Apparent monomer activity of saturated fatty acids in micellar bile salt solutions measured by a polyethylene partitioning system

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Abstract Partitioning of saturated fatty acids between discs of polyethylene film and aqueous buffer has been characterized and subsequently used to measure monomer activities of fatty acids in micellar solutions of bile salt.

Partitioning of fatty acids between polyethylene and buffer achieved equilibrium in about 24–48 hr. Partition coefficients for fatty acids 10:0 and 16:0 were essentially independent of concentration, as expected for true partitioning. Experiments with various pH buffers showed that only the protonated form of fatty acids 12:0 and 16:0 participated in partitioning, and the midpoints of the partition coefficients vs. pH curves were 4.5–5.0 and 6.5–7.0, respectively.

Experimentally determined partition coefficients at pH 7.4 ranged from 2.03 ± 0.09 for 9:0 to 56,100 ± 13,850 for 17:0. The addition of each methylene group increased the partition coefficient by a factor of about 3.75, corresponding to an incremental free energy change for each methylene group of −3433 $J \cdot mole^{-1}$ (−820 $cal \cdot mole^{-1}$).

Monomer activities of solutions of 14:0 and 16:0 dissolved in 20 mM taurodeoxycholate were linearly dependent on the total fatty acid concentration. 1 mM 14:0 and 16:0 in 20 mM taurodeoxycholate had monomer activities of 1.3×10^{-5} M and 5.6×10^{-7} M, respectively. Solutions prepared with a constant concentration ratio of fatty acid to taurodeoxycholate had essentially constant monomer activities between 8 and 20 mM taurodeoxycholate. These studies support the hypothesis that fatty acid interaction with bile acid micelles is similar to a phase distribution system, so that some measurable monomer activity of fatty acid exists in equilibrium with the mass of fatty acid contained in the micelle.

Supplementary key words distribution coefficient · incremental free energy · pK_a · aggregation · mixed micelle

Facilitation of intestinal fat absorption by micellar solubilization is well known even though the mechanism of this facilitation is poorly understood. Recent experiments have shown quite clearly that intact micelles do not penetrate the intestinal brush border membrane (1, 2), so that the micelle must have its role in facilitating fat absorption through some bulk phase phenomenon. However, despite intensive study of bile salt micellization (3, 4) and the ability of bile acid solutions to solubilize fatty acids (5, 6), no information is available concerning the nature of the interaction of fatty acids with bile acid micelles. This lack of knowledge has limited study of the rate determinants of fatty acid absorption.

It was previously assumed (7) that fatty acids interact with micelles according to a phase distribution phenomenon so that fatty acid exists in two phases in equilibrium with each other: (*a*) fatty acid monomer form in free aqueous solution and (*b*) fatty acid in the micelle. Of these two phases, only the fatty acid monomer was felt to contribute directly to absorption, while fatty acid in the micellar form constantly maintained that fatty acid monomer concentration during the absorption process. Facilitation of fatty acid absorption by micelles would then be explained by the ability of micelles to diffuse through the unstirred layer and maintain a maximal fatty acid monomer concentration adjacent to the brush border membrane.

Definitive testing of this assumption was not previously possible because of the lack of a technique to measure the fatty acid monomer activity in micellar solutions. The present experiments were therefore undertaken to fulfill two objectives: (*a*) to describe and validate a technique for measuring the monomer activity and (*b*) to characterize the relationship between monomer activity and total fatty acid concentration in a simple micellar solution.

The technique used is based upon the partitioning of fatty acids between true aqueous solution and a solid organic phase, a polyethylene disc. Partition coefficients for fatty acids may be determined using solutions without bile salt micelles and then used to calculate the activity of fatty acid in micellar solutions from the fatty acid content of polyethylene discs equilibrated with the complex solutions.

Abbreviations: *m*:*n*, number of carbon atoms:number of double bonds; TDC, taurodeoxycholate.

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The use of polyethylene rather than a liquid organic phase is necessary to preclude any alteration of the micellar solution.

Various experiments are first presented in this study to describe the interaction of fatty acids with polyethylene. Then the polyethylene system is used to study fatty acid activity in bile salt micellar solutions. In the discussion, the polyethylene system will be compared with partitioning of fatty acids between heptane and aqueous solution, which has been extensively studied by Goodman (8) and Smith and Tanford (9). The data are felt to provide strong evidence in support of partitioning rather than surface adsorption as a mechanism of interaction of fatty acids with polyethylene.

The studies with fatty acids in micellar solutions demonstrate: (*a*) that fatty acid monomer activity is linearly related to the total fatty acid concentration in a solution with constant concentration of bile salt and (*b*) that fatty acid monomer activity is relatively constant and independent of total concentration for solutions with a constant ratio of fatty acid to micellar bile salt concentration. The behavior of monomer activity in these two situations provides experimental support for the hypothesis that fatty acid interaction with micelles is similar to a phase distribution system, as assumed previously (7). Using the data presented in these experiments, it is also possible to calculate fatty acid monomer activities for taurodeoxycholate solutions of myristic acid (14:0) and palmitic acid (16:0), which will be valuable in analyzing intestinal uptake rates from these solutions.

METHODS

Distribution of ^{14}C-labeled fatty acids between aqueous phase and the polyethylene disc was determined after equilibration of one disc in 10 ml of buffer in a 25 × 100 mm screw-capped tube. The tubes were placed in an incubator at the appropriate temperature, usually with the shaking motor set at approximately 100 oscillations/min. After equilibration for the specified time, the disc was removed from the tube with forceps, rinsed vigorously in either buffer or 20 mM taurodeoxycholate solution, and placed in a counting vial, to which was added 1 ml of water and 15 ml of counting solution. ^{14}C activity in the aqueous phase was determined using an aliquot of either 50 μl or 1 ml, but all samples were counted under identical conditions where the counting vial contained 15 ml of counting solution and 1 ml of water. The counting solution contained 3 l of toluene, 1 l of Triton X-100 (Rohm and Haas Co.), 21 g of PPO (2,5-diphenyloxazole, Packard Instrument Co.), and 0.3 g of POPOP (1,4-bis-[2-(5-phenyloxazolyl)]-benzene, Packard). Counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer model 3320 using a setting of 12% gain and 50–1000 window divisions. Counting efficiency was approximately 80% with a background of about 18 cpm. Neither disc nor 50 μl of bile acid solution produced any measurable quenching.

The distribution coefficient was defined as the quantity of fatty acid contained in the disc as nanomoles when equilibrated with a 1 mM solution. The units nanomoles $\cdot disc^{-1} \cdot mM^{-1}$ reduced to the term microliter$\cdot disc^{-1}$. For those determinations in which only the high specific activity isotope was placed in the aqueous phase, the distribution coefficient was calculated as the counts per minute per disc divided by the counts per minute per microliter of solution. This calculation reduces to the identical terms as that described previously and allows experimental determination of distribution coefficients for fatty acids that are essentially insoluble in water.

The composite layered disc was prepared by heating to approximately 70°C a stack of polyethylene discs that were tightly clamped together. After about 1 hr, the discs were quenched with water, unclamped, and left at room temperature for several days. Finally, this layered disc was trimmed to size with a punch, and the outer layers were peeled away to expose a clean face and then mounted in a stainless steel holder that exposed only one surface. This surface was immersed in a solution containing the labeled fatty acid while the opposing surface was in contact with air. The 25 × 100 mm tube was placed in an incubator at 37°C and shaken at a slow rate for equilibration. After equilibration, the composite disc was removed from the holder and rinsed, and individual layers were peeled away with forceps and placed in counting vials. Discs weighed about 14 mg.

1-^{14}C-labeled fatty acids and the corresponding unlabeled compounds were obtained from Applied Science Laboratories, Inc., State College, Pa., and were used as supplied. Taurodeoxycholate was obtained from Calbiochem, Los Angeles, Calif.

Polyethylene discs, 0.5 inch in diameter, were punched from polyethylene film, Durethene, 0.006 inch thick, manufactured by Sinclair Koppers Co., Pittsburgh, Pa. Discs were washed in methanol and distilled water and were dried before use to remove oil and debris from the commercial punching operation. Average weight of one disc was 19.4 mg.

Most solutions were prepared using a phosphate-buffered saline, pH 7.4, which had the following composition: Na^+, 156 meq/l; Cl^-, 120 meq/l; and phosphate, 20 mM. Experiments in which the effects of pH were studied were conducted with various buffers that were titrated with HCl. In all cases, the buffering ions were present at a concentration of 20 mM each, with a sodium concentration of 150–180 meq/l. To decrease variability, tubes were rinsed with hydrochloric acid (approximately 1 N)

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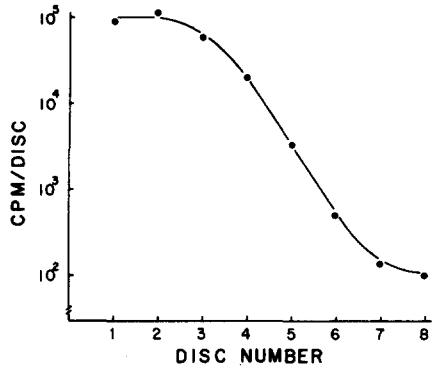

Fig. 1. Diffusion of 16:0 into successive thicknesses of polyethylene film. A composite disc consisting of eight layers of polyethylene film was mounted so that only one surface was exposed to an aqueous solution containing 1-^{14}C-labeled 16:0. After approximately 3 days of equilibration at 37°C, the disc was unmounted and rinsed, and individual layers were counted separately.

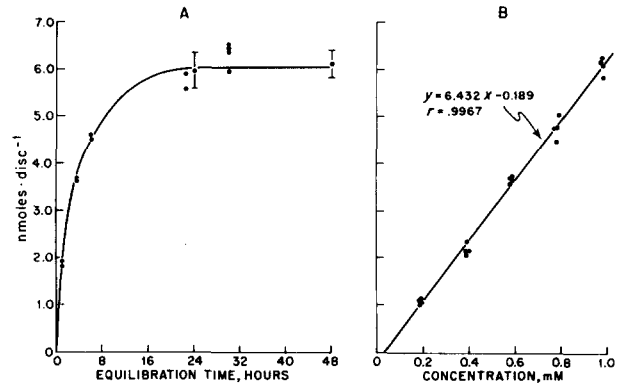

Fig. 2. Characteristics of partitioning of fatty acids into polyethylene discs. Panel *A* shows the time course of appearance of fatty acid in polyethylene discs exposed to various concentrations of 10:0 in 10 ml of phosphate-buffered saline, pH 7.4 (corrected to 1 mM). Mean values ± SD are plotted for time periods of 24 and 48 hr with 22 and 15 determinations, respectively. These values are not significantly different from each other. Panel *B* shows the relationship of the equilibrium content of fatty acid in polyethylene discs as a function of the aqueous phase concentration of 10:0. The intercept of the linear regression analysis is not significantly different from zero.

and then with distilled water before use in the pH studies with 16:0.

Statistical analysis was based upon the Student's *t* distribution, using test quotients and tables from Ref. 10. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Characteristics of fatty acid distribution between polyethylene and phosphate buffer

Either partitioning or surface adsorption might explain the interaction of fatty acid with polyethylene film. Fig. 1 shows that when only one surface of a composite layered disc was exposed to a solution containing 1-^{14}C-labeled 16:0, the fatty acid slowly diffused through the polyethylene into successive layers. The amount of fatty acid in each layer is very close to the expected logarithmic relationship. Surface adsorption cannot be a significant factor since the amount of fatty acid in disc no. 2 is even greater than the amount in the exposed disc. Thus, the fatty acid can penetrate the polyethylene as required for partitioning.

Theoretical analysis of partitioning systems requires partition coefficients determined at equilibrium. Further, partition coefficients should be independent of aqueous phase concentration. These relationships with time and concentration are shown in Fig. 2 for 10:0. In panel A it can be seen that approximately 24 hr is required for the distribution system to achieve equilibrium; 6 hr is clearly not satisfactory. Values determined at 48 hr are not significantly greater than those determined at 24 hr. Coefficients for 10:0 and 16:0, determined at 1, 2, and 7 days, are given in Table 1. Only a small increase for 16:0 at 48 hr is evident. This time course is consistent with the rate of diffusion within the polyethylene seen in Fig. 1.

In panel B of Fig. 2 it is demonstrated that the content of fatty acid in the polyethylene disc at 24 hr is linearly dependent upon the concentration of fatty acid in the

TABLE 1. Partition coefficients for 10:0 and 16:0 determined after various equilibration times

Equilibration Time	10:0[a]	16:0[b]
days	$\mu l \cdot disc^{-1}$	
1	9.76 ± 0.77	21,510 ± 3002
2	10.00 ± 0.83	29,540 ± 2375
7	8.33 ± 0.61	31,990 ± 2578[c]

Values are means ± SD for six determinations.

[a] 10:0 concentration was 1 mM.

[b] Only 1-^{14}C-labeled 16:0 with a specific activity of 55.7 mCi/mmole was added to tubes, producing an aqueous concentration of 2.1–3.5 × 10^{-7} M.

[c] This value is mean ± SD of only two determinations. Four excluded values of 1337 to 2846 were probably artifactually low because of bacterial metabolism of isotope during the long incubation period.

TABLE 2. Partition coefficients for 16:0 determined at various aqueous concentrations

Concentration[a]	Partition Coefficient
4.9 × 10^{-8} M	23,840 ± 862 (n = 4)
5.8 × 10^{-8} M	24,750 ± 770 (n = 5)
6.2 × 10^{-8} M	24,180 ± 631 (n = 3)
7.4 × 10^{-8} M	26,050 ± 2003 (n = 2)
2.6 (±0.2) × 10^{-7} M[b]	29,540 ± 2375 (n = 6)

In these experiments, two to five discs were impaled by a 22-gauge hypodermic needle and immersed in 10 ml of aqueous solution. All tubes contained the same amount of ^{14}C-labeled 16:0.

[a] Concentration was calculated using specific activity of 55.7 mCi/mmole as stated by Applied Science Laboratories and counting efficiency of 84%, determined using [^{14}C]toluene prepared in the same manner as a reference.

[b] These values were determined at 48 hr using the standard method of floating one disc on the solution in each tube. These values also appear in Table 1.

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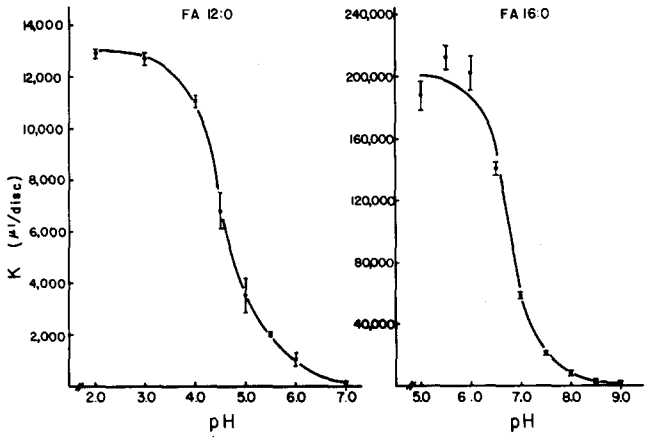

Fig. 3. Partition coefficients determined using different pH buffers. Values are means ± SE for eight or four (pH 5.5–7.0 for 12:0 and pH 8.0–9.0 for 16:0) determinations. Buffer for 12:0 contained citrate and phosphate while that for 16:0 contained citrate, phosphate, and borate, all at 20 mM. Experiments with single-component buffers were essentially identical.

aqueous–phosphate buffer up to 1 mM 10:0. The quotient of the content of the polyethylene disc and the concentration in the aqueous solution, that is, the partition coefficient, is therefore constant over the concentration ranges studied. In Table 2 are listed the partition coefficients determined for 16:0; these are essentially independent of the calculated concentrations with approximately a 10-fold range. The possibility of molecular aggregation in either aqueous phase or in organic phase that would alter this relationship will be considered subsequently.

The relationship between partitioning and the ionic state of fatty acid is demonstrated in Fig. 3 for 12:0 and 16:0. It is first evident that the protonated form of fatty acid is essentially the only form that interacts with polyethylene since the partition coefficient approaches zero as fatty acids are ionized to soaps at high pH. These curves, therefore, resemble titration curves for the fatty acids. The relationship for 12:0 is unremarkable since the midpoint of this curve is between pH 4.5 and 5.0, corresponding closely to a pK_a for short chain fatty acids of 4.8. However, the midpoint of the curve for 16:0 lies between 6.5 and 7.0, distinctly greater than that for 12:0. That only the protonated fatty acid form interacts with the polyethylene is additional strong support for partitioning rather than surface adsorption.

Molecular structure dependence is the next characteristic of partitioning systems to be considered. Partition coefficients have been determined for 9:0 through 18:0 at pH 7.4, and these values are presented in Table 3. It is evident that the distribution coefficients are extremely sensitive to the chain length of the fatty acid and increase by a relatively constant multiplying factor of approximately 3.75 for each additional methylene group. Since the partition coefficient is determined by the free energy of solution of the compound in the water phase and in the lipid phase

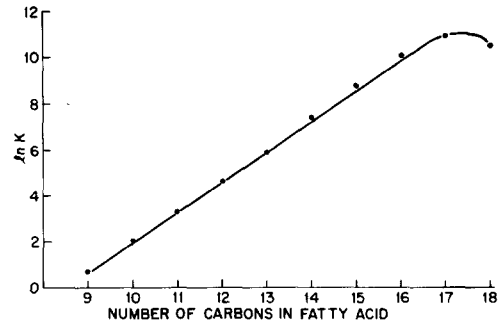

Fig. 4. Partition coefficients for fatty acids 9:0 through 18:0. Values from Table 1 are plotted as a function of the natural logarithm of the partition coefficients relative to the fatty acid chain length. Slope of line is equivalent to a 3.79-fold increase in K for each methylene group, or $\delta\Delta F_{w\to l} = -3433\ J\cdot mole^{-1}$ ($-820\ cal\cdot mole^{-1}$) per methylene group.

and this free energy term is a natural logarithmic function, it follows that the natural logarithm of the partition coefficient should be linearly related to the number of carbons in the fatty acid molecule. This linear relationship is shown in Fig. 4, using some of the data presented in Table 3. Distribution of fatty acids between the polyethylene discs and the aqueous–phosphate buffer is therefore behaving as expected for a partitioning type phenomenon. Other data on Table 3 will be considered in the Discussion.

The partition coefficients are only little influenced by temperature, as is seen in Fig. 5, although some deviation is seen with the longer chain length fatty acids. The value for 18:0 in Table 3 is deviant from the relationship at 37°C in a similar manner.

TABLE 3. Coefficients for partitioning of fatty acids between discs of polyethylene and aqueous solution

Fatty Acid	Aqueous Concentration	Partition Coefficient	
	mM	$\mu l \cdot disc^{-1}$	
9:0[a]	1.0	2.03 ± 0.09	(5)
10:0 (lot 1)[b]	0.2–1.0	6.14 ± 0.52	(46)
10:0 (lot 2)[b]	1.0 and *c*	9.35 ± 1.12	(20)
11:0[a]	*c*	27.2 ± 1.3	(6)
12:0[a]	*c*	99.8 ± 7.9	(16)
12:0	0.5	118.9 ± 4.6	(6)
13:0[a]	0.1	359 ± 77	(9)
13:0	0.5	744 ± 30	(6)
14:0[a]	*c*	1,640 ± 74	(6)
15:0[a]	*c*	6,500 ± 1,110	(9)
16:0[a]	*c*	24,660 ± 4,924	(39)
17:0[a]	*c*	56,100 ± 13,850	(15)
18:0[a]	*c*	36,800 ± 8,710	(6)

Values are means ± SD; number of determinations in parentheses.

[a] Value used in Fig. 4.

[b] Two separate lots of 1-^{14}C-labeled 10:0 prepared by Applied Science Laboratories were used. Experiments in Fig. 1 came from the first lot, all others from the second lot. Plotted on Fig. 2 is the mean of 6.14 and 9.35.

[c] Solutions contained only 1-^{14}C-labeled fatty acid with specific activity of 28.8–58.0 mCi/mmole. Calculated aqueous phase concentrations range from 4.5×10^{-8} M for 17:0 to 3.4×10^{-7} M for 12:0.

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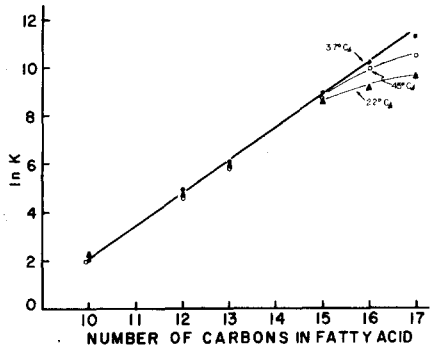

Fig. 5. Partition coefficients for fatty acids determined at three temperatures. Slope of line between 10 and 15 carbons is essentially identical with that of Fig. 4.

Apparent monomer activities of fatty acids in micellar solutions

The interaction of fatty acid with bile acid micelles was next investigated using the polyethylene disc partitioning technique. The fatty acid contents of polyethylene discs equilibrated with various concentrations of myristic acid (14:0) and palmitic acid (16:0) in 20 mM taurodeoxycholate solution are shown in Fig. 6, *A* and *B*, respectively. With the concentrations of fatty acids shown in this figure, the relationship of the fatty acid activity determining partitioning into the polyethylene disc is a linear function of the total fatty acid concentration and intercepts at zero.

Higher concentrations cannot be used because crystals form, presumed to be acid soap, and these adhere to the polyethylene. The linear relationship shown is important because it suggests that the interaction of fatty acid monomers with micelles is based upon partitioning of the fatty acid between the aqueous phase and the micellar lipid phase. If this hypothesis is true, then the fatty acid monomer activity should depend on the composition of the micelle rather than on the number of micelles in a given volume of the solution.

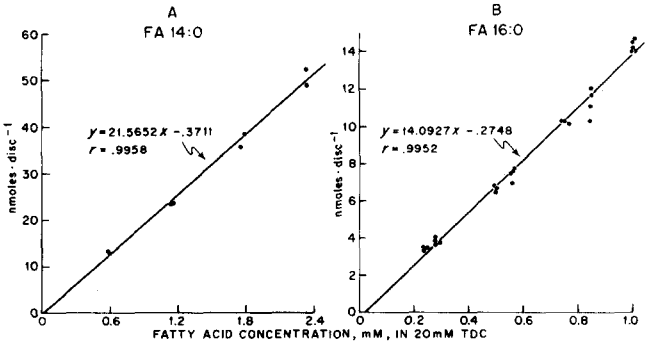

Fig. 6. Apparent monomer activities of fatty acids in 20 mM taurodeoxycholate micellar solutions. Panel *A:* various concentrations of myristic acid (14:0) are dissolved in 10 ml of 20 mM taurodeoxycholate and equilibrated with the polyethylene disc. The fatty acid content of the disc has been plotted relative to the total concentration of fatty acid in the aqueous micellar phase. Panel *B:* the experimental conditions were identical with those in panel *A* except that palmitic acid (16:0) was used. Intercepts described by linear regression analyses in the two panels are not significantly different from zero.

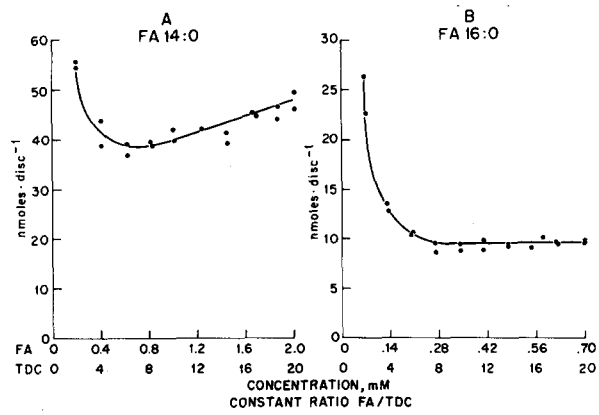

Fig. 7. Apparent monomer activities of fatty acids in taurodeoxycholate micellar solutions with constant ratio of fatty acid and bile salt concentrations. In panel *A* a solution of 2.0 mM myristic acid (14:0) was prepared in 20 mM taurodeoxycholate solution. This solution was then diluted with phosphate buffer to the various total concentrations shown. Fatty acid content of polyethylene discs equilibrated with 10 ml of these solutions are plotted relative to the total aqueous phase concentration of fatty acid and bile salt. In panel *B* the experimental conditions were identical with those in panel *A* except that palmitic acid (16:0) was used to prepare a solution of 0.7 mM concentration in 20 mM taurodeoxycholate solution.

In Fig. 7 total concentrations of fatty acid and bile salt have been varied at a constant ratio, keeping the micelle composition relatively constant from 8 to 20 mM taurodeoxycholate. From 0 to 8 mM bile salt, the fatty acid monomer activity actually decreases with increasing total concentration because near the bile salt critical micelle concentration (0.8 mM for taurodeoxycholate [3]) a large proportion of bile salt exists as monomers rather than micelles, resulting in an increased fatty acid to bile acid ratio in the micelle. Above this transition region, however, the fatty acid content of polyethylene discs is essentially constant for 16:0 and increases only slightly for 14:0.

DISCUSSION

Distribution of fatty acids between polyethylene and aqueous solution

Although the penetration of various compounds through membranes of organic polymers has been described (11), and the rate of solution of compounds in polymers has been used to study the process of diffusion in solid polymers including polyethylene (12, 13), it was still necessary to distinguish surface adsorption from the expected partitioning of fatty acids in polyethylene film. Fig. 1 conclusively demonstrates that the interaction of fatty acids with the polyethylene is not limited to the surface, but that fatty acid can traverse the membranes in succession. In addition, the concentration kinetics and pH dependence give strong support to partitioning rather than adsorption as the mechanism for fatty acid–polyethylene interaction.

However, this fatty acid–polyethylene interaction may be analyzed according to partitioning theory only if equi-

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librium has been achieved. This condition is validated for equilibration times of 24–48 hr by Fig. 2*A* and also by the data in Table 1. For the medium chain length fatty acid, 10:0, 24 hr is sufficient, but the partition coefficient determined at 48 hr for 16:0 is slightly higher than that determined at 24 hr. Equilibration for 1 wk produces no further change. The shape of this time relationship is similar to the time course described for diffusion into polyethylene (13) and is appropriate for the time course for diffusion seen in Fig. 1. At equilibrium, however, the measured partition coefficients are not influenced by this diffusion process, but are determined by the relative solubility of the fatty acid in polyethylene and in the aqueous phase. Although in many experiments discs were equilibrated for 24 hr, in most experiments with longer chain fatty acids they were equilibrated for 48 hr or longer.

Despite the linear concentration relationship expected for a simple partitioning system, the experimental partition ratios seen with fatty acids may be distorted by at least two reactions (8, 14). These reactions produce association complexes, presumably dimers, in both the hydrocarbon and aqueous phases as described by reactions 1 and 2, respectively, where HA and A^- represent the protonated and ionized forms of the fatty acid.

$$2HA \rightleftharpoons H_2A_2 \qquad \text{Eq. 1}$$

$$HA + A^- \rightleftharpoons HA_2^- \qquad \text{Eq. 2}$$

The product formed by reaction 2 has been termed an acid soap and may involve both hydrophobic bonding and a hydrogen bond to stabilize the dimer (14). As total concentration increases, formation of dimers is increased so that reaction 1 will produce an artifactually high ratio and reaction 2 an artifactually low ratio. Experimental results will depend not only on the concentration but also on the relative values of the equilibrium constants for the two reactions.

For the studies reported here, however, it is important that the concentration be sufficiently small so that the association reactions have very little influence on partition coefficient determinations. That this is the case may be seen in Fig. 2*B* for 10:0 and in Table 2 for 16:0. In these small concentration regions, the experimental partition coefficients are essentially independent of concentration. In Table 3 there are some indications that these dimerization reactions may have influence in the polyethylene system. Ratios determined for 12:0 and 13:0 at 0.5 mM are significantly higher than the values determined at lower concentrations. This observation suggests that dimerization within the polyethylene may occur, but in these two instances approximately 60 and 350 nmoles of fatty acid were contained in the disc, more than in any other experiments. Finally, Smith and Tanford (9) have stated that the equilibrium constant for dimerization in the organic phase should be little influenced by alkyl chain length. Thus, this reaction should be evident for any fatty acid only if the disc content is 60 nmoles or more. Since this value was achieved only with 12:0 and 13:0, the rest of the experiments reported here may be considered free from the influence of this reaction. Only the low value for 18:0 might be considered to indicate the influence of aqueous association complexes.

Experiments with buffers of various pH were undertaken to determine if only the protonated form of fatty acid participated in the partitioning process. Fig. 3 demonstrates that this is true for both 12:0 and 16:0, but it provides in addition a very interesting observation, i.e., the midpoint of the curves for the two fatty acids is different by approximately two pH units. The curves closely resemble typical titration curves, and indeed may be considered in essentially the same way. The apparent pK_a for 12:0 is between 4.5 and 5.0, appropriate for the water-soluble fatty acids (15). However, 16:0 appears to be distinctly different in its dissociation behavior since the midpoint of this curve is between pH 6.5 and 7.0. The dimerization reactions previously discussed are not responsible for this finding since both would produce artifactually low apparent pK_a values. Reaction 1 would increase the measured partition coefficient at low pH values where $[HA]^2_{HC}$ is high, and thereby shift the midpoint of the curve to a pH less than the true pK_a. On the other hand, the decrease of partition coefficients produced by reaction 2 would be maximal at the true pK_a since this reaction is proportional to the product of $[HA]_w$ and $[A^-]_w$. One possible explanation remains: a small amount of fatty acid exists in a monomolecular layer on the surface of the disc, so that ionization of this layer produces an electrostatic potential and, consequently, an apparent pK_a shift due to local hydrogen ion accumulation (16). This possibility is presently being considered in further experiments.

Theoretical analysis of distribution coefficients for homologous series of compounds has been carried out by Diamond and Wright (17) using data from Collander (18, 19), by Mukerjee (20) using data from Goodman (8), and by Smith and Tanford (9). The essence of these analyses for the homologous series of fatty acids are contained in the following equations where K and K' are the distribution coefficients for fatty acids of m and $(m + 1)$ carbon atoms, respectively.

$$K = e^{-\Delta F_{w\rightarrow 1}/RT} \qquad \text{Eq. 3}$$

$$K' = e^{-(\Delta F_{w\rightarrow 1}/RT) - (\delta\Delta F_{w\rightarrow 1}/RT)} \qquad \text{Eq. 4}$$

The term $\Delta F_{w\rightarrow 1}$ is equal to the free energy change necessary to transfer 1 mole of fatty acid from the aqueous phase into the lipid phase, and $\delta\Delta F_{w\rightarrow 1}$ is the incremental free energy change associated with one methylene

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group (-CH_2-). Eq. 5 shows that a plot of the natural logarithm of the distribution coefficient versus the number of carbons in the fatty acid should yield a linear relationship with the slope of the relationship mathematically equal to the incremental free energy for each -CH_2- group.

$$\delta\Delta F_{u\to 1} = -RT \ln\frac{K'}{K} = (-RT)\,\text{slope} \qquad \text{Eq. 5}$$

The slope of this relationship shown in Fig. 4 describes an incremental free energy of −3433 J (−820 cal) per mole per -CH_2- group. This quantity is essentially identical with the published value of −3453 J (−825 cal) per mole obtained with the heptane–water system (9, 20).

The small effect of temperature on this partitioning system (Fig. 5) is not surprising since only disproportionate changes of solubilities in the two phases would appear here. Partition coefficients of only 16:0 and 17:0 are significantly influenced by temperature. Although 18:0 was not included in this study, the shape of the curve is similar to that in Fig. 4. At least two factors may be operative in this temperature sensitivity: (*a*) partitioning of fatty acid may be altered by the "melting" of the alkyl chain hypothesized for the Krafft point of micelle formation (45°C for 16:0 [21]), and for the expansion temperature of a monolayer film (28.5°C for 16:0 [22, 23]), and (*b*) acid soap formation or possibly even dissociation of fatty acid may change with temperature and thus change the concentration of fatty acid that partitions. These effects may not be presently explained, but the lack of temperature effects on fatty acids shorter than 16 carbons enforces the concept of partitioning rather than surface adsorption.

Characteristics of fatty acid/bile salt micellar solutions

Although much quantitative information about micellization of bile salts is available (3, 4), the interaction of fatty acids with bile salt micelles is less well understood. Hofmann (5, 6) has measured the ability of various bile salt micellar solutions to solubilize fatty acids and other lipolytic products; however, of necessity, all of his experiments have dealt with solutions that are saturated with respect to fatty acid content. He emphasizes that the myriad aggregation reactions possible in physiological solutions make the fatty acid/bile salt solutions very complex indeed.

The use of polyethylene discs as a measure of the chemical activity of fatty acid in solution is the first technique potentially capable of distinguishing the monomer form of the fatty acid from the total concentration present in solution. This technique has therefore been used to describe the nature of the fatty acid interaction with bile salt in relatively dilute solutions of fatty acid dissolved in the presence of only taurodeoxycholate. Two types of situations have been used to characterize this fatty acid/bile salt interaction: (*a*) various concentrations of fatty acid have been dissolved in solutions having the same concentration (20 mM) of TDC, and (*b*) solutions have been prepared that contain various concentrations of fatty acid but the concentration of TDC has been changed to maintain a constant concentration ratio of the two constituents of the solution. Bile salt in the first situation would provide a "constant volume of micellar lipid," but the fatty acid content of that lipid would vary with the total concentration of fatty acid in the solution. In the second situation, however, the "volume of micellar lipid" would vary as the concentration of bile salt changed, tending to keep relatively constant the net content of fatty acid in that micellar lipid. Results of experiments using these two types of situations show that, first, the apparent monomer activity of fatty acid is a linear function of the total concentration of fatty acid when the total bile salt concentration is held constant. This suggests that the fatty acid, similar to a phase distribution phenomenon, is equilibrating with that micellar lipid in the solution. In the second situation, the relatively constant fatty acid monomer activity between the ranges of total concentration of TDC between 8 and 20 mM suggests that the determinant of fatty acid monomer activity in the aqueous solution is the concentration of fatty acid in the micellar lipid and not the volume of that micellar lipid. These two concentration relationships are essentially identical with the dependence of intestinal uptake on concentration under similar conditions (7). Thus, in TDC solutions, dilute with respect to fatty acids, the interaction of fatty acids with micelles should be considered similar to an immiscible phase distribution system that is characterized by a partitioning coefficient, producing a monomer form of fatty acid active for both partitioning and absorption.

Since accurate values for the partition coefficients of fatty acids between polyethylene and buffer have been determined, it is possible to calculate actual values for monomer activity of fatty acid in certain solutions. This is accomplished by solving the regression equation for the desired total concentration of fatty acid in 20 mM TDC to obtain the corresponding equilibrium content of the polyethylene disc (nanomoles per disc) and subsequently dividing that content by the partition coefficient.

For 1 mM 16:0 in 20 mM TDC at pH 7.4, the calculations are as follows:

$$\text{nmoles/disc} = 14.09\,(1\text{mM}) - 0.275 = 13.815$$

$$\text{Monomer activity} = \frac{13.815}{24660} = 0.00056\ \text{mM}$$

$$\text{Monomer activity} = 5.6\times10^{-7}\,\text{M}$$

This value is very much lower than the total concentration of fatty acid in the solution and is also lower than the

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determined maximum solubility of palmitic acid in a similar solution without bile salt.[1]

14:0 (1 mM) in 20 mM TDC has a monomer activity of 1.3×10^{-5} M, demonstrating the lower micelle/monomer partition coefficient for 14:0 as compared with 16:0. For any TDC concentration between 8 and 20 mM, the monomer fatty acid activity may be estimated by solving the regression equation in Fig. 3 for the concentration of fatty acid in 20 mM TDC that has the same fatty acid/TDC ratio. This procedure is validated by Fig. 4.

It should be pointed out that in Fig. 4 the initial decrease in fatty acid monomer activity as the total concentration is increasing is due to micelle formation near the critical micelle concentration of TDC. With low concentrations the proportion of bile acid that exists in a monomer form is much greater than at higher concentrations so that the true ratio of fatty acid concentration to micellar bile acid concentration is changing in this concentration range. Above 8 mM, the proportion of bile acid in the monomer form becomes negligible, and monomer activity of fatty acid is relatively constant. The slight increase in this region for 14:0 in TDC has not been explained, although it is also seen with uptake by intestinal tissue.[2]

Data presented in these experiments have shown that monomer activity of fatty acids in micellar solutions of bile acid varies as expected for a phase distribution system. In addition, it has been shown that the measured polyethylene/buffer partition coefficient may be used to calculate the actual value of monomer activity in various micellar solutions. The incremental free energy for one methylene group may therefore be calculated for the micelle/monomer partition ratio according to Eq. 6, which assumes that the volume of micellar lipid is the same in the two situations and that the proportion of fatty acid as monomer is negligible. Using the previously calculated values for monomer activity of 1 mM 14:0 and 16:0 in 20 mM TDC, $\delta\Delta F_{w\to l} = -4056$ J·mole^{-1} (-969 cal·mole^{-1}) per methylene group. This value, similar to the previously discussed values for both the heptane/buffer and the polyethylene/buffer partitioning systems, supports the hypothesis that the interaction of fatty acid with bile salt micelles is a partitioning system with an extremely hydrophobic micelle core.

$$\delta\Delta F_{w\to l} = -RT\,(0.5 \ln \frac{[\text{mon}]_{14}}{[\text{mon}]_{16}}) \qquad \text{Eq. 6}$$

Finally, it is appropriate to point out some limitations with this system. Fatty acid crystals tend to adhere to the surface of the polyethylene, invalidating the use of saturated solutions. For certain solutions, the apparent dimerization of fatty acid in the polyethylene obscures the true partitioning ratio, but this does not seem to influence fatty acid studies with 14:0 or longer chain length where the disc content of fatty acid does not exceed 60 nmoles/disc. The relationship of activity vs. total concentration for 12:0 and 13:0 in 20 mM TDC is distorted, however, apparently by this mechanism (data not presented). For these fatty acids, a detailed study of partitioning as a function of concentration will be necessary to quantitate the dimerization and correct the subsequent studies for this reaction.

This work was supported by U.S. Public Health Service General Research Support Grant 5 S01 RR 05426-10.

Manuscript received 29 September 1972 and in revised form 25 July 1973; accepted 14 September 1973.

[1] Approximately 8–10 μmoles of 1-^{14}C-labeled 16:0 of known specific activity was equilibrated in 100 ml of Krebs-Ringer bicarbonate buffer at 37°C, pH 7.4, for 24 hr with stirring. The solution was then filtered through a Millipore filter (0.22 μm pore size) and duplicate aliquots were counted. Solubility values of 1.3×10^{-6} M and 8.4×10^{-7} M were obtained in two separate experiments, giving a mean solubility of 1.07×10^{-6} M.

[2] Sallee, V. L. Unpublished observations.

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