinaric acid with K_d = 0.412 ± 0.033 μM and B_{max} = 1.65 ± 0.44 mol/mole of protein and RdB-stearate with K_d = 0.201 ± 0.004 μM and B_{max} = 3.30 ± 0.08 mol/mole of protein (the latter B_{max} was probably an overestimate due to the tendency of RdB-stearate to adsorb to glass surfaces in the cuvette). However, the 12-carbon chain length NBD-lauric acid was bound more weakly than NBD-stearic acid since it had a nearly 50-fold lower K_d near 1.05 μM and appeared to bind

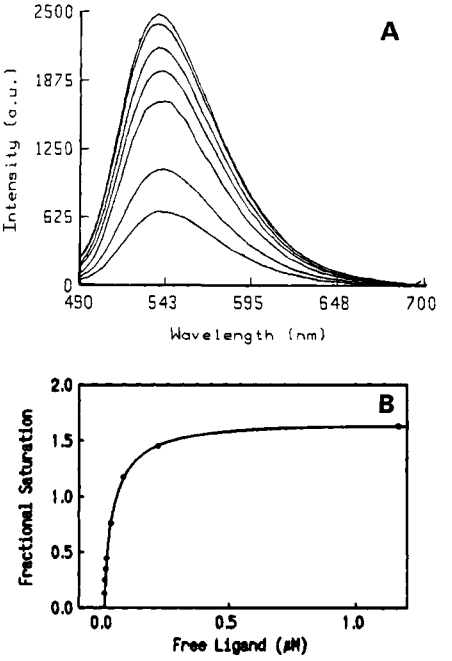

FIGURE 8: Emission spectra of NBD-stearic acid interacting with L-FABP. Interaction of NBD-stearic acid with L-FABP was measured as described in Materials and Methods. Panel A: 0.3 μM NBD-stearic acid and from bottom to top 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, and 0.8 μM L-FABP. Panel B: fractional saturation plot of data in panel A.

to only one of the two L-FABP fatty acid binding sites as indicated by a B_{max} of 0.88 mol/mole of L-FABP.

Data showing the effect of increasing SCP-2 in the presence of constant NBD-stearic acid are indicated in Figure 9. NBD-stearic acid interaction with SCP-2 displayed a concentration dependent fluorescence intensity increase (Figure 9A). SCP-2 also showed saturation binding of NBD-stearic acid (Figure 9B). When the data were transformed into a Lineweaver–Burke plot, straight lines indicative of a single class of binding sites were obtained (not shown). Lineweaver–Burke analysis of NBD-stearic acid binding to SCP-2 yielded a single high affinity site with K_d = 0.234 ± 0.047 μM and B_{max} = 0.983 ± 0.075 mol/mole (Table 1). In addition, SCP-2 also bound other 18-carbon fatty acids with similar affinity and stoichiometry: *cis*-parinaric acid with K_d = 0.400 ± 0.019 μM and B_{max} = 0.67 ± 0.30 mol/mole of protein and RdB-stearate with K_d = 0.259 ± 0.056 μM and B_{max} = 1.20 ± 0.08 mol/mole of protein. Thus, SCP-2 bound both naturally occurring fluorescent fatty acid (*cis*-parinaric acid) and synthetic fatty acids with extrinsic reporter groups (NBD-stearic acid, RdB-stearate) with K_ds in the submicromolar range with a stoichiometry near 1. The binding affinities of the SCP-2 for the respective probes were

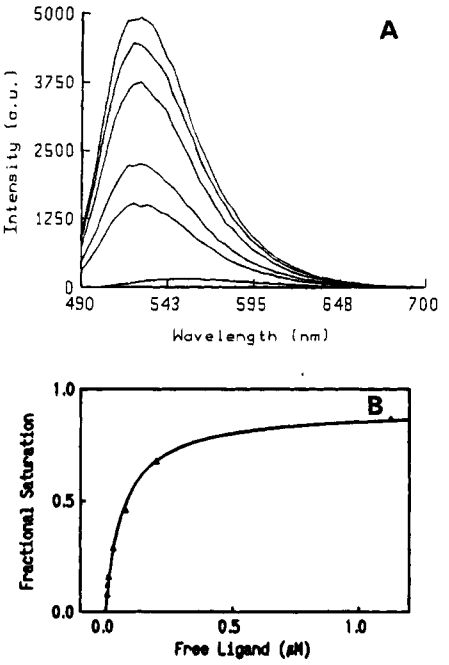

FIGURE 9: Effect of increasing SCP-2 concentration on NBD-stearic fluorescence emission. NBD-stearic acid was 0.3 μM. Panel A: SCP-2 concentrations from bottom to top, none and 0.033, 0.067, 0.13, 0.20, and 0.27 μM. Panel B: fractional saturation plot of data in panel A.

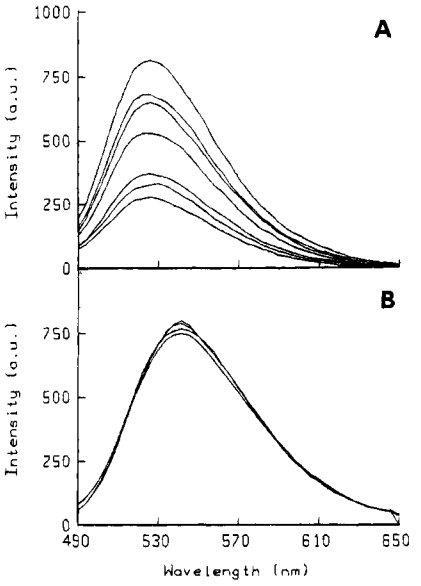

FIGURE 10: Effect of increasing ethanol concentration on NBD-stearic acid–SCP-2 and NBD-stearic acid-L-FABP fluorescence emission. NBD-stearic acid was 0.1 μM while SCP-2 and L-FABP were 0.1 μM. Panel A: SCP-2. Ethanol concentrations indicated by solid lines were from top to bottom: none and 25, 50, 200, 400, 600, and 800 mM. Panel B: L-FABP. At the same concentrations of ethanol as in Panel A, the curves nearly superimposed on one another.

very similar to those of L-FABP, except that the L-FABP had a 10-fold higher affinity for NBD-stearic acid than SCP-2.

Effect of Ethanol in Vitro on Binding of NBD-Stearic Acid and RdB-Stearic Acid to SCP-2 and L-FABP. Ethanol *in vitro* dramatically decreased the interaction of NBD-stearic acid with SCP-2 (Figure 10A). The fluorescence intensity decreased with increasing ethanol concentration (Figure 11). The decrease in fluorescence intensity was significant at

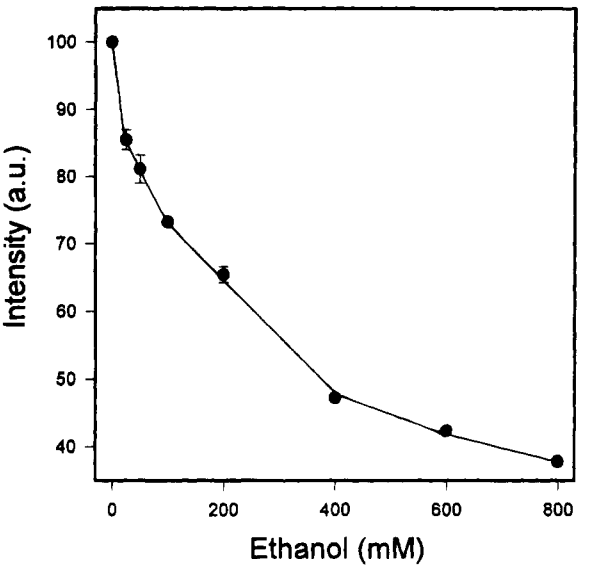

FIGURE 11: Effect of ethanol on NBD-stearic acid–SCP-2 emission intensity. All data were obtained as described in the legend to Figure 10. Intensity is in arbitrary units normalized to 100%.

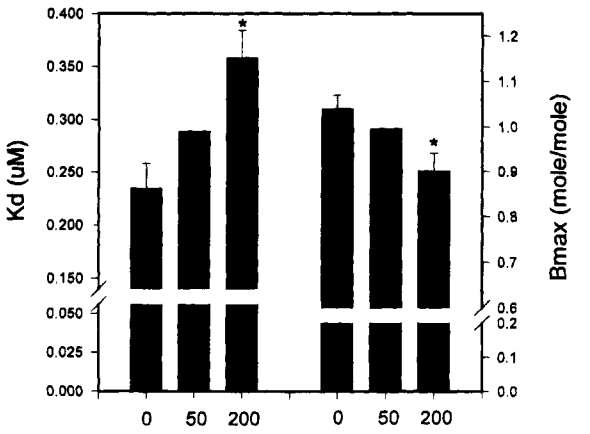

FIGURE 12: Effect of ethanol on NBD-stearic acid–SCP-2 binding parameters. The bars from left to right represent 0, 50, and 200 mM ethanol. Dissociation constants and maximal binding stoichiometry are on the left and right, respectively. Where indicated, values represent the mean ± the standard error of the mean, n = 4.

ethanol clinically relevant concentrations. At 25 mM ethanol, the fluorescence intensity decreased 14.5 ± 1.5% (Figure 11). The effect of acute ethanol over the range 0–200 mM on fractional saturation of NBD-stearic acid binding to SCP-2 was to increase the K_d 1.6-fold (0.36 ± 0.03 vs 0.23 ± 0.02, $p < 0.025$) and decrease the B_{max} 14% (0.90 ± 0.04 vs 1.04 ± 0.03, $p < 0.05$) (Figure 12). Finally, it was important to note that these effects of ethanol on NBD-stearic acid binding were specific to SCP-2. Binding on NBD-stearic acid to L-FABP was not significantly altered by ethanol (Figure 10B).

To determine whether the effect of ethanol on the interaction of fatty acids with these proteins was specific for the NBD-stearic acid, a second probe RdB-stearic acid was tested. Ethanol *in vitro* also decreased the interaction of RdB-stearic acid with SCP-2. The fluorescence intensity of RdB-stearic acid bound to SCP-2 decreased with increasing ethanol concentration such that, at 25 mM ethanol, the fluorescence intensity decreased 27% (data not shown). In contrast, the effects of ethanol on RdB-stearic acid binding

to L-FABP were significantly less. The fluorescence intensity of RdB-stearic acid interacting with L-FABP decreased only 12% at 25 mM ethanol (data not shown).

DISCUSSION

Ethanol not only disrupts the membrane protein/lipid interface (Wood et al., 1995; Schroeder et al., 1994, 1995; Colles et al., 1995; Rottenberg, 1992) but also affects lipid trafficking (Slomiany et al., 1992). Nevertheless, almost nothing is known regarding direct effects of ethanol on cytosolic proteins involved in lipid trafficking. That direct effects of ethanol on the interaction of proteins with their ligands are possible is based on two reports indicating that ethanol disrupts the activity of the cytosolic protein luciferase (Franks & Lieb, 1984, 1994). In addition, chronic ethanol consumption modified amounts of intracellular lipid transfer proteins, L-FABP and SCP-2 (Pignon et al., 1987; Wood et al., 1995). Direct effects of ethanol on either protein have not previously been reported. Such studies are important because a number of *in vitro* studies have shown that SCP-2 and L-FABP may be involved in intracellular trafficking of a variety of ligands [review in Schroeder et al. (1991), Billheimer and Reinhart (1990), and Moncecchi et al. (1991)]. More important, in transfected L-cell fibroblasts expressing L-FABP, the uptake and intracellular transfer of cholesterol (Jefferson et al., 1991) and fatty acids (Schroeder et al., 1993) are enhanced in intact cells. SCP-2 not only binds sterols (Schroeder et al., 1990a; Vahouny et al., 1987; Sams et al., 1991), phospholipids (Nichols, 1987, 1988; Gadella & Wirtz, 1991), and long chain isoprenoids such as dolichol (Ericsson et al., 1991) but also enhances the intermembrane transfer of cholesterol (Schroeder et al., 1990a, 1991; Billheimer & Reinhart, 1990; Moncecchi et al., 1991; Butko et al., 1992; Hapala et al., 1994; Woodford et al., 1994), phospholipids, sphingomyelin, gangliosides, and neutral glycosphingolipids [review in Schroeder et al. (1991), Billheimer and Reinhart (1990), and Moncecchi et al. (1991)]. L-FABP binds fatty acids (Nemecz et al., 1991a,b), fatty acyl CoAs (Hubbell et al., 1994), sterols [review in Nemecz and Schroeder (1991)], retinol, heme, hematin, lysophospholipids, bilirubin, prostaglandins, and other amphipathic ligands [review in Paulussen and Veerkamp (1990)]. L-FABP stimulates sterol and fatty acid transfer between membranes (Jefferson et al., 1991; Schroeder et al., 1993; Peeters et al., 1989; Spener et al., 1989). Thus, by direct disruption of their interaction with SCP-2 or L-FABP, ethanol may dramatically modulate intracellular trafficking of a large variety of hydrophobic ligands involved in cell growth regulation, membranogenesis, steroidogenesis, lipid storage, and energy metabolism in the cell.

The data show that ethanol *in vitro* distinctly disrupts the interaction of NBD-stearic acid with SCP-2, but not with L-FABP. Specifically, ethanol at low concentrations altered the interaction of NBD-stearic acid with SCP-2. NBD-stearic acid, as well as other naturally occurring or extrinsically labeled fluorescent fatty acids, bound with high affinity, K_d = 0.23−0.40 μM and 1:1 stoichiometry, to SCP-2. The apparent dielectric of the microenvironment sensed by NBD-stearic acid in SCP-2, near 2, was indicative of a very hydrophobic binding environment in the vicinity of the NBD fluorophore. The ethanol-induced disruption of the NBD-stearic acid binding to SCP-2, but not L-FABP, could be due to one of two effects.

First, ethanol may disrupt the interaction of NBD-stearic acid with SCP-2 due to a decrease in the dielectric value of water. This is unlikely because, at 25 mM ethanol (the lowest dose tested), the dielectric of water only shifted slightly, from 80 to 79. Thus, simple repartitioning of NBD-stearic acid from the SCP-2 binding site to the buffer (containing ethanol) did not account for the ethanol effects on NBD-stearic acid binding to SCP-2.

Second, ethanol may directly bind within the ligand binding pocket to disrupt the ligand/protein interaction in SCP-2, but not in L-FABP. Although the spectra in Figure 10A do not show an obvious red shift in NBD-stearic acid emission maximum wavelength with increasing ethanol, ethanol may be disrupting NBD-stearic, acid interactions with SCP-2 at positions along the acyl chain farther away than carbon 12 at which the NBD fluorophore is attached (Figure 1). With the exception of some site-directed mutagenesis investigations and preliminary NMR data on apo-SCP-2, very little is known regarding the structure of the SCP-2 lipid binding pocket (Seedorf et al., 1994; Szyperski et al., 1993). Thus, a description of the SCP-2 ligand binding site is not available.

In contrast to the paucity of tertiary structural information on the SCP-2 ligand binding site, many of the fatty acid binding proteins have been crystallized with and without bound ligand, and the structure of the binding pocket is well-known [review in Peeters et al. (1989), Paulussen and Veerkamp (1990), and Schroeder et al. (1993)]. Both ionic and hydrophobic interactions appear important. For example, the fatty acid binding site of I-FABP encloses 24 and 18 ordered water molecules in the apo-I-FABP and holo-I-FABP, respectively (Scapin et al., 1993). The fatty acid is bound to I-FABP with the carboxyl facing the interior of a deep binding pocket, wherein it formed a salt bridge with ARG 108 (Scapin et al., 1993). An analogous ARG residue occurs in L-FABP (Schroeder et al., 1993), and this salt bridge may be a key difference between L-FABP vs SCP-2 binding of NBD-stearic acid and other ligands. Indeed, L-FABP has a 10-fold higher affinity than SCP-2 for NBD-stearic acid. Furthermore, in general, L-FABP binds uncharged hydrophobic ligands less well than does SCP-2. In addition to the ionic interaction, the hydrophobic chain of the fatty acid interacts with hydrophobic amino acid residues lining the I-FABP binding pocket closer to the opening of the ligand binding site [review in Scapin et al. (1993)]. Molecular modeling of L-FABP based on the X-ray crystal structure of I-FABP basically yielded a very similar structure (Schroeder et al., 1993).

The use of NBD-stearic acid as a probe for SCP-2 and L-FABP interaction with ligands has been especially useful not only because both proteins bind the same ligand but also because the SCP-2 binding site has been difficult to saturate (Nichols, 1987, 1988; Gadella & Wirtz, 1991; Sams et al., 1991; Schroeder et al., 1990). For example, the highest binding stoichiometries that have been reported for a number of fluorescent phospholipids that interact with SCP-2 are near 0.08 fluorescent phospholipids per mole of SCP-2 (Nichols, 1987; Gadella & Wirtz, 1991). In contrast to the low stoichiometry of NBD-phospholipid binding to SCP-2, NBD-stearic acid and the other naturally occurring (*cis*-parinaric acid) and synthetic (RdB-stearate) fluorescent fatty acids bound to SCP-2 with stoichiometry near 1. This readily

allowed for detection of ethanol effects on ligand−SCP-2 interaction.

Finally, the data presented herein with both naturally occurring and synthetic fluorescent fatty acids indicated for the first time that liver SCP-2 can specifically bind a variety of native and extrinsic fluorescent fatty acids, this binding was saturable, and the K_ds were in the submicromolar range. With the exception of NBD-stearic acid, which bound 10-fold more tightly to L-FABP than SCP-2, the other fluorescent fatty acids bound with similar affinity to L-FABP and SCP-2 (Table 1). Earlier studies using the Lipidex 1000 radiolabeled fatty acid binding assay reported that rat and chicken liver SCP-2 (also called nsLTP) either did not or only weakly bound radiolabeled fatty acids as compared to liver L-FABP (Scallen et al., 1985; Sams et al., 1991). The latter studies used propylene glycol (1,2-propanediol) as a [^{3}H]oleic acid vehicle such that the final solvent concentration in the Lipidex 1000 assay was 150 mM (Scallen et al., 1985). At 200 mM concentrations, both propylene glycol and ethanol inhibited NBD-stearic acid binding to SCP-2 34 and 33%, respectively (data not shown).

It is important to recognize that simple binding of a lipid ligand does not necessarily imply that the binding protein functions in ligand metabolism (Sziegoleit, 1982; Habig et al., 1974; Nemecz et al., 1991a,b; Hubbell et al., 1994). SCP-2 and L-FABP have distinctly different functions (Scallen et al., 1985; Vahouny et al., 1987). Some of these functions may overlap. For example, there are connections between L-FABP as well as SCP-2 and cholesterol metabolism. L-FABP expression in transfected L-cell fibroblasts enhanced cholesterol uptake and lowered the cell surface membrane cholesterol content (Jefferson et al., 1991; Woodford et al., 1993). Likewise, SCP-2 gene expression correlates with the free cholesterol content of peritoneal macrophages (Hirai et al., 1994). In contrast, these two proteins differ more than 10-fold in their ability to stimulate acyl-CoA cholesteryl acyl transferase *in vitro* (Scallen et al., 1985; Nemecz & Schroeder, 1991; Schroeder et al., 1990a) or transfer sterols between membranes (Schroeder et al., 1991; Scallen et al., 1985; Moncecchi et al., 1991). Thus, the physiological role of SCP-2 in binding fatty acids remains to be elucidated.

In conclusion, NBD-labeled lipids provided useful probe molecules for comparing the binding sites of SCP-2 and L-FABP. The NBD-stearic acid proved especially useful in demonstrating that ethanol disrupted the NBD-stearic acid−SCP-2 binding site. This effect was specific since ethanol had no effect on the NBD-stearic acid−L-FABP interaction. These findings now clearly show that anesthetics such as ethanol can act selectively on lipid/protein interactions.

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REFERENCES

Billheimer, J. T., & Reinhart, M. P. (1990) in *Subcellular Biochemistry, Vol. 16, Intracellular Transfer of Lipid Molecules* (Hilderson, H. J., Ed.) pp 301−331, Plenum Press, New York.

Butko, P., Hapala, I., Nemecz, G., & Schroeder, F. (1992) *J. Biochem. Biophys. Methods 24*, 15−37.

Colles, S., Wood, W. G., Myers-Payne, S., Joseph, J., & Schroeder, F. (1995) *Biochemistry 34*, 5945−5959.

Ericsson, J., Scallen, T. J., Chojnacki, T., & Dallner, G. (1991) *J. Biol. Chem. 266*, 10602−10607.

Fager, R. S., Kutina, C. B., & Abrahamson, E. W. (1973) *Anal. Biochem. 53*, 290−294.

Franks, N. P., & Lieb, W. R. (1984) *Nature 310*, 599−601.

Franks, N. P., & Lieb, W. R. (1994) *Nature 367*, 607−614.

Gadella, T. W. J., & Wirtz, K. W. A. (1991) *Biochim. Biophys. Acta 1070*, 237−245.

Habig, W. H., Pabst, M. J., Fleischner, G., Gatmaitan, Z., Aria, I. M., & Jakoby, W. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3879−3882.

Hapala, I., Kavecansky, J., Butko, P., Scallen, T. J., Joiner, C. H., & Schroeder, F. (1994) *Biochemistry 33*, 7682−7690.

Hirai, A., Kino, T., Tokinaga, K., Tahara, K., Tamura, Y., & Yoshida, S. (1994) *J. Clin. Invest. 94*, 2215−2223.

Hubbell, T., Behnke, W. D., Woodford, J. K., & Schroeder, F. (1994) *Biochemistry 33*, 3327−3334.

Jefferson, J. R., Slotte, J. P., Nemecz, G., Pastuszyn, A., Scallen, T. J., & Schroeder, F. (1991) *J. Biol. Chem. 266*, 5486−5496.

Lange, Y., Echevarria, G., & Steck, T. L. (1991) *J. Biol. Chem. 266*, 21439−21443.

Lohman, T. M., & Bujalowski, M. (1991) *Methods Enzymol. 208*, 258−290.

Lowe, J. B., Sacchettini, J. C., Laposata, M., McQuillan, J. J., & Gordon, J. I. (1987) *J. Biol. Chem. 262*, 5931−5937.

Matsuura, J. E., George, H. J., Ramachandran, N., Alvarez, J. G., Strauss, J. F., III, & Billheimer, J. T. (1993) *Biochemistry 32*, 567−572.

Moncecchi, D., Nemecz, G. N., Schroeder, F., & Scallen, T. J. (1991) The participation of sterol carrier protein-2 in cholesterol metabolism, in *Physiology and Biochemistry of Sterols* G. W. (Patterson, G. W., & Nes, W. D., Eds.) pp 1−27, American Oil Chemistry Society Press, Champaign, IL.

Myers-Payne, S. C., Fontaine, R. N., Loeffler, A. L., Hubbell, T., Pu, L., Rao, A. M., Kier, A. B., Wood, W. G., & Schroeder, F. (1995) *J. Neurochem.* (in press).

Nemecz, G. N., & Schroeder, F. (1991) *J. Biol. Chem. 266*, 17180−17186.

Nemecz, G. N., Hubbell, T., Jefferson, J. R., Lowe, J. B., & Schroeder, F. (1991a) *Arch. Biochem. Biophys. 286*, 300−309.

Nemecz, G. N., Jefferson, J. R., & Schroeder, F. (1991b) *J. Biol. Chem. 266*, 17112−17123.

Nichols, J. W. (1987) *J. Biol. Chem. 262*, 14172−14177.

Nichols, J. W. (1988) *Biochemistry 27*, 1889−1886.

Paulussen, R. J. A., & Veerkamp, J. H. (1990) in *Subcellular Chemistry: Intracellular Transfer of Lipid Molecules* (Hilderson, H. J., Ed.) pp 175−226, Plenum Press, New York.

Peeters, R. A., Veerkamp, J. H., & Demel, R. A. (1989) *Biochim. Biophys. Acta 1002*, 8−13.

Pignon, J.-P., Bailey, N. C., Baraona, E., & Lieber, C. S. (1987) *Hepatology 7*, 865−871.

Rao, A. M., Igbavboa, U., Semotuk, M., Schroeder, F., & Wood, W. G. (1993) *Neurochem. Int. 23*, 45−52.

Rottenberg, H. (1992) *Biochemistry 31*, 9473−9481.

Sams, G. H., Hargis, B. M., & Hargis, P. S. (1991) *Comp. Biochem. Physiol. 99B*, 213−219.

Scallen, T. J., Noland, B. J., Gavey, K. L., Bass, N. M., Ockner, R. K., Chanderbhan, R., & Vahouny, G. V. (1985) *J. Biol. Chem. 260*, 4733−4739.

Scapin, G., Young, A. C. M., Kromminga, J. H., Veerkamp, J. H., Gordon, J. I., & Sacchettini, J. C. (1993) *Mol. Cell. Biochem. 123*, 1−13.

Schroeder, F. (1988) in *Methods for Studying Membrane Fluidity* (Aloia, R. C., Curtain, C. C., & Gordon, L. M., Eds.) pp 193−217, Alan R. Liss, New York.

Schroeder, F., & Wood, W. G. (1992) in *Alcohol and Neurobiology: Receptors, Membranes, and Channels* (Watson, R. R., Ed.) pp 161−184, CRC Press, Boca Raton, FL.

Schroeder, F., Butko, P., Nemecz, G., & Scallen, T. J. (1990a) *J. Biol. Chem. 265*, 151−157.

Schroeder, F., Butko, P., Hapala, I., & Scallen, T. J. (1990b) *Lipids 25*, 669−674.

Schroeder, F., Jefferson, J. R., Kier, A. B., Knittel, J., Scallen, T. J., Wood, W. G., & Hapala, I. (1991) *Proc. Soc. Exp. Biol. Med. 196*, 235−252.

Schroeder, F., Jefferson, J. R., Powell, D., Incerpi, S., Woodford, J. K., Colles, S. M., Myers-Payne, S., Emge, T., Hubbell, T., Moncecchi, D., Prows, D. R., & Heyliger, C. E. (1993) *Mol. Cell. Biochem. 123*, 73−83.

Schroeder, F., Colles, S. M., Kreishman, G. P., Heyliger, C. E., & Wood, W. G. (1994) *Arch. Biochem. Biophys. 309*, 369−376.

Schroeder, F., Hubbell, T., Colles, S. M., & Wood, W. G. (1995) *Arch. Biochem. Biophys. 316*, 343−352.

Seedorf, U., Scheek, S., Engel, T., Steif, C., Hinz, H.-J., & Assman, G. (1994) *J. Biol. Chem. 269*, 2613−2618.

Slomiany, A., Grabska, M., Grzelinska, E., Yamaki, K., Kasimathan, C., Slomiany, B. A., & Slomiany, B. L. (1992) *Alcoholism: Clin. Exp. Res. 16*, 1152−1161.

Spener, F., Borchers, T., & Mukherjea, M. (1989) *FEBS Lett. 244*, 1−5.

Sziegoleit, A. (1982) *Biochem. J. 261*, 273−276.

Szyperski, T., Scheek, S., Johansson, J., Assmann, G., Seedorf, U., & Wuthrich, K. (1993) *FEBS Lett. 335*, 18−26.

Turner, D. C., & Brand, L. (1968) *Biochemistry 7*, 3381−3390.

Vahouny, G. V., Chanderbhan, R., Kharroubi, A., Noland, B. J., Pastuszyn, A., & Scallen, T. J. (1987) *Adv. Lipid Res. 22*, 83−113.

Wood, W. G., Schroeder, F., Hogy, L., Rao, A. M., & Nemecz, G. N. (1990) *Biochim. Biophys. Acta 1025*, 243−246.

Wood, W. G., Schroeder, F., Rao, M. A., Igbavboa, U., & Avdulov, N. (1995) Membranes and ethanol: lipid domains and lipid-protein interactions, in *Effects of Ethanol on the Central Nervous System* (Deitrich, R. A., Ed.) CRC Press, Boca Raton, FL (in press).

Woodford, J. K., Jefferson, J. R., Wood, W. G., Hubbell, T., & Schroeder, F. (1993) *Biochim. Biophys. Acta 1145*, 257−265.

Woodford, J. K., Hapala, I., Jefferson, J. R., Knittel, J. J., Kavecansky, J., Powell, D., Scallen, T. J., & Schroeder, F. (1994) *Biochim. Biophys. Acta 1189*, 52−60.

Zannoni, C., Arcioni, A., & Cavatorta, P. (1983) *Chem. Phys. Lipids 32*, 179−250.

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