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Fatty Acid and Alcohol Partitioning with Intestinal Brush Border and Erythrocyte Membranes

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Summary. Relative partition coefficients of fatty acids and alcohols between aqueous buffers and biological membranes have been determined from the linear relationship between isotope content of sedimented membranes and aqueous concentration. This technique allows study of highly lipid soluble compounds such as long-chain saturated fatty acids. Rat intestinal brush border membranes and erythrocyte ghost membranes were studied by using homologous series of saturated fatty acids, mono-unsaturated fatty acids and 10, 12, and 14 carbon normal alcohols. The influence of chain length on partitioning was similar in the three series with an incremental free energy of −820 cal/mole per methylene group in brush borders for the saturated fatty acids. Incremental enthalpy and entropy were −1331 cal/mole and −1.64 cal/mole, °K respectively. Decrease in the partition coefficient due to the double bond (monounsaturated relative to saturated) had an incremental free energy of +1178 cal/mole, incremental enthalpy of −3453 cal/mole, and incremental entropy of −7.34 cal/mole, °K, while substitution of the hydroxyl for the ionized carboxyl group (pH 7.4) increased the partition coefficient by 72-fold. From these data it must be concluded that the lipid phase of the membrane bilayer is extremely hydrophobic, similar to heptane or polyethylene in polarity.

Membrane partition coefficients have been rarely measured; however, Seeman [20] has reviewed the pharmacological actions of various anesthetics including the partition coefficients of the anesthetics with the erythrocyte membrane. More recently Katz and Diamond [4, 9–11] have described the partitioning of various solutes with synthetic lecithin liposomes. Although such measurements are extremely important for the thermodynamic analysis of passive membrane permeation processes, these direct studies have only recently been utilized significantly in permeation analysis [2, 23]. Earlier studies relating to the absorption of fat from the intestine compared permeation of short-chain fatty acids to bulk solvent partition coefficients, and concluded the membrane was highly polar compared to solvents such as ether or olive oil [19]. Later work with

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permeation of long-chain fatty acids has suggested a nonpolar membrane similar to cell membranes of algae or partitioning in heptane [17, 18]. This author is unaware of any direct determinations of membrane partition coefficients of lipids in intestinal brush border membranes despite the fact that these membranes may be relatively easily prepared.

Deviations of small members of homologous series from the theoretically predicted permeation rates have not been explained, although the possible contribution of permeation through parallel polar routes has been suggested [5, 8] as has the relative selective influence of rate of movement within the phase of the membrane [12, 22]. The possible variation of partition coefficients with size of molecule should not be overlooked despite the lack of selectivity in bulk solvent systems. Since membrane lipid phases are highly structured molecular arrays, it is possible that variations of partition coefficients due to molecular configuration occur in homologous series and should be experimentally studied.

In the present study, a very simple method for obtaining partition coefficients in biological membrane systems is described as well as the effect on partitioning of the methylene group in homologous series of saturated fatty acids, unsaturated fatty acids, and normal alcohols. These series may be compared to describe the influence of substitution of the carboxyl group for the hydroxyl group, and the influence of the double bond in the acyl chain of the fatty acid. Measurements are reported for compounds which have been shown elsewhere [17][1] to deviate from the predicted pattern of permeation rate in the intestine.

Materials and Methods

Rat intestinal brush border membranes were prepared by the method of Forstner *et al.* [6]. Our preparations corresponded to Forstner's purified brush border fraction which contains sheets of brush border membrane with core material intact. Sucrase activity measured according to the technique of Dahlqvist [3] was about .5–.6 units per mg protein, while TPNH-cytochrome C-oxido-reductase measured by the method of Masters *et al.* [14] was not detected. Membranes were suspended in phosphate buffered saline at pH 7.4 with a phosphate concentration of 20 mM and NaCl concentration of 120 mM.

Erythrocyte ghosts were prepared from 20 ml fresh human blood after washing two times with normal saline. Five ml of packed cells were hemolyzed in 250 ml cold .015 M NaCl, stirred for 15 min at 0 °C with a magnetic stirrer, and then centrifuged at $15{,}000 \times g$ for 20 min. After decanting the supernatant, the fluffy precipitate was poured into a separate centrifuge bottle. The precipitate was resuspended in approximately 100 ml of hypotonic saline and centrifuged under the same conditions again. The precipitate was finally suspended in sufficient phosphate-buffered saline to yield $1-2\,\mu g$ protein per μl solution.

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Protein analysis was done according to the technique of Lowry *et al.* [13], and phosphorus was determined on membranes in saline by the method of Bartlett [1].

Partition coefficients were determined by the equilibrium distribution of isotopic solute between aqueous buffer and sedimented membranes, by using a series of concentrations of solute. Volumes of stock solution and buffer were added to 1.5 ml polyproplene centrifuge tubes to yield final desired concentrations when 0.2 ml of membrane preparation were added to yield a total volume of 1 ml. Five concentrations of solute were routinely used with duplicate determinations. Following incubation at 37 °C for the desired time period, the tubes were removed, placed in the microcentrifuge (Brinkman Model 3200) and spun for 2 min. The $12{,}000 \times g$ force represents over twice the force-time product used in preparation of brush border. An aliquot of the supernatant was then removed, followed by vacuum removal of the remaining supernatant. Membranes were transferred into a scintillation vial using 1 ml of normal saline. Sufficient saline was added to the aliquot of the supernatant to bring the final volume to 1 ml. Counting cocktail was three volumes toluene containing 7 g/liter PPO and 0.1 g/liter POPOP and 1 vol Triton-X 100. Fifteen ml cocktail was added to each vial. All samples were counted to at least 2000 counts in a Packard Model 3320 liquid scintillation spectrometer. Quench was monitored by external standard ratio, although no correction was necessary since all vials had essentially the same degree of quench.

Tritiated water (THO) was added to some experiments in order to correct for trapped water in the membrane pellet. It was determined that this was unnecessary for fatty acids with chain lengths greater than 10 or 11 carbons. Data calculated as nmoles of solute contained in the membrane precipitate and nmoles of solute per 100 μl in the supernatant were plotted and linear regression analysis by least squares was performed. The slope of this relationship corresponds to partitioning between buffer and membrane lipid and has the units of nmoles in the membrane per unit aqueous concentration.[2] This ratio preferably would be based on equivalent volumes of lipid and aqueous phases; however, the procedure normally used was to express data relative to 100 μg protein contained in the membrane preparation. When only an isotope was added, calculations were based on cpm in the two phases, but the slope, as a ratio, has equivalent units to the above procedure. Thus, K membrane equals nmoles/100 μg protein per 10^{-5} M, or μl/μg membrane protein. To allow comparison of the present data with those of Seeman *et al.* [15, 20, 21], data may be multiplied by 726 since membranes with 100 μg protein have a dry wt of about .13781 mg. After multiplying, the units of K membrane are μl/mg.

Results

The first experiment necessary to characterize the technique used for partitioning was the time required for equilibrium to be established

2 Since a true partition coefficient is a ratio of the equilibrium concentrations in two immiscible solvents, the coefficient has no units. In these experiments, however, the effective volume of membrane lipid is unknown so that K_{membrane} has units depending on the method chosen to express the membrane quantity, i.e., 100 μg membrane protein. The true partition coefficient is related to K_{membrane} as expressed by

$$K = \alpha K_{\text{membrane}}$$

where α is a constant equal to the number of μg of membrane protein required for the effective volume of membrane lipid to be one μl. A similar constant is implicit in the work of Seeman [21] where α is mg of membrane per μl volume and in the work of Katz and Diamond [9] where α is mg of phospholipid per μl effective volume.

Table 1. Time relationship of membrane partitioning

Fatty acid	$K_{membrane}$ Equilibration time			
	5 min	10 min	20 min	30 min
8:0	−0.015	0.00844	–	0.0113
14:0	4.85	4.74	4.77	5.42

Units for $K_{membrane}$ are nmoles/100 μg protein per 10^{-5} M.

between the solute in buffer and in the membrane phase. Table 1 shows the apparent membrane partition coefficients for fatty acids 8:0 and 14:0 measured at 5, 10, 20, and 30 min. Other than the negative value for fatty acid 8:0 at 5 min, which is a physical impossibility, there is essentially no difference between the time periods, implying that the equilibration time is very short. The routine time period used for equilibration in most experiments was 15 min, although experiments with polar solutes were equilibrated from 1 to 4 hr. Figure 1 shows typical results for fatty acid partitioning. Fatty acid 13:0 aqueous concentration varied from 12×10^{-6} to 60×10^{-6} M, and the content of fatty acid in the membrane phase of 196 μg protein increased linearly up to approximately 20 nmoles. Linear regression analysis of the 10 data points gives a slope of 3.21 when the aqueous concentration is expressed as 10^5 times the molar concentration. The regression correlation coefficient for this analysis is .993, showing high linearity. Intercepts were quite near zero in almost all cases. Also shown in Fig. 1 is the regression analysis for fatty acid 15:0 where the scale for nmoles of fatty acid in the membrane has been expanded. The slope of the regression analysis is increased 12.1-fold by the addition of two methylene groups, and the regression correlation coefficient is still high at .995, supporting the concept of partitioning as the basis for this relationship. Regression correlation coefficients for lipid solutes were rarely less than 0.9.

With THO volume correction the true membrane content of solute will be underestimated by an amount equal to $^{14}C_S \cdot V_{ns}$ where $^{14}C_S$ is the aqueous concentration of ^{14}C labeled solute and V_{ns} is the volume of "non-solvent" water. When a series of solute concentrations is used to calculate the membrane lipid partition coefficient, the resulting relationship is still linear but of less slope than the true regression. The

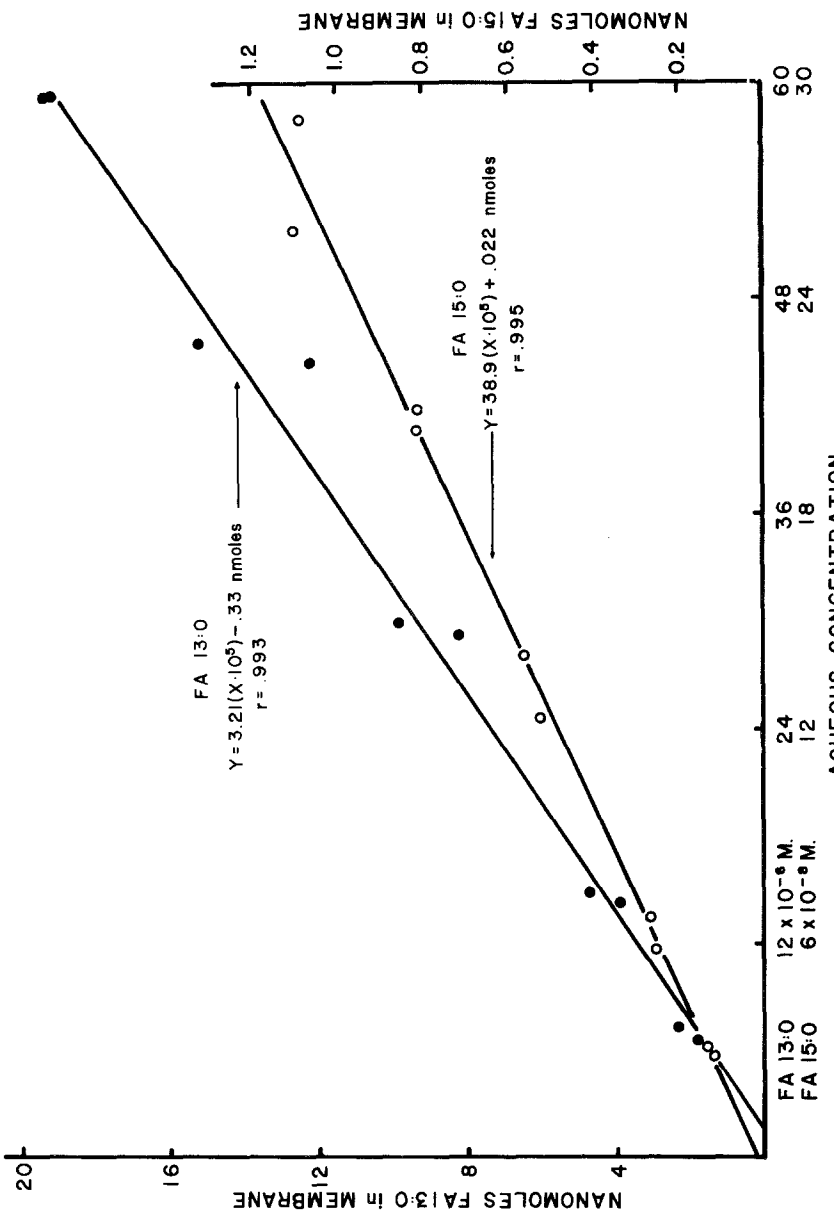

Fig. 1. Intestinal brush border membrane (196 μg protein) content of fatty acids 13:0 and 15:0 at various aqueous concentrations. Linear regression analysis was performed with molar concentrations multiplied by 10^5

apparent regression equation will be:

$$^{14}C_M = {}^{14}C_S \cdot K_m - {}^{14}C_S \cdot V_{ns} + \text{constant.} \quad (1)$$

Since this would result in a negative slope for a solute with $K_m = 0$, the nonsolvent water volume can be evaluated by very polar solutes.

Table 2.

Solute	Equil. time	Slope	Intercept	r	K_m
FA6:0	1 hr	0.00935	4.3	0.968	0.0062
FA6:0	4 hr	0.00685	26.3	0.757	0.0046
Antipyrine	1 hr	0.0096	−9.1	0.958	0.0064
Antipyrine	4 hr	0.0119	−9.5	0.902	0.0079
Glycerol	1 hr	0.0116	−4.2	0.984	0.0077
Glycerol	4 hr	0.0249	31.4	0.998	0.0166
Urea	1 hr	0.0061	−1.4	0.980	0.0041
Urea	3 hr	0.0064	7.3	0.973	0.0043
Urea*	1 hr	0.004	10.0	0.976	0.0027
Urea*	4 hr	0.003	25.2	0.552	0.0020

All ^{14}C-labeled solutes were added to aqueous buffer as supplied; unlabeled solute, 0.8–.75 mM, was added only for urea studies marked*. Intestinal brush border membranes containing 150 μg protein were added for each determination. Linear regression analysis by least squares was performed on one determination at five concentrations.

Table 2 presents results of experiments in intestinal brush border membranes for four solutes which should have very low K_m. Sucrose is not appropriate because of the presence of sucrase. None of the four solutes have zero or negative slopes as would be seen if the nonsolvent water volume were significant. In the studies for .75 mM urea, the correction of isotope in trapped water accounted for 89 to 97% of the total urea in the pellet. Thus no correction for nonsolvent water is necessary in the brush border membrane experiments.

The method of normalization of data to account for the quantity of membrane added was by relating all values to 100 μg membrane protein. Since correction to the amount of phospholipid actually present in the membrane preparation would more accurately reflect the lipid volume, phosphate determinations were performed and total phosphate used as a normalization factor. These two procedures are compared in Fig. 2, and it can be seen that either normalization scheme is appropriate. Finally, eight determinations of K_m for FA10:0 by using different preparations of brush border membranes gave a value of 0.38 ± 0.006 (SEM) nmoles/100 μg protein per 10^{-5} M even though the amount of membrane protein varied from 180 to 1500 μg per tube.

Figure 3 shows the data for saturated fatty acids of chain lengths of 8 to 18 carbons as well as the alcohols of 10, 12, and 14 carbon chain lengths. These experiments show that, with the intestinal brush border membrane, increase in membrane partition coefficients with addi-

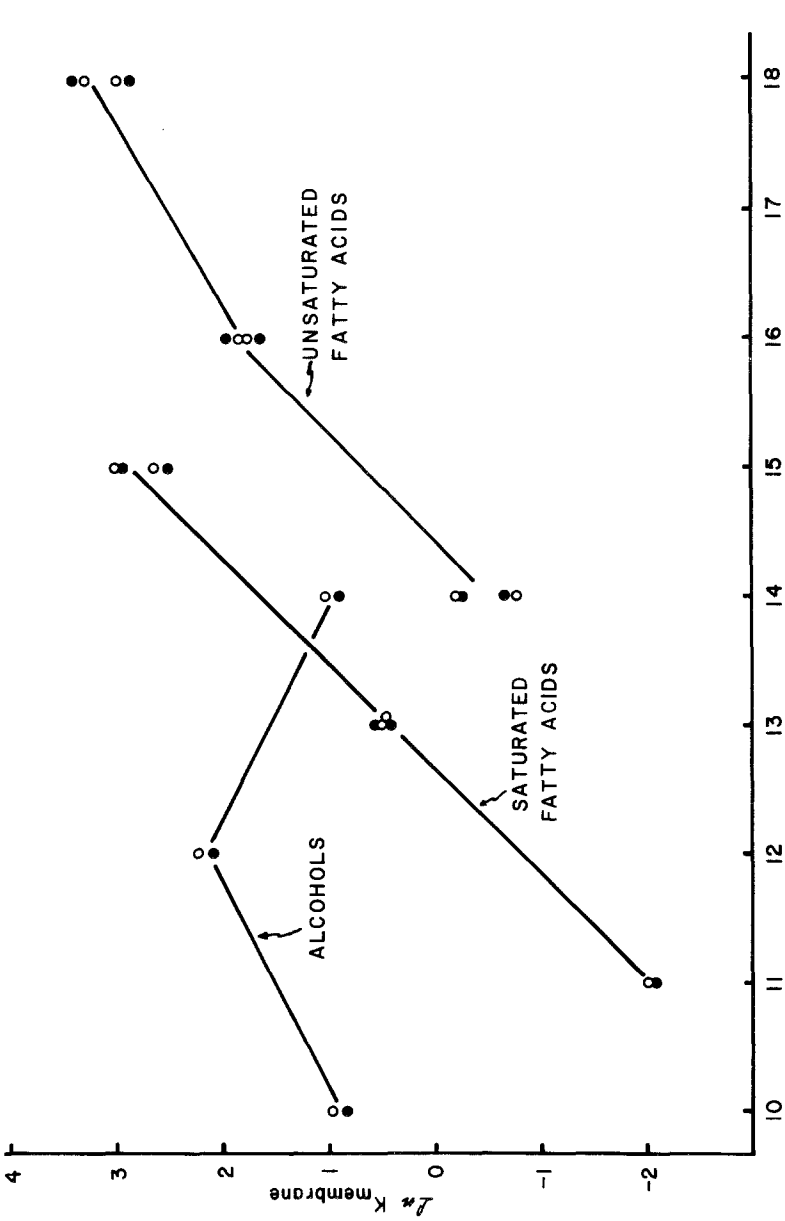

Fig. 2. Intestinal brush border membrane partition coefficients for fatty acids and alcohols. Values are plotted as natural logarithm K relative to the compound chain length. Open circles are normalized per 100 μg protein, while closed circles are normalized per μg total phosphate

tion of methylene groups is appropriate compared to other partitioning systems. Fall-off in the incremental increase of the partition coefficient at higher chain lengths occurs in this system at about 15 to 16 carbons. The relationship of natural logarithm K_{membrane} *vs.* the Number of Carbons is essentially linear down to fatty acid 8:0, although experimental variation for 8:0 makes this judgment difficult. As anticipated, substitu-

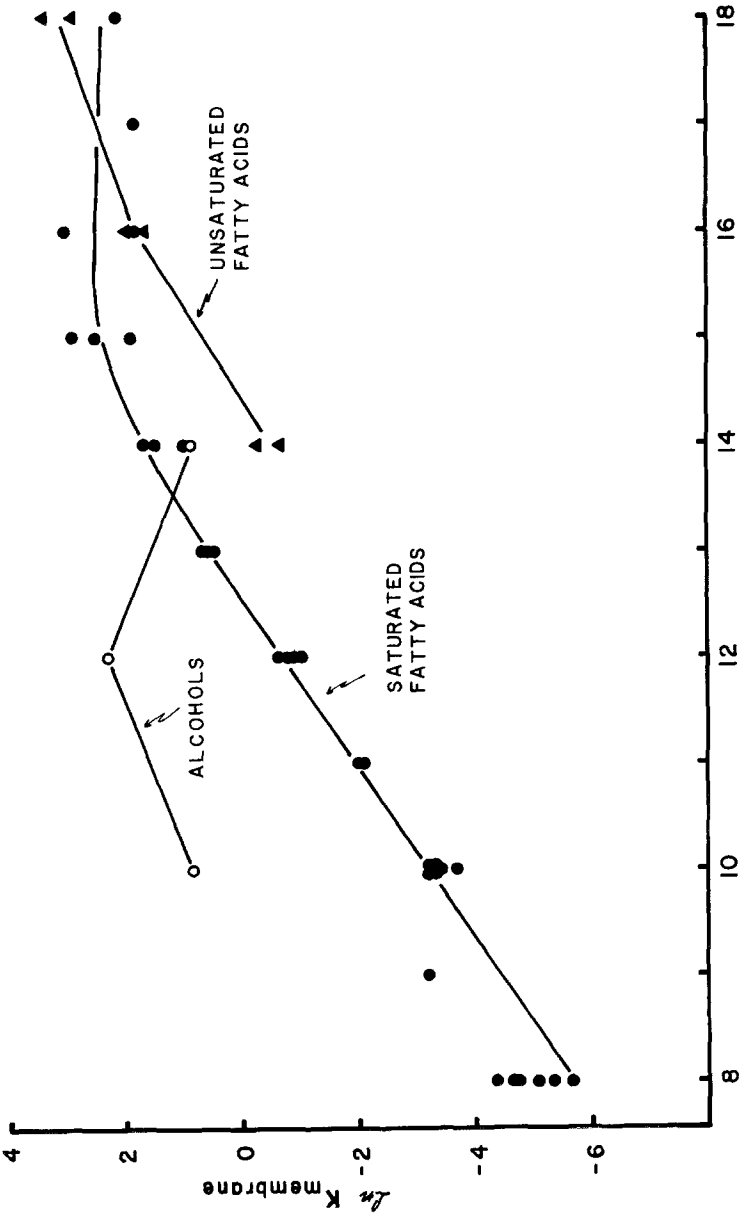

Fig. 3. Intestinal brush border membrane partition coefficients for fatty acids and alcohols. Values are plotted as natural logarithm K relative to the compound chain length. Each point represents a linear regression of two determinations at each of five concentrations. Essentially constant increase in $\ln K$ is seen for each additional methylene group. Deviation from this generalization is seen for tetradecanol and the fatty acids 16:0, 17:0, and 18:0. Incremental free energies are -821 cal/mole per methylene group and $+1178$ cal/mole per double bond

tion of a hydroxyl group for the ionized carboxyl group increases partition coefficients markedly, as seen by the increase of the alcohol partition coefficients over those of the saturated fatty acids. The low value for alcohol 14 cannot be explained at the present time. Introduction of the double bond decreases the membrane partition coefficient and is most readily seen with fatty acid 14:1, compared to fatty acid 14:0.

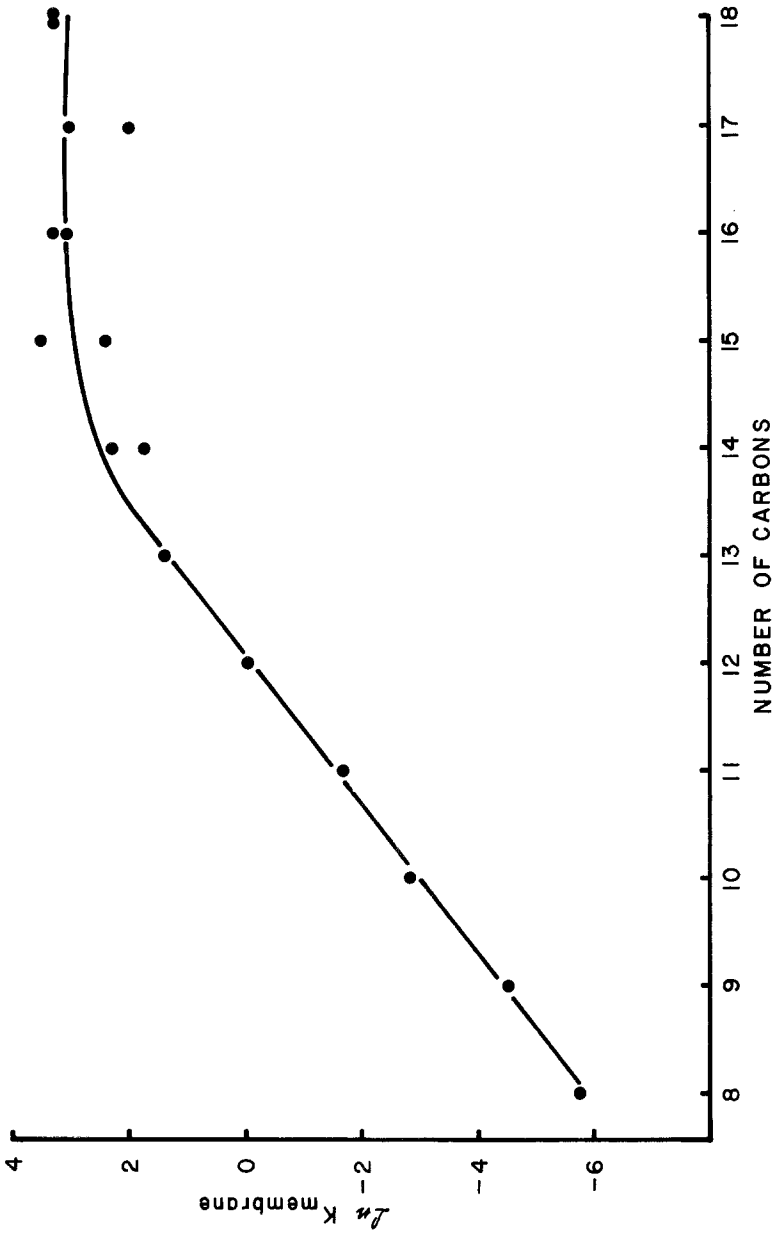

Fig. 4. Erythrocyte ghost membrane partition coefficients for saturated fatty acids. Values are plotted as natural logarithm K relative to the compound chain length. Each point represents a linear regression of two determinations at each of five concentrations. Incremental free energy for methylene group addition is −883 cal/mole per methylene group

In all cases, the incremental influence of the methylene group in the region of the linear relationship is essentially the same for homologous series of alcohols, saturated fatty acids, or unsaturated fatty acids.

Partition coefficient measurements for saturated fatty acids have been performed for erythrocyte ghost preparations, and these results are presented in Fig. 4. Again it may be seen that for saturated fatty acids

Table 3. Thermodynamic functions of brush border partitioning

Lipid	Linear regression of log K_m *vs.* $1/T$			$\Delta H^\circ_{w\to1}$ (cal/mole)	Incremental functions			
					$\delta\Delta H^\circ_{w\to1}$	$\delta\Delta F_{w\to1}$	$\delta\Delta S^\circ_{w\to1}$	
	Slope	Int.	r					
FA10:0								
	−333.9	−0.575	0.957	1527	−1331	−821	−1.64	cal/mole per $-CH_2-$
FA14:0	830.1	−2.13	0.998	−3797	+3453	+1178	+7.34	cal/mole per $-HC=CH-$
FA14:1	75.26	−0.409	0.406	−344	−1236	−800	−1.41	cal/mole pr $-CH_2-$
FA16:1	615.6	−1.30	0.953	−2816				
FA18:1	2658.2	−7.51	0.996	−12159	−4671	−800	−12.48	cal/mole per $-CH_2-$
10 ol	645.7	−1.75	0.987	−2954	−2136	−382	−5.66	cal/mole per $-CH_2-$
12 ol	1579.8	−4.01	0.997	−7226				

A linear regression of partition coefficients at four temperatures was evaluated for each lipid. $\Delta H^\circ_{w\to1}$ was calculated as $-2.303\ R$ (slope). $\delta\Delta F_{w\to1}$ was derived from Fig. 3. $\delta\Delta S^\circ_{w\to1}$ was calculated as $(\delta\Delta H^\circ_{w\to1} - \delta\Delta F_{w\to1})/T$ where T was 310 °K.

from 8 to 14 carbons the relationship of $\ln K$ to Number of Carbons is essentially linear, while a plateau for $\ln K$ is seen for fatty acids 15:0 to 18:0.

Since partial molar enthalpies and entropies are helpful in evaluating the membrane lipid/buffer interactions of lipids, a series of experiments was carried out with intestinal brush border membranes at 27, 32, 42, and 47 °C. K_m determined at each temperature was plotted as log K_m versus 1/T, and linear regression by least squares performed. These data are presented in Table 3 along with the calculated thermodynamic functions. Values for $\delta\Delta F_{w\to1}$ were derived from data presented in Fig. 3, performed at 37 °C.[3]

Discussion

Because partitioning is the description of the relative solubilities of a solute in two separate solvents, dilute (ideal) solutions should exhibit

3 Thermodynamic analysis depends on the relationship of K_m with K discussed earlier. $\ln K_m$ will deviate from $\ln K$ by the constant $-\ln\alpha$. This will not influence the slope analysis from which $\Delta H^\circ_{w\to1}$ is derived; however, the intercept cannot be used to determine $\Delta S^\circ_{w\to1}$. Still, since $\ln\alpha$ is constant for different solutes, the values $\delta\Delta S^\circ_{w\to1}$ can be determined and are appropriate to compare to other investigators' values which are based on molal (or molar if very dilute) concentrations.

a linear relationship between the concentration of solute present in one phase compared to that in the other. This relationship was chosen to be central to the technique for determination of membrane partition coefficients because of the numerous other factors that could distort single equilibrium concentration measurements. One of these factors, protein binding to solute, could distort values producing a positive intercept of the linear regression analysis. Although all the intercepts are very near zero, protein binding is not ruled out. Even the high degree of linearity does not prove the mechanism of membrane/aqueous phase interaction to be partitioning, although it is strong support for this interpretation. The finding of incremental free energies for methylene groups of about -800 cal/mole is entirely consistent with partitioning, but a conformational change of protein in binding might produce a more negative value. Seeman *et al.* [21] evaluated the mechanism of membrane interaction of alcohols and concluded that partitioning was a better model than the finite site model.

The predominant observation in these studies is the influence of the chain length of the partition coefficient with the membrane. This relationship was anticipated on the basis of permeation across the intestinal brush border membrane, as well as studies in other tissues and partitioning with organic solvents. The magnitude of the influence, however, was unknown based upon previous studies [19], which indicated that shorter chain length fatty acids had incremental free energies of permeation close to -300 cal/mole. These studies show, however, that the partition coefficient with the membrane has an incremental free energy of approximately -820 cal/mole per methylene group, essentially identical to the incremental free energy for partitioning in organic solvents such as heptane and polyethylene. This is an extremely important observation to show that the intestinal brush border membrane and the erythrocyte membrane have lipid phases which are as hydrophobic as the major organic solvents in sharp contrast to the concept that the membrane is a relatively polar structure. Other support for this observation comes from Seeman and coworkers [15, 21] in experiments with anesthetics partitioning into erythrocytes, where the incremental free energy for partitioning of the alcohol from methanol to decanol averages -695 calories per mole per methylene group. The value calculated by Diamond and Katz [4] of approximately -530 is not as high as these values, however, but is significantly higher than the average value determined for the shorter chain length fatty acids in intestinal permeation.

Incremental thermodynamic functions for the methylene group seen

in these studies are very similar to those described by Diamond and Katz [4] for liposomes. The negative incremental enthalpy is probably due to the greater formation of van der Waal's forces in the membrane as compared to attractive forces in water. The negative incremental enthalpies are partially offset by the negative incremental entropies. The accentuation of these values for the comparison of FA18:1 to FA16:1 is intriguing since the eighteen carbon chain probably matches the bulk chain length of the acyl chains of the phospholipids. Van der Waal's forces are probably maximized, while immobilization of the FA18:1 is also increased. These compounds have not been studied for thermodynamic properties of hydration, so the thermodynamic functions for solution in lipid cannot be determined.

The nonpolar nature of the brush border membrane is also demonstrated by permeation coefficients determined for long-chain fatty acids [17][4]. The average incremental free energy of -604 cal/mole per methylene group for FA12:0 through FA18:0 and -657 cal/mole per methylene group for FA12:0 through FA17:0 shows that the area of membrane through which these lipids permeate is very hydrophobic. Thus, the nature of the permeation barrier is quite similar to the bulk membrane lipid as studied by the present partition coefficient measurements. The appropriate question to ask with respect to the discrepancy between the relationship of fatty acid chain length to membrane partitioning and to intestinal permeation is, "By what mechanism is intestinal uptake of short-chain fatty acids more rapid than expected from solute polarity?"

Also of extreme importance to consideration of membrane polarity is the partitioning selectivity seen with addition of the unsaturated bond. Comparison of fatty acid 14:1 with fatty acid 14:0 in the intestinal membrane preparation shows that the double bond reduces the partition coefficient by a factor which corresponds to an incremental free energy of $+1178$ cal/mole. This value is substantially higher than was seen in the data of Goodman [7] for partitioning in heptane where the comparison is between oleic acid and linoleic acid. This higher selectivity is reasonable considering the structured nature of the bilayer membrane such that the van der Waal forces of attraction are reduced, and, in addition, the volume occupied by the unsaturated fatty acid in the membrane is greater than the volume occupied by the saturated acid so that net solubility in the hydrocarbon phase of the membrane would be reduced. The positive incremental enthalpy is consistent with this

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interpretation since the van der Waal forces would be reduced because of the double bond distortion of the acyl chain. The positive incremental entropy partially compensates for the enthalpy by causing a disordering of the phospholipids. The influence of the double bond has not been evaluated in other membrane partitioning systems.

The conclusion that must be drawn from the consideration of incremental free energy for partitioning with the methylene group and with the unsaturated bond is that the biological membrane lipid phase is an extemely hydrophobic phase and not relatively polar. In fact, the membrane polarity may be compared to bulk solvents such as heptane or polyethylene which have yielded the greatest selectivity of any of the bulk solvents. In addition to this polarity selectivity must be added the influence due to the molecular organization of the membrane where the ordered arrangement of the phospholipid acyl chains produces steric selectivity of partitioning. This has been described for branched compounds as well as double bonds.

Partitioning of fatty acids occurs as the protonated form as shown in partition measurements with bulk solvents [16] and in intestinal permeation studies [19]. This effect is of a magnitude which suggests that the predominant difference between saturated fatty acids and alcohols is the removal of the charge of the ionized carboxyl group rather than a primary effect of substitution of a hydroxyl group. It is impossible with these experiments to determine what the actual incremental free energy for substitution of the hydroxyl for the protonated carboxyl group would be. Partitioning of the alcohols may be directly compared to studies where the shorter chain length alcohols were partitioned into the erythrocyte membrane system. Incremental free energy for the methylene group in those studies [15, 21] was -695 cal/mole, which would compare reasonably well to the already plateauing incremental free energy of -382 cal/mole seen from 10 to 12 carbons in the present study. Whether the low value of partitioning of alcohol 14 is an artifact or is a real number is difficult to determine at the present time. Various artifacts could explain this low value, such as an isotope impurity; however, this isotope lot partitioned with polyethylene in an appropriate manner [18]. It has also been shown [20] that the anesthetic effect of the alcohols decreases above a limiting chain length, perhaps indicating some change in the membrane partition coefficient.

To compare data with the studies carried out by Seeman and coworkers [20, 21] where partition coefficients were expressed as nmoles/kg membrane per nmoles/liter water, the data in this study must be multi-

plied by 726, yielding equivalent units for the partition coefficients. Only decanol was utilized in both the present study in intestinal brush borders and by Seeman *et al.* [21] in erythrocyte membranes. The present value of 1928 μl/mg compares very favorably to that of Seeman of 1226 μl/mg. Careful examination of Seeman's data suggests that a more appropriate value to compare with might be 2233, however, since this is the linear extrapolation of his ln "P" values for shorter chain alcohols. Thus, the results of this study are quite comparable to Seeman's data.

It should be emphasized that the partition coefficients presented here do represent partition with the total lipid in the membrane preparation and, therefore, an average of all of the lipid/aqueous partition relationships occurring. It is unknown whether more than one partition relationship does exist, but some data of Forstner [6] may be useful in that the lipid composition of microvillus membranes has been determined to reside almost totally within the plasma membrane fraction and not within the fibrous filament core of the microvilli. Further, data with erythrocyte membranes show that the same general relationships are observed when only the plasma membrane fraction is present. Probably of greater significance than the consideration of other cell fractions present in the preparation is the consideration of whether the micro-environments of different lipid fractions in the membrane all partition with solutes in the same manner. This question cannot be resolved with the technique utilized in this study; however, the similarity of these data to those observed in the intestinal permeation of fatty acids *in vitro* [17][5] indicates that the values determined for the average partition coefficient are appropriate for analysis of determinants of passive membrane permeation of larger molecules which almost certainly permeate through the bulk lipid bilayer of the membrane.

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