

Activation of Membrane Cholesterol by 63 Amphipaths[†]

Yvonne Lange,^{*,‡} Jin Ye,[‡] Mark-Eugene Duban,[§] and Theodore L. Steck^{||}

[‡]Department of Pathology, Rush University Medical Center, Chicago, Illinois 60612, [§]Department of Chemistry and CLP Institute, Northwestern University, Evanston, Illinois 60208, and ^{||}Department of Biochemistry & Molecular Biology, University of Chicago, Chicago, Illinois 60637

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ABSTRACT: A few membrane-intercalating amphipaths have been observed to stimulate the interaction of cholesterol with cholesterol oxidase, saponin and cyclodextrin, presumably by displacing cholesterol laterally from its phospholipid complexes. We now report that this effect, referred to as cholesterol activation, occurs with dozens of other amphipaths, including alkanols, saturated and cis- and trans-unsaturated fatty acids, fatty acid methyl esters, sphingosine derivatives, terpenes, alkyl ethers, ketones, aromatics and cyclic alkyl derivatives. The apparent potency of the agents tested ranged from 3 μ M to 7 mM and generally paralleled their octanol/water partition coefficients, except that relative potency declined for compounds with > 10 carbons. Some small amphipaths activated cholesterol at a membrane concentration of \sim 3 mol per 100 mol of bilayer lipids, about equimolar with the cholesterol they displaced. Lysophosphatidylserine countered the effects of all these agents, consistent with its ability to reduce the pool of active membrane cholesterol. Various amphipaths stabilized red cells against the hemolysis elicited by cholesterol depletion, presumably by substituting for the extracted sterol. The number and location of cis and trans fatty acid unsaturations and the absolute stereochemistry of enantiomer pairs had only small effects on amphipath potency. Nevertheless, potency varied \sim 7-fold within a group of diverse agents with similar partition coefficients. We infer that a wide variety of amphipaths can displace membrane cholesterol by competing stoichiometrically but with only limited specificity for weak association with phospholipids. Any number of other drugs and experimental agents might do the same.

This study probed the state of cholesterol in the RBC¹ membrane by using a wide variety of intercalating amphipaths to displace sterol molecules from phospholipid complexes. Sterols have evolved as universal constituents of eukaryotic plasma membranes, where they serve multiple functions (see, for review, refs (1–3).) The associations of sterols with bilayer phospholipids are ordered to some degree, with the sterol 3- β -hydroxyl group hydrogen-bonded to headgroup polar atoms and the steroid ring system and branched octyl tail aligned with the fatty acid chains. These associations are generally weak, short-lived, and of limited structural specificity, especially those involving phospholipids bearing (poly)unsaturated chains (4–7). Phospholipids bearing large head groups (*e.g.*, phosphorylcholine) and, especially, saturated fatty acyl chains form stronger complexes with sterols (8–12). These complexes appear to have characteristic proportions; for example, 2 sphingomyelins per cholesterol (13), 2 dimyristoylphosphatidylethanolamines per cholesterol (14), 3 dimyristoylphosphatidylcholines per 2 cholesterol (15) and one dioleoylphosphatidylcholine per cholesterol (16). The shielding

of sterols from the water phase by the large polar head groups of phospholipids also contributes to their stoichiometric association (17, 18).

The cholesterol present in excess of the complexing capacity of membrane phospholipids is relatively free to interact with other molecules, a property we shall refer to as its *activity* (6). For example, excess plasma membrane cholesterol reacts more readily with the lytic intercalator, saponin, and with probes in the aqueous phase such as cholesterol oxidase and cyclodextrin (19). The enhanced reactivity of uncomplexed sterols would seem to derive from the increased rate and extent of their partial projection into the aqueous medium. This mechanism would cause plasma membrane cholesterol in excess of the phospholipid equivalence point to be removed through both passive transfer and metabolic (homeostatic) pathways, thereby setting the membrane level of the sterol at stoichiometric equivalence with the phospholipids (6). The properties of excess membrane cholesterol have been interpreted to reflect the elevated chemical activity or fugacity of the unassociated sterol molecules, meaning that they have a higher escape tendency or activity coefficient than those in complexes (10, 20).

Like sterols, amphipaths are small molecules with polar and nonpolar ends that can intercalate into bilayers in an oriented fashion; for examples, see refs (21–24). This class includes a vast number of metabolites, drugs, detergents and natural products with myriad cellular effects. Of importance here, a few amphipaths have been observed to activate bilayer cholesterol, apparently by displacing the sterol from its phospholipid partners (6). For example, 1-octanol [9], C6:0-ceramide [61] and di(C8:0)glyceride

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^{*}To whom correspondence should be addressed at Department of Pathology, Rush University Medical Center, 1653 W. Congress Pkwy., Chicago, IL 60612. Tel: 312-942-5256. Fax: 312-563-3115. E-mail: ylange@rush.edu.

Abbreviations: HMGR, hydroxy-3-methylglutaryl coenzyme A reductase; LPS, 1- α -lyso(palmitoyl)phosphatidyl-L-serine; PBS, 0.15 M NaCl containing 5 mM Na Pi at pH 7.4; rac-, racemic; RBC, red blood cell; SEM, standard error of the mean.

promote the attack of cholesterol oxidase on membranes and stimulate the transfer of plasma membrane cholesterol to cyclodextrin (19). The observation that these intercalators promote the partition of cholesterol from red cell membranes to cyclodextrin is consistent with their postulated ability to increase the chemical activity of the sterol (10, 19, 20). Furthermore, diverse intercalators increase the susceptibility of bilayer cholesterol to saponin (19) and other sterol-specific membrane lysins (25, 26); intercalators also oust cholesterol from rafts (27, 28). We have hypothesized that the displaced cholesterol remains dispersed in the bilayer where it exhibits the same behavior as cholesterol in excess of its stoichiometric equivalence point (6). Related to the displacement of cholesterol from phospholipids by amphipaths is their ability to rescue of RBCs from hemolysis following the depletion of their cholesterol; presumably, the amphipaths take the place of the missing sterol (19). In addition, some amphipaths have been shown to mimic cholesterol, at a similar stoichiometry, in promoting lateral phospholipid condensation into liquid-ordered bilayer phases (23, 29, 30).

The activation of membrane cholesterol has only been reported for a few amphipaths, and those characterizations were incomplete. We now ask: At what *membrane* concentration do these amphipaths act? Is cholesterol activation driven by competitive displacement? Is there structural specificity for such displacement reactions that sheds light on the association of cholesterol and/or amphipaths with phospholipids? To address these questions, we have determined the relative potencies of 63 structurally diverse amphipaths using several measures of cholesterol activation (19).

EXPERIMENTAL PROCEDURES

Materials. Amphipaths were chosen to allow the evaluation of relevant functional groups and the comparison of related structures. We primarily selected uncharged agents that rapidly equilibrate across bilayers (31, 32). Unless noted, chemicals and biochemicals, including cholesterol oxidase (*Streptomyces* sp.) and saponin mixture #S4521, were the highest quality provided by the general inventory or the DiscoveryCPR area of Sigma-Aldrich. C6:ceramide [61] was obtained from Avanti Polar Lipids. The amphipaths are listed in Table 1 according to their ClogP values and referenced in the text with bracketed bold numerals. We purchased 1 α ,2 α -[3 H]cholesterol from Amersham/GE and hydroxymethylglutaryl-CoA bearing a DL-3-[glutaryl-3- 14 C] label from American Radiolabeled Chemicals, Inc.

Whole blood was drawn from a healthy human volunteer and stored on ice. Just prior to use, the cells were separated from the plasma and buffy coat and washed thrice with 10 volumes of PBS. It was estimated that 1 μ L of packed cells contained 1.0×10^7 cells and 2.5 nmol of cholesterol. One of the many advantages of erythrocytes is their indifference to the various physiologic stimuli some amphipaths exert on nucleated cells.

Potency of Agents in Promoting Hemolysis by Cholesterol Oxidase (19). Aliquots containing 0.24 μ L of washed and packed RBC suspended in 200 μ L of PBS (final) were distributed in 96-well plates. Serial concentrations of amphipaths in DMSO or ethanol (< 1% v/v final) were then added to the wells, and the plates were preincubated for 5 min at 37 °C. (By adding agents in solvent to RBC suspensions, we fostered their direct uptake and thereby minimized losses due to limited aqueous solubility.) Neither 1% DMSO nor 1% ethanol had a significant effect in any of our assays. Cholesterol oxidase (0.4 IU) or a buffer blank

was added and the samples were incubated at 37 °C. At intervals over 1–2 h, the samples were mixed by inverting the plates and optical absorbance was determined at 500 nm. Decreasing absorbance, reflecting diminished light scattering, was shown to report hemolysis. (Under our conditions, none of the amphipaths caused hemolysis in the absence of cholesterol oxidase.) Fractional hemolysis was plotted as a function of incubation time (see Figure 1A); the interval required for a 50% decrease in absorbance from the highest reading (minus amphipath) to the lowest reading (reaction plateau) was obtained by interpolation or extrapolation. These values were replotted to estimate the total concentration of an agent that caused 50% hemolysis by cholesterol oxidase after 1 h at 37 °C (see Figure 1B). We used **H** to denote these values and calculated potencies as $-\log H$ (in molar units; see Table 1). True estimates of potency would, in principle, be given by the aqueous concentration of agents at equilibrium. Fortunately, the free concentrations are the nearly the same as the total concentration for amphipaths with ClogP up to 4 (e.g., 1-decanol [31]) because only a small fraction of those agents is taken up by the membranes under these conditions. Two to 5 independent determinations were made for each agent. Note that a 10 μ M amphipath delivers ~ 1 mol of agent/mol of RBC cholesterol.

Potency of Agents in Promoting Hemolysis by Saponin (19). As with the cholesterol oxidase method described above, 0.24 μ L of packed RBCs were treated with 4 μ g of saponin plus amphipaths in 200 μ L of PBS (final) in microtiter wells (this time at room temperature) and optical absorbance determined periodically at 500 nm to measure hemolysis. (None of the amphipaths caused hemolysis in the absence of saponin.) The interval required for a 50% decrease in absorbance from the highest reading (minus amphipath) to the lowest reading (plateau) was obtained by interpolation or extrapolation. These values were replotted as a function of amphipath concentration (see Figure 1D). **S** denotes the concentration required for half-lysis in 15 min at room temperature. The potency of an agent determined by the saponin method was expressed as $-\log S$, in molar units. Two or 3 independent determinations were made on each agent.

Effect of Amphipaths on the Transfer of [3 H]Cholesterol from RBCs to Cyclodextrin (19). 160 μ L of packed RBCs was pre-equilibrated at room temperature in 16 mL of PBS containing 160 mg of methyl- β -cyclodextrin plus 1.68 mg of cholesterol, proportions that maintain cell cholesterol content near their normal level. The cells were pelleted and the supernatants saved. The cells were then pulse-labeled with [3 H]cholesterol and washed. Various amphipaths were added to aliquots of the pre-equilibrated cyclodextrin-cholesterol acceptor. 60 μ L aliquots of the packed [3 H]cholesterol-labeled cells were then mixed with 5.94 mL aliquots of the acceptor, the mixtures were incubated at 10 °C and the label appearing in the supernatant was determined periodically after centrifugation as in Figure 4.

Protection by Amphipaths against the RBC Hemolysis by Cholesterol Depletion (19). 15 μ L of packed cells was mixed with 2.5 mL of PBS at room temperature. Methyl- β -cyclodextrin was added to 6.6 mg/mL so as to extract about half of the cell cholesterol. 200 μ L aliquots of this mixture were immediately placed in wells containing varied amounts of amphipath, and optical absorbance was then followed at 500 nm. Percent lysis at 15 min was estimated using controls for zero and complete hemolysis; these values were plotted against the agent concentration as in Figure 6.

Table 1: Potencies and Partition Coefficients of Amphipaths

	agent ^a	ClogP ^b	H ^c	SEM, %	potency ^d	S ^e
1	hexanol, 1-	1.88	7200		2.14	
2	heptanol, 1-	2.41	1260		2.90	1380
3	heptanoic acid	2.45	1200		2.92	
4	decyn-1-ol, 5-	2.64	500		3.30	630
5	octanol, <i>rac</i> -2-	2.72	1447	8	2.84	1630
6	octanol, <i>rac</i> -3-	2.72	1427	23	2.85	1100
7	diethylene glycol monoethyl ether	2.82	420		3.38	
8	methyl heptanoate	2.83	1080		2.97	848
9	octanol, 1-	2.94	498	6	3.30	400
10	ethylene glycol monoethyl ether	2.96	350	13	3.46	
11	nonanone, 2-	2.97	1000	6	3.00	
12	geraniol, <i>trans</i> -; <i>trans</i> -3,7-dimethyl-octa-2,6-dien-1-ol	2.97	605	7	3.22	450
13	geraniol, <i>cis</i> -; <i>cis</i> -3,7-dimethyl-octa-2,6-dien-1-ol	2.97	430	2	3.37	320
14	nonen-1-ol, <i>cis</i> -3-	2.98	340	9	3.47	280
15	octanoic acid	2.98	370		3.43	
16	butyl ether	2.99	2433	14	2.62	2500
17	nonen-1-ol, <i>cis</i> -2-	3.18	290	5	3.54	178
18	nonen-1-ol, <i>trans</i> -2-	3.18	263	7	3.58	270
19	thymol; 2-isopropyl-5-methylphenol	3.20	225		3.65	
20	nonanol, (+)-2-	3.25	527	9	3.28	380
21	nonanol, (-)-2-	3.25	323	6	3.49	260
22	citronellol, (+)- β -; (<i>R</i>)-3,7-dimethyl-6-octen-1-ol	3.25	290	8	3.54	220
23	citronellol, (-)- β -; (<i>S</i>)-3,7-dimethyl-6-octen-1-ol	3.25	255	10	3.60	200
24	methyl octanoate	3.36	450		3.35	
25	nonanol, 1-	3.47	109		3.96	
26	nonanoic acid	3.51	1600		2.80	
27	decen-1-ol, <i>trans</i> -5-	3.51	120		3.92	140
28	dimethyl-1-octanol, 3,7-	3.74	63	14	4.20	
29	decanol, 2-	3.78	120		3.92	130
30	propofol; 2,6-diisopropylphenol	3.93	125		3.90	
31	decanol, 1-	4.00	23		4.64	42
32	decanoic acid	4.04	90		4.05	
33	cyclododecanone	4.22	139	17	3.86	70
34	methyl decanoate	4.41	50		4.30	
35	undecanol, 1-	4.53	25		4.60	10
36	cyclododecanol	4.62	48		4.32	70
37	dodecanol, 2-	4.84	27	6	4.57	24
38	farnesol, <i>trans,trans</i> -; <i>trans,trans</i> -3,7,11-trimethyl-2,6,10-dodecatrien-1-ol	5.00	10		5.00	12
39	dodecanol, 1-	5.06	11		4.97	18
40	hexyl ether	5.10	50		4.30	100
41	lauric acid; <i>dodecanoic acid</i>	5.10	38		4.42	
42	methyl laurate	5.47	16	11	4.79	
43	tridecanol, 1-	5.58	11	5	4.96	11
44	tridecanoic acid	5.63	64		4.19	
45	chlorpromazine	5.80	122		3.91	31
46	ethyl-2-methylundecan-1-ol, 7-	5.85	21		4.68	
47	sphingosine, D-erythro-	5.94	11		4.96	
48	tetradecanol, 1-	6.11	11	9	4.96	15
49	myristic acid; <i>tetradecanoic acid</i>	6.15	7.6		5.12	
50	methyl myristate	6.53	16		4.80	
51	palmitoleic acid; <i>cis</i> -9-hexadecenoic acid	6.73	4.2		5.38	
52	linolenic acid, γ -; <i>cis,cis,cis</i> -6,9,12-octadecatrienoic acid	6.82	21	2	4.68	11
53	linolenic acid, α -; <i>cis,cis,cis</i> -9,12,15-octadecatrienoic acid	6.82	12	5	4.93	13
54	hexadecanol, 1-	7.17	8.0	11	5.1	
55	palmitic acid; <i>hexadecanoic acid</i>	7.21	6.0		5.22	
56	octyl ether	7.22	10	41	5.00	
57	linoleic acid; <i>cis,cis</i> -9,12-octadecadienoic acid	7.30	3.1		5.51	
58	arachidonic acid; <i>cis,cis,cis,cis</i> -5,8,11,14-eicosatetraenoic acid	7.39	7.2		5.14	
59	elaidic acid; <i>trans</i> -9-octadecenoic acid	7.79	6.3	3	5.21	
60	oleic acid; <i>cis</i> -9-octadecenoic acid	7.79	4.0	5	5.40	7.0
61	sphingosine, N-hexanoyl-D-erythro-	8.25	7.5		5.12	
62	stearic acid; <i>octadecanoic acid</i>	8.27	17		4.77	
63	phytanic acid; 3,7,11,15-tetramethyl hexadecanoic acid	8.81	3.0	2	5.52	3.0

^aItalicized names following semicolons are chemically descriptive synonyms of the common names. ^bClogP, literature calculated octanol/water partition coefficient (see Experimental Procedures). ^cH, micromolar concentration promoting hemolysis in the cholesterol oxidase assay. ^dPotency, $-\log H$ (molar concentration). ^eS, micromolar concentration promoting hemolysis in the saponin assay.

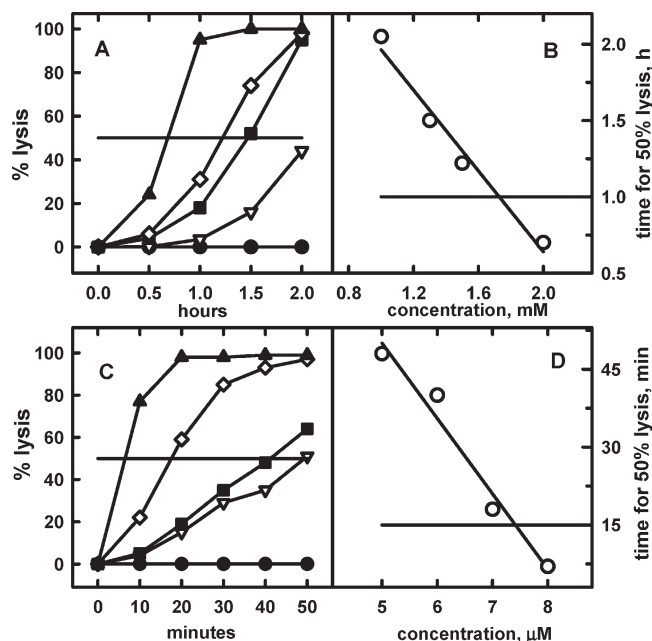


FIGURE 1: Effect of amphipaths on red cells. Panel A: cholesterol oxidase lysis assay. Washed RBCs ($0.24 \mu\text{L}$) suspended in 0.2 mL of PBS (pH 7.4, final volume) were pretreated in wells with 0 (\bullet), 1.0 (∇), 1.3 (\blacksquare), 1.5 (\diamond) and 2.0 (\blacktriangle) mM nonanoic acid [26] for 5 min at 37°C . Cholesterol oxidase (0.4 IU) was then added, the plate incubated at 37°C , optical absorbance determined periodically and fractional hemolysis calculated. Panel B: The time intervals for 50% lysis (horizontal line in panel A) were replotted so as to estimate H , the concentration required to achieve 50% lysis at one hour (horizontal line in panel B). Panel C: saponin lysis assay. Red cells were pretreated as in panel A with oleic acid [60] at $0 \mu\text{M}$ (\bullet), $5 \mu\text{M}$ (∇), $6 \mu\text{M}$ (\blacksquare), $7 \mu\text{M}$ (\diamond), and $8 \mu\text{M}$ (\blacktriangle). Saponin ($4 \mu\text{g}$) was added to each well; the plates were incubated at room temperature, optical absorbance determined periodically and fractional hemolysis calculated. Panel D: The time intervals for 50% lysis by saponin (horizontal line in panel C) were replotted as a function of oleic acid concentration so as to estimate S , the concentration required to achieve 50% lysis at 15 min (horizontal line in panel D).

Promotion of the Inactivation of HMGR by Amphipaths (33). Human fibroblasts were cultured as described (34). Flasks were incubated overnight in medium containing 5% lipoprotein-deficient serum to induce HMGR activity. The flasks were then incubated at 37°C for 1 h with 2 mL of PBS containing the test agent or solvent blank ($<1\%$ ethanol or DMSO). Enzyme activity was determined on homogenates of the cells; duplicate values were averaged and expressed in pmol of mevalonate/min/mg of cell protein (as in Figure 7).

Lipophilicity Estimation. ClogP values expressing the log of octanol/water partition coefficients were calculated using the Biobyte algorithm in ChemDraw Ultra 9.0.1 (CambridgeSoft, Inc.). Values for fatty acids were for the undissociated (protonated) form. Estimates were validated using standard sets of measured octanol/water partition coefficients (35). Pairs of agents differing in ClogP values by ≤ 0.5 were not considered useful for comparison; for comparisons involving sphingosine derivatives, a difference ≥ 0.7 log unit was required.

RESULTS

Hemolysis by Cholesterol Oxidase Provides an Assay for Cholesterol Activation by Amphipaths. Cholesterol oxidases interact only superficially with bilayers, and the deeply seated cholesterol in plasma membranes is typically a very poor substrate for the enzyme (36). However, cholesterol susceptibility

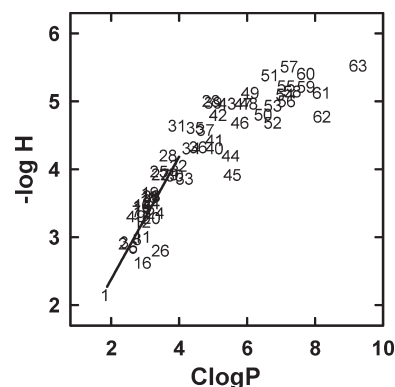


FIGURE 2: Relationship of the potency of amphipaths in promoting red cell lysis by cholesterol oxidase to their partition coefficients. $-\log H$ (potency, molar) was plotted versus ClogP (computed log octanol/water partition coefficient) for 63 agents numbered as in Table 1. The linear least-squares fit to the 31 points with ClogP < 4 had a slope of 0.90 and an R^2 of 0.68.

is dramatically increased when membranes are slightly enriched with exogenous cholesterol (37), treated with small amounts of certain amphipaths (19, 33) or otherwise mildly perturbed (38–40). It appears that the action of the amphipaths is to increase its availability to the enzyme at the membrane surface (see introductory comments). Because the consequent oxidation of cholesterol promotes RBC lysis, the resulting drop in light scattering provides a facile microwell indicator for the activation of membrane cholesterol by amphipaths (19).

Figure 1A illustrates the method. Here, increasing concentrations of the amphipath, nonanoic acid [26], promoted hemolysis with progressively shorter lag times. Replotting the time required for 50% hemolysis as a function of amphipath concentration gave a reasonably straight line (Figure 1B) from which the concentration causing half-lysis after one hour, H , was estimated (Table 1). Assay precision was evaluated by performing 3 or 4 experiments on different days on each of 23 representative agents. From these data, the average SEM for the assay was estimated to be 8.1%. We calculated from a two-tailed t test that a difference of 33% between the H values of two agents was significant at a confidence level of $p = 0.05$.

Comparative Analysis. The agents we tested included ten 1-alkanols, ten saturated fatty acids, five unsaturated C18 fatty acids, the methyl esters of five fatty acids, 5 terpenes, 3 alkyl ethers, 2 ketones, 2 cyclic alkyl derivatives, several enantiomeric and cis/trans pairs, and various other agents (including sphingosine derivatives and membrane-active aromatics). The formula weights of these compounds ranged between 110 and 400. A few very water-insoluble compounds gave erratic results and were not presented.

As shown in Table 1, 63 amphipaths promoted hemolysis by cholesterol oxidase. H values varied inversely with amphipath partition coefficients from $3 \mu\text{M}$ to 7.2 mM . Cholesterol-activating potencies ($-\log H$) are also listed in Table 1. As shown in Figure 2, amphipath potency varied linearly with ClogP with a slope of 0.90 up to a ClogP ~ 4 , beyond which point the slope decreased toward a plateau. Primary alcohols tended to be more effective than other classes of intercalators at a given ClogP, while 2-nonanone [11] and butyl ether [16] were substantially less potent than their ClogP values would suggest. (Their relatively low H values were statistically significant in that their SEM values were 6% and 14% ($n = 3$), respectively.) Branches and rings tended to reduce cholesterol activation potency compared

Table 2: Comparison of Isomeric Pairs of Amphipaths

agent	no. ^a	ClogP ^b	H ^c	SEM ^d	% SEM	p ^e
octanol, <i>rac</i> -2-	5	2.72	1447	107	7.5	0.96
octanol, <i>rac</i> -3-	6	2.72	1427	325	23	
geraniol, <i>trans</i> -	12	2.97	605	44	7.3	0.031
geraniol, <i>cis</i> -	13	2.97	430	10	2.3	
nonen-1-ol, <i>cis</i> -2-	17	3.18	290	15	5.2	0.30
nonen-1-ol, <i>trans</i> -2-	18	3.18	263	17	6.5	
nonanol, (+)-2-	20	3.25	527	47	8.9	0.016
nonanol, (-)-2-	21	3.25	323	20	6.5	
citronellol, (+)- β -(<i>R</i>)-	22	3.25	290	23	7.3	0.39
citronellol, (-)- β -(<i>S</i>)-	23	3.25	255	25	9.8	
linolenic acid, γ -	52	6.82	21	0.5	2.4	0.0001
linolenic acid, α -	53	6.82	12	0.6	5.1	
elaidic acid	59	7.79	6.3	0.17	2.7	0.015
oleic acid	60	7.79	4.0	0.2	5.0	

^aCompounds numbered as in Table 1. ^bClogP, literature calculated octanol/water partition coefficient (see Experimental Procedures). ^cH, micromolar concentration promoting hemolysis in the cholesterol oxidase assay. ^dN \geq 3. ^ep, the probability that the two H values reported for an isomer pair are the same, based on a two-tailed *t* test. For 95% confidence, *p* \leq 0.05.

with normal aliphatic chains; for example, cyclododecanone [33] was less potent than either 1-decanol [31] or 1-undecanol [35]. The two phenol derivatives tested, thymol [19] and propofol [30], had potencies roughly commensurate with their ClogP values. The relatively weak effect of chlorpromazine [45], the only amine tested, may be related to its positive charge.

Scaled to their ClogP values, the saturated and unsaturated C18 fatty acids in Table 1 had comparable potencies that were surprisingly insensitive to the number, position and *cis/trans* orientation of their double bonds. [Inexplicably, nonanoic acid [26] was a disproportionately poor effector.] The ClogP values of fatty acids were computed for their undissociated (neutral) form. The aqueous pK_a values for fatty acids are near 5 and should render them fully deprotonated at the assay pH of 7.4; however, they appear to intercalate in the protonated form (32, 41, 42). If so, the fatty acids would be far more potent than indicated here, presumably because of strong hydrogen bonding through their protonated carboxyl groups (43).

Because ClogP values are subject to experimental and computational errors, we tested seven pairs of isomeric compounds, since they have identical ClogP values, at least for partition into octanol. Table 2 shows that three of the matched pairs had statistically indistinguishable H values at 95% confidence. While four other isomer pairs showed significant differences in their H values, these were less than 2-fold in each case.

Hemolysis by Saponin Provides a Complementary Assay for Cholesterol Activation by Amphipaths. Saponins complex with the cholesterol in plasma membranes to form lytic pores (44). It has been observed that increasing membrane cholesterol or adding amphipaths reduces the amount of saponin required for this hemolysis (19, 45). Apparently, in all these cases, the uncomplexed (hence, free and active) sterol molecules preferentially ligand with saponin to build oligomeric pores. It follows that saponins compete with phospholipids for association with sterol molecules.

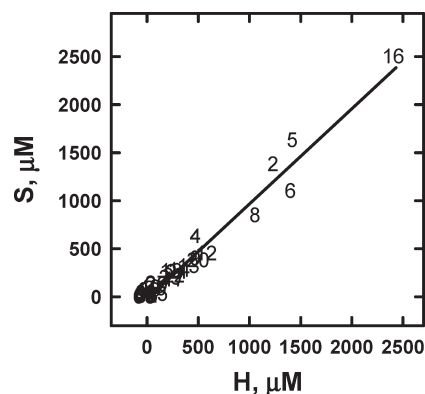


FIGURE 3: Correlation of the ability of 33 amphipaths to promote red cell lysis by cholesterol oxidase (H) and saponin (S). The linear least-squares fit had a slope of 0.958 and an *R*² of 0.992.

The utility of the saponin lysis assay in assessing the ability of amphipaths to activate cholesterol is illustrated in Figure 1, panels C and D. The values obtained with saponin paralleled those with cholesterol oxidase quite closely. As shown in Figure 3, a plot of values for H versus S for 33 randomly selected compounds from Table 1 gave a line with a least-squares best fit slope of 0.958 and an *R*² of 0.992. (Short chain fatty acids were omitted from the saponin data set because they interfered with this assay.) That the concentrations of amphipaths causing hemolysis by saponin were nearly the same as those using cholesterol oxidase merely reflects our choice of experimental conditions. On the other hand, the linearity and high *R*² value in Figure 3 shows that the two independent assays report on the same parameter.

A few compounds gave divergent results in the two assays. Notably, chlorpromazine [45] was conspicuously weak in the cholesterol oxidase assay (two independent H values of 120 and 122 μ M) relative to its ClogP, while its S value (namely, 31 and 33 μ M in two independent determinations) was more in line with its ClogP.

Effect of Amphipaths on Cholesterol Transfer to Cyclodextrin. It has been shown that the exit of cholesterol from phospholipid monolayers and plasma membranes to aqueous cyclodextrin proceeds several times more rapidly when the sterol level is increased above its apparent stoichiometric equivalence point (15, 33). The presence of 1-octanol [9], di(C8:0)glyceride or C6:0-ceramide [61] also stimulates the rate and extent of cholesterol transfer (19). It was postulated that these treatments activate cholesterol. To test the generality of this hypothesis, we tested the effect of agents in two other chemical classes on the rate of transfer of [³H]cholesterol from RBCs to cyclodextrin. In the experiments shown in Figure 4, the rate of cholesterol transfer was stimulated 1.8-fold by hexyl ether [40] and 3.0-fold by nonanoic acid [26]. Even greater stimulation was observed with higher concentrations of these amphipaths in other experiments.

LPS Counters the Amphipaths. Our working hypothesis postulates that active (free, uncomplexed) cholesterol can arise when it exceeds the capacity of its phospholipid partners or when displaced from them by competing amphipaths (6). Supporting that view is the observation that lysophosphatides oppose the effect of amphipaths in promoting cholesterol activation, as if complexing with the active sterol (19). We therefore tested whether LPS reversed the action of several of the amphipaths listed in Table 1. The data in Figure 5A support the hypothesis: increasing amounts of LPS progressively reduced the cholesterol

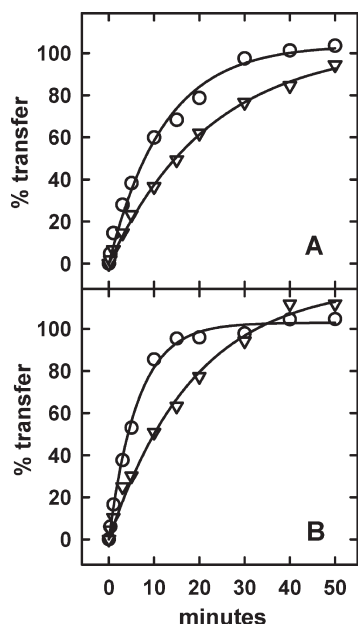


FIGURE 4: Effect of amphipaths on the transfer of [^3H]cholesterol from red cells to cyclodextrin. Panel A: RBCs were pre-equilibrated in PBS (pH 7.4) with cyclodextrin-cholesterol mixtures in proportions that do not appreciably alter RBC cholesterol (see Experimental Procedures). The mixtures were centrifuged and the pellet and supernatant saved. The equilibrated cells were pulse-labeled with [^3H]cholesterol and washed. Aliquots of the pre-equilibrated cyclodextrin-cholesterol acceptor were mixed with ethanol alone (0.1% final, ▽) or containing 0.14 mM hexyl ether [40] (final, ○). 60 μL aliquots of packed [^3H]cholesterol-labeled cells were then added to 5.94 mL of the pre-equilibrated acceptor, and the transfer of label was followed at 10 $^{\circ}\text{C}$ and plotted as percent of input radioactivity transferred. Panel B: As in panel A, except that the treatments were either ethanol alone (0.1% final, ▽) or containing 4 mM nonanoic acid [26] (final, ○), and the PBS was at pH 6 to increase the membrane uptake of the fatty acid.

oxidase-dependent hemolysis induced by linoleic acid. Similarly, Figure 5B summarizes the evidence that LPS protects RBCs from hemolysis by cholesterol oxidase induced by two primary alcohols, a fatty acid and an ether.

Replacement of Cholesterol by Amphipaths. In an earlier study, the removal of about half of the cholesterol from erythrocytes caused their rapid lysis but the immediate addition of 1-octanol, a diglyceride or a ceramide arrested this process (19). It was inferred that the amphipaths substituted for the missing cholesterol molecules. To test the generality of this phenomenon, we examined the effects of several alkanols and fatty acids in this assay (Figure 6). The ten new amphipaths tested all protected the cholesterol-depleted RBC from hemolysis with an order of potency that paralleled that found in the foregoing tests.

Inactivation of HMGR by Amphipaths. Elevating plasma membrane cholesterol slightly above its physiological rest point increases the pool of intracellular cholesterol (46) and stimulates the rapid inactivation of the rate-determining enzyme for cholesterol biosynthesis, HMGR (33). It was subsequently found that 1-octanol [9], C6:0-ceramide [61] and di(C8:0)glyceride have similar effects (19). To further test the premise that amphipaths can displace plasma membrane cholesterol from phospholipids and thereby promote its transfer to the cytoplasm of tissue cells, we assessed the acute effects of two alcohols, a fatty acid and an ether on the activity of HMGR activity in human fibroblasts. As shown in Figure 7, all of these agents reduced the activity of the

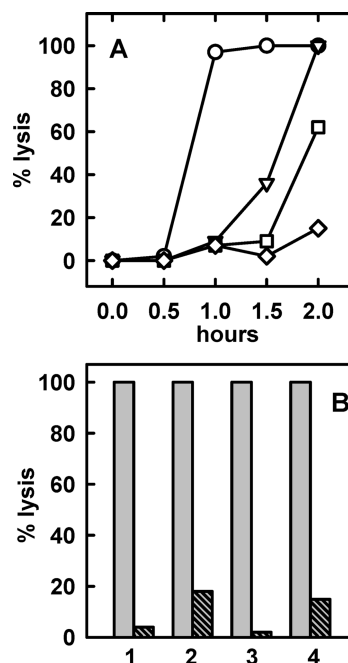


FIGURE 5: Inhibition of hemolysis by lysophosphatidylserine (LPS). Panel A: Washed RBCs (0.24 μL) were suspended in wells containing 0.2 mL of PBS (final volume) plus 1 nmol of linoleic acid [57] and 0 (○), 0.2 (▽), 0.4 (□) or 0.8 (◇) nmol of LPS. Cholesterol oxidase (0.4 IU) was added, and the samples were incubated at 37 $^{\circ}\text{C}$. Optical absorbance was determined periodically and fractional hemolysis calculated. Panel B: To paired aliquots of 0.24 μL of packed RBCs in wells containing 200 μL of PBS (final) was added (1) 70 nmol of 1-octanol [9]; (2) 16 nmol of hexyl ether [40]; (3) 2 nmol of 1-dodecanol [39] and (4) 1 nmol of linoleic acid [57]. LPS (0.3–0.6 nmol) was added to one of each pair (right, striped bars). Finally, 0.4 IU of cholesterol oxidase was added and the plate incubated at 37 $^{\circ}\text{C}$ for 2 h before optical absorbance was determined and fractional hemolysis calculated.

enzyme at concentrations a few-fold greater than those required for their action on RBCs, described above.

Other Agents. It was reported previously that decane stimulates the action of cholesterol oxidase on red cell membranes (39). We now report that hexane has a potency of ~ 9 mM in both the cholesterol oxidase and saponin assays. This value is close to that obtained for 1-hexanol [1]. Given that the ClogP for hexane is 3.9 (i.e., 2 orders of magnitude greater than that for 1-hexanol), we infer that this alkane is a far weaker activator of cholesterol than the corresponding agents in Table 1. We also found that two detergents, cetyltrimethylammonium bromide (CTAB) and dodecyl maltoside, were not effective in either the cholesterol oxidase or the saponin assay up to concentrations that were lytic by themselves (approximately 20 and 50 μM , respectively). Triton X-100 (with a log partition coefficient of 3.14) was unusually potent; it promoted cholesterol oxidase and saponin susceptibility at $\text{H} = 45$ μM and $\text{S} = 18$ μM . These levels are far below its critical micelle concentration and its hemolytic concentration. However, Triton X-100 did not replace extracted cholesterol in assays akin to that shown in Figure 6 and may act by a different mechanism that is not relevant here.

DISCUSSION

1. Many Amphipaths Activate Cholesterol by Displacing It from Phospholipids. Incrementing plasma membrane cholesterol by a few percent above its physiological rest-point has been shown to create a pool of active sterol (6). The properties of

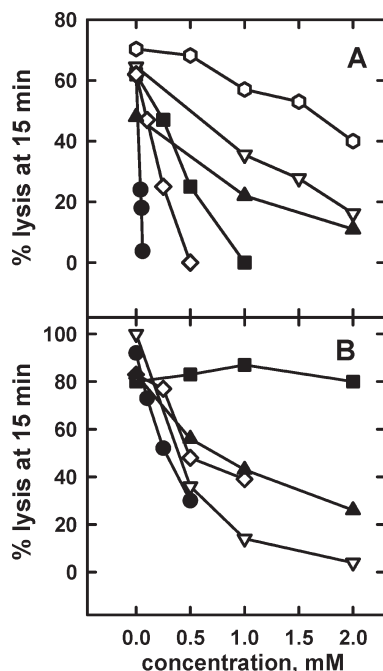


FIGURE 6: Amphipaths protect red cells from hemolysis by cholesterol depletion. Panel A: To deplete RBC cholesterol by roughly half, replicate aliquots of 1.2 μ L of packed cells were incubated in wells containing 1.33 mg of methyl- β -cyclodextrin in 200 μ L of PBS (final) for 8 min at room temperature. Amphipaths were added immediately, and optical absorbance was determined 15 min later. Fractional hemolysis was calculated relative to undepleted controls. The *n*-alcohols were heptanol [2] (○), octanol [9] (▽), decanol [31] (▲), tridecanol [43] (◇), tetradecanol [48] (■), and hexadecanol [54] (●). Panel B: As in panel A, but with these fatty acids: lauric [41] (■), elaidic [59] (▲), linoleic [57] (◇), palmitic [55] (▽), and oleic [60] (●).

excess cholesterol were mimicked by all 63 amphipaths listed in Table 1 when tested in any of our six assays. It follows that these intercalators activate cholesterol; that is, they make the sterol more interactive with two probes and promote its partition to exogenous cyclodextrin, as if increasing its chemical activity (19). In contrast to this class of agents, a few alkanes and detergents were weak or ineffective. It is conceivable that some amphipaths (perhaps, for example, Triton X-100) act by perturbing the molecular organization of the bilayer; see refs (47–49). However, our evidence for the 63 amphipaths in Table 1 favors the hypothesis that they displace cholesterol from its association with phospholipids, as follows.

(a) That all of the amphipaths tested stimulated the attack of cholesterol oxidase on the RBC membrane suggests that they increased the transient partial projection of cholesterol molecules into the aqueous compartment (50), thereby increasing sterol accessibility to the enzyme at the membrane surface (36).

(b) That amphipaths promoted saponin hemolysis suggests that they competitively displace cholesterol laterally from its association with phospholipids so as to increase its availability to the lytic agent. A similar case was recently made for perfringolysin O (26).

(c) The exit of sterol molecules from membranes to cyclodextrin has been taken as a good indicator of their chemical activity, escape tendency or fugacity (10, 20). We showed previously that 1-octanol [9], 1-hexadecanol [54], C6:0-ceramide [61] and di-(C8:0)glyceride all promote the rate and extent of cholesterol transfer in this system (19, 33). The data in Figure 4 generalize the evidence for membrane cholesterol activation by amphipaths.

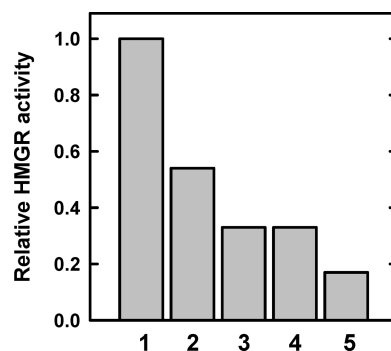


FIGURE 7: Effect of amphipaths on HMG-CoA reductase activity. Fibroblasts were preincubated overnight in growth medium containing 5% lipoprotein-deficient serum. The medium was replaced by 2 mL of PBS containing 0.5–0.8% ethanol \pm agents, the concentrations of which were scaled to their relative potency, given in Table 1. The flasks were incubated at 37 $^{\circ}$ C for 1 h, following which HMGR activity and cell protein were determined in duplicate and HMGR activities (pmol of mevalonate/min/mg of cell protein) plotted relative to controls lacking agents: (1) ethanol control; (2) 160 μ M 1-decanol [31]; (3) 160 μ M hexyl ether [40]; (4) 20 μ M linoleic acid [57]; (5) 80 μ M 1-dodecanol [39]. Composite of representative experiments.

(d) Lysophosphatides have been shown to reduce the effects of elevated plasma membrane cholesterol and to counter cholesterol activation by three amphipaths (19, 33). This action was ascribed to the ability of lysophosphatides to associate with sterols, just as do bilayer phospholipids (6). The present results show that lysophosphatides also counter the activation of cholesterol by a variety of other amphipaths (Figure 5). The reversal of amphipath activation by lysophosphatides provides reassurance that the intercalators are not simply activating cholesterol oxidase directly.

(e) A variety of primary alcohols and fatty acids were shown to forestall the lysis of RBCs induced by the depletion of their cholesterol, as if replacing the lost sterol (Figure 6). It is plausible that the amphipaths associate with the phospholipids in a manner akin to cholesterol and thereby stabilize the membrane (19). Support for this premise is the evidence that 1-hexadecanol [54] condenses and stabilizes phospholipid monolayers, duplicating the action of cholesterol mole for mole (23). That higher levels of the intercalators were required to rescue depleted cells from hemolysis than to stimulate the action of cholesterol oxidase and saponin is consistent with the fact that activation of only a small percentage of the cholesterol is required for the latter effects; presumably, far larger amounts of amphipaths are needed to replace the substantial fraction of cholesterol removed in the depletion experiments.

(f) Excess cholesterol exits the plasma membrane not only to extracellular acceptors but also to the cytoplasm (6). The cholesterol pool in the endoplasmic reticulum serves as a homeostatic signal that restores plasma membrane cholesterol to its physiological set point. 1-Octanol [9] also increases the size of the cholesterol pool in the endoplasmic reticulum (19, 33), presumably through the displacement of the plasma membrane sterol (6). Active plasma membrane cholesterol goes to the mitochondria as well. There it is hydroxylated (51), and the 27-hydroxycholesterol product thereupon activates the proteolytic destruction of HMGR in the endoplasmic reticulum, another homeostatic feedback function (52, 53). It was previously shown that not only excess plasma membrane cholesterol but the intercalating amphipaths, 1-octanol [9], C6:0-ceramide [61] and

di(C8:0)glyceride, all stimulate the down-regulation of HMGR (19, 33). Figure 7 demonstrates that two other alkanols, a fatty acid and an ether do likewise, strengthening our core hypothesis. [It is worth considering that, under some circumstances, various pharmaceuticals and experimental agents might similarly drive plasma membrane cholesterol to the cytoplasm where it could falsely signal sterol excess to homeostatic proteins therein (6).]

While not every agent was examined with each of the six assays, there was persuasive consistency among the tests performed. Although a detailed understanding of molecular mechanism is lacking, a common final pathway appears to underlie all of our observations; namely, that the association of amphipaths with phospholipids leads to the displacement and replacement of the cholesterol in those complexes with consequent activation of the displaced sterol.

2. The Membrane Concentration of Amphipaths Required for Cholesterol Activation. We estimated this parameter using a molecular weight for cholesterol of 386, a mean molecular weight of RBC phospholipids of 750, the presence of 0.8 mol of cholesterol/mol of phospholipids (54, 55) and 5.1 mg of membrane lipid/packed mL of cells (56); hence, 8.7 nmol of membrane lipid/ μ L of packed RBC. From Table 1 and published partition coefficients for RBC membranes (57, 58), we calculated the membrane concentrations (in mole percent of RBC lipid) of 5 primary alkanols required for the activation of cholesterol at the observed *H* values: hexanol [1], 4.3 mol %; heptanol [2], 2.2 mol %; octanol [9], 3.5 mol %; nonanol [25], 2.9 mol %; and decanol [31], 1.3 mol %. The mean of these values is 2.8 mol % of RBC membrane lipid or \sim 6 mol of amphipath/100 mol of RBC cholesterol. This value is similar to the amount of extra cholesterol needed to render RBC membranes susceptible to cholesterol oxidase (37). It can therefore be inferred that about one mole of any of these amphipaths in the RBC membrane displaces and thereby activates one mole of cholesterol. This inference is in accord with the finding that 1-hexadecanol substitutes for cholesterol one-for-one in phospholipid monolayer studies (23). Given that the phospholipid species in the plasma membrane are very diverse (55), the weakest cholesterol complexes would presumably be the first to be disrupted by modest bilayer concentrations of amphipaths. The small mole fraction of intercalators required for cholesterol activation suggests that they act in a specific fashion, rather than through gross membrane perturbations.

3. The Driving Force for Amphipath Uptake. The partition of lipophiles into unstructured organic solvents is driven by the increase in the entropy of the water phase accompanying their transfer: the classical hydrophobic effect (59). In contrast, the transfer of a variety of amphipaths to bilayers is accompanied by a favorable enthalpy of solvation arising from weak interactions between the intercalators and the phospholipids (60–62). For example, the enthalpy change for the transfer of *n*-alkanols from water to synthetic vesicles and plasma membranes grows more favorable and the entropy change less favorable with increasing chain length (63). This enthalpy-driven mechanism, termed the “bilayer” or “non-classical” hydrophobic effect (64), suggests that the effects of the various agents studied here involves their weak chemical interaction with phospholipids.

4. Energetics of Cholesterol–Phospholipid Associations. The transfer of cholesterol from aqueous cyclodextrin complexes to phospholipid bilayer vesicles is also accompanied by a favorable enthalpy change and an unfavorable entropy

change befitting a nonclassical hydrophobic mechanism (65, 66). It has also been shown that the free energy change associated with the transfer of cholesterol from one bilayer phospholipid species to another is relatively small. In particular, highly favored cholesterol associations (e.g., with sphingomyelins) are stronger by only \sim 1 kcal/mol than weak associations (e.g., with polyunsaturated phosphatidylethanolamine) (66–68). Furthermore, despite evidence for some structural specificity, the differences among sterols for association with bilayer phospholipids are often minor (4–7). The evidence that, once in the bilayer, sterols interact weakly and rather nonspecifically with phospholipids is consistent with the premise that numerous small amphipaths displace them laterally from their complexes.

5. Competition among Sterols and Amphipaths for Phospholipids. The presence of cholesterol reduces 5–10-fold the uptake of various amphipaths by both synthetic bilayers and biological membranes (57, 63, 69–71). Cholesterol also suppresses the uptake into synthetic bilayers of merocyanine 540, tetracaine and Triton X-100 (72–74). Furthermore, amphipaths tend to be excluded from cholesterol-rich liquid-ordered phases (70). Conversely, ceramides (which we show to be cholesterol-activating agents) displace sterols from such domains (75). Amphipaths not only can order bilayer phospholipid chains themselves (23) but also can competitively reverse the even stronger ordering effects of cholesterol (76). Consistent with the premise that small amphipaths displace large sterol molecules from their associations with phospholipids is the observation that the favorable enthalpy change accompanying the uptake of alkanols into bilayers is significantly reduced by the presence of cholesterol (64). There is therefore strong evidence for the mutual exclusion of sterols and amphipaths in their association with phospholipids.

6. Must Amphipaths Displace Cholesterol To Be Taken up by Plasma Membranes? As stated above, the uptake of an amphipath by the plasma membrane may be constrained by cholesterol competition for association with phospholipids. While uncomplexed amphipath molecules could remain in the aqueous phase, the sterol molecules they displace would be dispersed in the continuum of bilayer phospholipid–sterol complexes, where they would increasingly oppose further uptake of amphipath by mass action. This speculation is in accord with the observation that the capacity of plasma membranes for amphipaths is limited. In particular, the uptake of several 1-alkanols reaches a plateau at 8–9 mol % of RBC bilayer lipids (57, 77). Our studies were performed well below this apparent saturation level (see section 2 of the Discussion).

The putative cholesterol:phospholipid mole ratios in complexes of varied composition were found to be on the order of 1:1 to 1:2 (10, 16). Also, the cholesterol content of plasma membranes is typically \sim 0.8 mol/mol of phospholipid (54, 55). It follows that most or all of the phospholipids in plasma membranes may normally reside in cholesterol complexes. We pointed out above that plasma membrane cholesterol may normally be titrated homeostatically to stoichiometric equivalence with phospholipids, instructed by the complexation mechanism. If so, the sites for potential associations of amphipaths with phospholipids may all be occupied by cholesterol in unperturbed plasma membranes. (A corollary would be that one of the evolved roles for sterols is to minimize such phospholipid binding vacancies so as to help exclude xenobiotics from cell membranes.)

7. Determinants of Amphipath Potency. Figure 2 makes it clear that lipophilicity is a dominant factor in the activation of

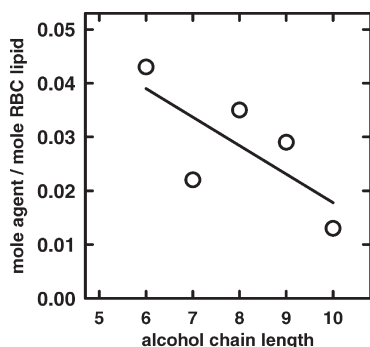


FIGURE 8: Variation of alcohol efficacy with chain length. The mole fractions of 1-hexanol through 1-decanol in red cell membranes needed to promote their susceptibility to cholesterol oxidase were calculated from *H* values and RBC partition coefficients. $R^2 = 0.53$. See section 2 of Discussion.

cholesterol by amphipaths. The lack of strong structural specificity in the action of this broad set of agents is evident in the similar potencies of diverse compounds with comparable ClogP values (Table 1), including isomer pairs with identical ClogP values (Table 2). These findings agree with an earlier report that plasma membrane cholesterol is displaced and activated equally well by four stereoisomers of C8:0-ceramide (25).

That the activation of cholesterol does not depend strongly on the structural features of an amphipath is also supported by the calculation presented in Discussion section 2; that is, 1-decanol was only ~ 3 times more effective per molecule incorporated than was 1-hexanol, despite being roughly 100 times more lipophilic. The implication is that, once in the membrane, the efficacies of the various amphipaths—i.e., their effectiveness per molal concentration in the bilayer—are similar. This conclusion is supported even more strongly by the initial slope of the data in Figure 2, namely, 0.90. It can be postulated that the potencies of all agents with values falling on a line of slope 1.0 will vary precisely inversely with their partition coefficients. Thus, assuming that their partition into RBC membranes parallels that into octanol, all agents on that line should have equal efficacy regardless of their chemical nature (58, 69).

8. Structural Specificity. Nevertheless, the data suggest a degree of structural specificity in amphipath–phospholipid interactions. First, note the weakly positive correlation between membrane efficacy and alkanol chain length (Figure 8). Second, there were small but statistically significant differences between potencies in some isomer pairs (Table 2). In addition, fatty acids and normal-chain compounds with terminal hydroxyl groups generally had higher potencies than their counterparts (Table 1).

The dispersion in the potencies of agents with ClogP ~ 3 is instructive (Table 1 and Figure 2). Compounds [9] through [16] had statistically indistinguishable ClogP values (i.e., 2.94 to 2.99) yet exhibited a highly significant ~ 7 -fold spread in their *H* values. In particular, butyl ether [16], with ClogP = 2.99, had a ~ 5 -fold higher *H* value (i.e., weaker potency) than 1-octanol [9] with ClogP = 2.94. This may be because ethers have a compromised hydrogen bonding potential: their oxygen atoms are weak acceptors and not donors, as is the case for alkanols (43). The two short chains of butyl ether may also limit its membrane penetration and, therefore, reduce its van der Waals contacts with phospholipids. In addition, its interchain (C–O–C) bond angle of 110° could impose an unfavorable molecular shape. The relatively low cholesterol-activating potencies of methyl heptanoate [8], 2-nonanone [11] and methyl octanoate [24], scaled to

ClogP, are also likely to reflect weak hydrogen bonding potential (43) as well as possible shielding of their polar moieties by their terminal methyl groups. The reduced potency of cyclodecanone [33] presumably reflects both weak hydrogen bonding and an unfavorable molecular shape.

9. Decline in Amphipath Potency with Chain Length. Why did cholesterol activation plateau for agents with ClogP > 4 (Figure 2)? This phenomenon could conceivably reflect the depletion of the aqueous pool of those amphipaths because of their strong partition into the membranes; i.e., not enough agent. (The true measure of potency is not the total amount of the amphipath causing the effect, as scored here by *H* and *S* values, but its less easily determined aqueous concentration at equilibrium.) This problem is unlikely here, however, because the amounts of amphipaths used in all cases should greatly exceed that needed to load the membrane for cholesterol activation, calculated above for small alcohols to be ~ 3 mol % of total RBC lipid. More likely, aggregation, precipitation, micelle formation or adsorption could have reduced the apparent potency of these low-solubility amphipaths (71). It is also possible that these agents actually have relatively small partition coefficients (60) or that their efficacy per molecule in the bilayer is low (78, 79). Finally, highly lipophilic amphipaths might themselves form complexes with cholesterol, instead of or in addition to displacing the sterol from phospholipids; this effect could undermine their ability to activate the sterol (14).

10. Parallels between Activators of Cholesterol and Intravenous General Anesthetics. (a) The kinds of agents that evoke these two phenomena overlap strongly, and the hierarchies of their potencies correspond closely; compare the present data with refs (58, 71, 80).

(b) The membrane concentration of cholesterol-activating agents, as calculated above, is about the same as that for anesthetic agents: roughly, 3 mol % of plasma membrane bilayer lipids (58).

(c) There is a notable lack of chemical specificity in both classes of agent (47, 81). No structural or stereochemical feature predominates in determining potency.

(d) Each of the two classes shows a pronounced change in potency beyond ClogP ~ 4 (i.e., around dodecanol). This effect is termed the “cutoff” for anesthetics (47, 48, 58, 71, 78, 80). There is a difference, however: the cholesterol-activating potency of long-chain amphipaths simply plateaus (Figure 2) but does not decline, as is the case for anesthetics.

(e) In both classes, plots of potency versus lipophilicity for small amphipaths have slopes close to unity; hence, their efficacy is rather independent of chemical structure (58, 71, 80).

Thus, in these several respects, cholesterol activation by amphipaths parallels the century-old Meyer–Overton rule for general anesthetics: “Narcosis commences when any chemically indifferent substance has attained a certain molar concentration in the lipoids of the cell” (58). Might amphipaths therefore elicit general anesthesia through their displacement of cholesterol from phospholipid complexes? Such a mechanism could involve the interaction of active cholesterol with susceptible membrane proteins; e.g., ion channels (82–84). If so, diverse proteins in other membranes might similarly be perturbed by active cholesterol. Cells might then contrive to maintain their plasma membrane cholesterol at its equivalence point with phospholipids in order to minimize untoward effects of active cholesterol on integral proteins (6).

There is evidence that myristic acid forms cholesterol complexes by itself (14). This mechanism could underlie the aforementioned

cutoff (drop) in the anesthetic potency of large lipophiles. The difference between anesthetic cutoff and the plateau without decline in potency demonstrated for the action of long-chain amphipaths on cholesterol activation (Figure 2) might then signify that cholesterol complexes with long-chain amphipaths do not interact with critical membrane proteins but only partially diminish cholesterol activity in the modes studied here (e.g., its projection from the membrane surface and pore formation with saponin).

11. Conclusions. Any number of small, uncharged amphipaths associate with membrane phospholipids, not at discrete loci but rather through weakly structured and dynamic interactions befitting fluid bilayers. These intercalators displace (and replace) plasma membrane cholesterol from its weak associations with the phospholipids, imparting to the sterol enhanced reactivity with exogenous probes and intracellular pathways. Because of mutual exclusion between cholesterol and other membrane molecules, the various agents have similar membrane efficacies despite broad differences in their chemical form; apparently on the order of one mole of cholesterol is activated per mole of amphipath intercalated. Displacement of cholesterol suggests a molecular mechanism for the otherwise poorly explained action of intravenous general anesthetics.

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