Effect of Polysorbate 60 on Interphase Transport of Cholesterol

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Abstract □ Interphase cholesterol transport was investigated at 24 ± 1° in a stirred diffusion cell and in various oil-in-water emulsions. Cholesterol uptake by vegetable oil from a cholesterol–surfactant-rich aqueous phase was extremely slow in the stirred cell; no measurable transport had occurred after 500 hr. Cholesterol transport in oil-in-water emulsions following dilution with a cholesterol–surfactant-rich aqueous phase was much faster due to the greatly increased interfacial area available for mass transfer. Equilibration half-lives, $t(50)$, varied from 2.02 to 28.1 hr. Variations in the $t(50)$ were due to: *(a)* differences in the mean oil droplet diameter among various emulsions, and *(b)* differences in cholesterol–polysorbate 60 micelle sizes among various dilution media. When polysorbate 60 was omitted from the dilution medium, transport occurred in a two-stage process. In the first stage, transport was extremely rapid, with the $t(50)$ less than 30 sec; in the second stage, transport was comparable to previous emulsion rates, with the $t(50)$ varying from 7.9 to 8.1 hr. The significance of this two-stage transport to mechanisms of interphase cholesterol transport is briefly discussed.

Keyphrases □ Polysorbate 60—effect on interphase transport of cholesterol, mechanism □ Cholesterol—effect of polysorbate 60 on interphase transport, mechanisms and equations □ Transport, interphase—cholesterol, effect of polysorbate 60, stirred diffusion cell, mechanism □ Emulsions—vegetable oil–surfactant–water, effect of polysorbate 60 on interphase transport of cholesterol

Much research has been devoted to investigating the transport of various solute molecules in aqueous media across biomembranes, using both *in vivo* and *in vitro* techniques. Such studies have enhanced the understanding of the mechanisms of absorption, distribution, and metabolism of biologically important compounds in living systems.

Recent studies (1, 2) attempted to elucidate the mechanism (or mechanisms) of *in vivo* transport of cholesterol and related steroids by investigating interphase transport in liquid hexadecane oil-in-water emulsions stabilized by various surfactants. The results of these studies led to the postulation of two possible mechanisms of transport: "free drug delivery" and "micelle delivery." The term "drug" is used because the investigators regard the steroids as model drug compounds. The term "free drug" denotes a steroid dissolved in monomeric form in aqueous solution in contrast to the aggregated or micellar form consisting of steroid–surfactant micelles dispersed throughout the aqueous medium.

The free drug delivery model is based on that proposed by Sylvén and Borgström (3) for absorption and digestion of fat, in which monomeric or free cholesterol diffuses from an oil droplet into the intestinal aqueous phase, where it associates with the bile salts and the polar split products of the oil phase formed by the action of lipase. This micelle transports cholesterol to the "vicinity" of the cell membrane, where the micelle dissociates and monomeric cholesterol is absorbed by the cell while the bile salts return to the bulk aqueous phase. According to Surpuriya and Higuchi (2), the micelle may not take part in the transfer of solute across the hexadecane–water interface and only the free drug is involved in the rate-determining step.

The micelle delivery model proposes that only the micelle takes part in the transfer of drug across the hexadecane–water interface, although free drug is present in solution. Surpuriya and Higuchi (2) suggested that the micelle: "interacts with the hexadecane–water interface and unloads the solute, which passes into the oil phase, and the micelle returns to the aqueous phase."

The objective of this investigation is to present data from a related study (4), which suggest an alternative interpretation of interphase cholesterol transport.

EXPERIMENTAL

Stirred Diffusion Cell Experiment—Cholesterol[1] uptake by vegetable oil[2] containing 1.5% (w/v) sorbitan monostearate[3] from an aqueous phase containing 5% isopropanol[4] and 1.1% polysorbate 60[5] was studied at 24 ± 1° in a 1-liter aspirator bottle[6]. The initial aqueous cholesterol concentration, C_{wi}, was 500 μg/ml. The water was double distilled and deionized.

The upper oil phase (200 ml) was stirred at 12.5 rpm by an umbrella stirrer[7] driven by a variable speed motor[8]. The lower aqueous phase (200 ml) was stirred at 52 rpm by a 25.4 × 7.9-mm octagonal magnetic stirring bar powered by a stirrer[9]. Both stirrers were hooked to variable autotransformers[10] to provide sensitive stirring speed control.

Transport was measured periodically by withdrawing 0.5 ml of the aqueous phase (through the stirred cell side arm sealed with a serum cap) in a 0.5-ml tuberculin syringe fitted with a 50.8-mm 22G hypodermic needle. The samples were mixed with 2 ml of isopropanol and analyzed by colorimetry[11] at 550 nm using a standard procedure (5). Full experimental details were described elsewhere (4).

Emulsion Experiments—*Emulsion Preparation*—Liquid oil-in-water emulsions were prepared using vegetable oil and the sorbitan monostearate–polysorbate 60 emulsifier system (4). The emulsifier hydrophilic–lipophilic balance (HLB) was 11.8, and the concentration was 2.67% (w/v) of oil plus emulsifier. Emulsification was by the agent-in-oil method (6), and the initial oil volume fraction, ϕ_i, was 0.5.

The oil droplet size was varied by using emulsification treatments of increasing severity: *(a)* propeller mixing in a blender[12] at an autotransformer setting of 30 v, *(b)* propeller mixing in the

[1] Eastman grade, Eastman Organic Chemicals, Rochester, N.Y.
[2] Vegetable oil (Wiley mp <0°, ~80% soybean and 20% cottonseed oil) was obtained from Hunt-Wesson Foods Inc., Fullerton, Calif.
[3] Span 60 (HLB = 4.7), ICI America Inc., Wilmington, Del.
[4] Reagent grade, Merck & Co., Rahway, N.J.
[5] Polyoxyethylene (20) sorbitan monostearate (Tween 60, HLB = 14.9), ICI America Inc., Wilmington, Del.
[6] No. 1220, Corning Glass Works, Corning, N.Y.
[7] No. 14-514, Fisher Scientific Co., Medford, Mass.
[8] No. 59880-10, Matheson Scientific Co., Stoneham, Mass.
[9] No. 69302, Precision Scientific Co., Chicago, Ill.
[10] No. 116B, Superior Electric Co., Bristol. Conn.
[11] AutoAnalyzer, No. AA1, Technicon Inc., Tarrytown, N.Y.
[12] No. 1001, Waring Products Co., Winsted, Conn.

blender at an autotransformer setting of 100 v, and *(c)* treatment *(b)* followed by piston homogenization at 140 kg/cm^2 (1 pass) in a single-stage laboratory homogenizer[13]. Particle size, d_{vs}, and distribution, d_m/d_n (*i.e.*, polydispersity), were measured[14] (4), where d_{vs} is the volume to surface mean droplet diameter, and d_m and d_n are the mass and number average diameters, respectively.

Preparation of Aqueous Dilution Media—Cholesterol-rich aqueous phases were prepared using different solvents—*viz.*, ethanol[15], isopropanol, and polysorbate 60 (4) (Table I). The cholesterol–polysorbate 60 micelle size was estimated by filtration through 25-mm diameter filters[16] with pore sizes of 0.025, 0.05, 0.1, 0.22, 0.3, and 0.45 μm. The filters were contained in a microsyringe filter holder[17] attached to a 5-ml hypodermic luer-lok syringe. Samples in the syringe were forced under manual pressure through the filter by the syringe plunger. Approximately 1 ml of filtrate was collected in a 3-ml vial and was analyzed for cholesterol concentration as described previously.

Measurement of Cholesterol Transport—Ninety-five milliliters of the appropriate aqueous dilution medium at 24 ± 1° was weighed into a 125-ml erlenmeyer flask, which was placed on a rotary shaker. Then 5 ml of emulsion (ϕ_i = 0.5) at 24 ± 1° in a 5-ml graduated cylinder was rapidly poured into the dilution medium at zero time. (This procedure gave an emulsion dilution factor, DF_{em}, of 20.) The shaker was immediately switched on at 150 rpm.

The sampling procedure involved: *(a)* withdrawing ~3 ml of diluted emulsion in a 5-ml syringe attached to a 102-mm 15G stainless hypodermic needle, *(b)* inverting the syringe and detaching the needle, *(c)* attaching the syringe to the filter holder, and *(d)* filtering the sample through 0.22-μm pore size filters. The earliest separation time was 30 sec. Aqueous filtrates of unlabeled cholesterol were analyzed as described previously. Cholesterol-4-^{14}C[18] was analyzed by liquid scintillation counting[19] after 0.5 ml of aqueous filtrate was pipetted into 10 ml of scintillation cocktail[20] in a 20-ml scintillation vial.

RESULTS AND DISCUSSION

Stirred Cell Experiment—Transport rate may be evaluated from Eq. 1, which applies to one-dimensional unsteady-state mass transfer across an oil–water interface in cylindrical stirred diffusion cells (4, 7):

$$\ln \frac{C_{we} - C_w}{C_{we} - C_{wi}} = -\frac{K_w A}{V_w}\left[\frac{V_w + V_o K_p}{V_o K_p}\right]t = -S_{sc}t \quad \text{(Eq. 1)}$$

where $(C_{we} - C_w)/(C_{we} - C_{wi})$ = fraction of unattained equilibrium; C_{we} and C_{wi} = equilibrium and initial aqueous concentrations, respectively; K_w = overall mass transfer coefficient accounting for the diffusional resistance in each phase expressed in terms of the aqueous concentration gradient; A = interfacial area; V_o and V_w = oil and aqueous volumes, respectively; S_{sc} = stirred cell transport rate; and t = time. The partition coefficient, K_p, is defined as follows:

$$K_p = C_{oe}/C_{we} \quad \text{(Eq. 2)}$$

where C_{oe} and C_{we} = concentrations in the oil and aqueous phases at equilibrium, respectively.

Figure 1 shows that no perceptible uptake of cholesterol by vegetable oil from an aqueous phase containing 5% isopropanol and 1.1% polysorbate 60 was detected over 500 hr. The extremely slow uptake rate was surprising, particularly since transport rates equivalent to equilibration half-lives of 2.0–13.5 hr were reported for normal alcohols (C_3–C_8) in a similar system (4, 7) and the estimated diffusion coefficients of cholesterol in vegetable oil and water were only slightly less than *n*-octanol diffusivities (4).

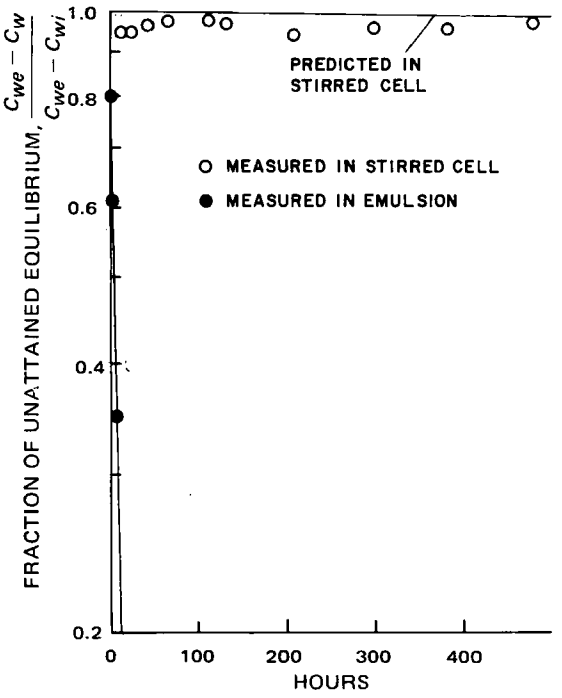

Figure 1—*Comparison of cholesterol transport rates in the stirred diffusion cell (experimental and predicted) and in a vegetable oil-in-water emulsion (Experiment 4) at 24°.*

Prediction of Cholesterol Transport in Emulsions—A scale-up model was developed (4, 8) on the assumption that Eq. 1 also applies to three-dimensional diffusion to spherical oil droplets in oil-in-water emulsions following instantaneous aqueous dilution, *i.e.*:

$$\psi = S_{em}/S_{sc} = t_{sc}(50)/t_{em}(50) \quad \text{(Eq. 3)}$$

where ψ is the scale-up factor (*i.e.*, transport rate ratio) and $t(50)$ is the time for the attainment of 50% equilibration. Equation 1 can be written in a form more suitable for mass transfer in diluted emulsions when the following relationships are used:

$$DF_{em} = \phi_i/\phi \quad \text{(Eq. 4)}$$

$$V_w = (1 - \phi)V_{ow} \quad \text{(Eq. 5)}$$

$$d_{vs} = 6\phi V_{ow}/A \quad \text{(Eq. 6)}$$

where DF_{em} = emulsion dilution factor; ϕ_i and ϕ = initial and diluted oil volume fractions, respectively; and V_{ow} = emulsion volume. Rewriting Eq. 1 gives:

$$\ln \frac{C_{we} - C_w}{C_{we} - C_{wi}} = -\frac{K_w}{d_{vs}}\left[\frac{6\phi_i/DF_{em}}{1 - \phi_i/DF_{em}}\right] \times \left[\frac{K_p - 1 + DF_{em}/\phi_i}{K_p}\right]t = -S_{em}t \quad \text{(Eq. 7)}$$

If $K_{p,em} = K_{p,sc}$ and $K_{w,em} = K_{w,sc}$, an isothermal scale-up factor, ψ, can be expressed in measurable stirred cell and emulsion parameters by substituting Eqs. 1 and 7 into Eq. 3 as follows:

$$\psi = \left[\frac{V_{ow}(1 - \phi)}{A}\right]_{sc}\left[\frac{6\phi_i/DF_{em}}{d_{vs}(1 - \phi_i/DF_{em})}\right]_{em} \times \left[\frac{K_p - 1 + DF_{em}/\phi_i}{K_p - 1 + 1/\phi_{sc}}\right] \quad \text{(Eq. 8)}$$

If 50% equilibration has been attained in the diluted emulsion, Eqs. 3 and 7 can be combined to give:

$$t_{em}(50) = 0.693/\psi S_{sc} = 0.693/S_{em} \quad \text{(Eq. 9)}$$

[13] No. 15M-8TBA, Manton-Gaulin Manufacturing Co., Everett, Mass.
[14] Coulter counter, model B, No. 3303, Coulter Electronics Inc., Hialeah, Fla.
[15] Reagent grade, U.S. Industrial Chemical Co., New York, N.Y.
[16] Millipore Corp., Bedford, Mass.
[17] No. XX30-025-00, Millipore Corp. Bedford, Mass.
[18] 0.344 mg (0.05 mCi) was obtained in 2.5 ml of benzene from New England Nuclear Corp., Boston, Mass.
[19] No. LS 250, Beckman Instruments Inc., Fullerton, Calif.
[20] The cocktail consisted of 2,5-diphenyloxazole (7 g), naphthalene (100 g), and dioxane (*q.s.* 1000 ml).

Table I—Interphase Cholesterol Transport in Oil-in-Water Emulsions at 24°

Experiment	Aqueous Dilution Medium, % Polysorbate 60	Isopropanol	Ethanol	C_{wi}, μg/ml	$\frac{C_{wd}}{C_{we}}$	K_p	d_{vs}, μm	$\frac{d_m}{d_n}$	$S_{em} \times 10^4$, 1/sec	t_{soem}, hr
1	0.106	5.0	0	500	NM[a]	NM	5.78	9.27	NM	NM
2	1.06	5.0	0	500	3.12	82.6	5.78	9.27	0.0685	28.1
3	1.06	5.0	0	500	3.33	90.8	3.28	3.14	0.178	10.8
4	1.06	5.0	0	500	2.84	71.4	1.56	1.94	0.447	4.31
5	5.0	0	0	500	1.20	7.8	1.56	1.94	0.956	2.02
6	1.1	0	0.15	0.134	2.12	43.6	1.56	1.94	0.725	2.66
7	0	0	0.15	0.134	5.40	172	1.56	1.94	NM	(*a*) <30 sec (*b*) 8.1 hr
8	0	0	0.15	0.134	5.61	180	1.56	1.94	NM	(*a*) <30 sec (*b*) 7.9 hr

[a] NM = not measured.

Hence the time for the attainment of 50% equilibration in the emulsion can be predicted from Eq. 9 once ψ is evaluated from Eq. 8, provided K_p and S_{sc} are known. However, due to the slow cholesterol transport in the stirred diffusion cell, S_{sc} and K_p could not be evaluated from Eqs. 1 and 2 because of insufficient data. Therefore, emulsion transport rates could not be predicted.

Emulsion Experiments—Filtration of the aqueous dilution media used in Experiments 1–5 (Table I) revealed that some cholesterol was retained by the filters and that the extent of cholesterol retention increased as both filter pore size and polysorbate 60 concentration decreased (Fig. 2). These results were expected, since micelle size in the micellar solutions increased sharply as the polysorbate 60 concentration decreased, as was indicated by an increase in turbidity. It is clear from Fig. 2 that micelle size in the case of the 0.106% polysorbate 60 (P60) concentration level was extremely large; for example, approximately 485 out of 500 μg/ml cholesterol was retained on a 0.1-μm (1000 Å) filter. When the polysorbate 60 concentration is further reduced, cholesterol precipitates out of solution (4).

The extent of cholesterol uptake by emulsion oil droplets was evaluated using Eq. 10, derived from the model of McNulty and Karel (9):

$$\frac{C_{wd}}{C_{we}} = \frac{\phi_i(K_p - 1) + DF_{em}}{DF_{em} - \phi_i} \qquad \text{(Eq. 10)}$$

where C_{wd} = aqueous concentration immediately after emulsion dilution. If K_p is not known, C_{wd} and C_{we} may be estimated by extrapolating C_w *versus* t plots to zero and infinite times, respectively. Then K_p may be evaluated using Eq. 10.

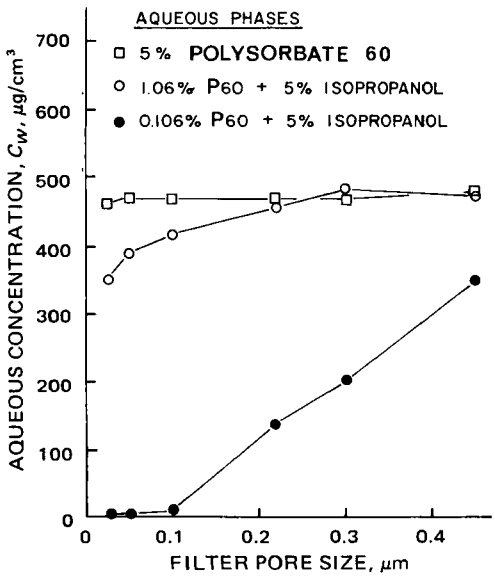

Figure 2—*Effect of aqueous phase composition on the size of cholesterol–polysorbate 60 (P60) micelles* (C_{wi} = *500 μg/ml) at 24°.*

Results of emulsion experiments revealed that the extent of cholesterol transport, C_{wd}/C_{we}, increased as the concentration of polysorbate 60 in the dilution media decreased (Table I). This finding was expected, since the cholesterol equilibrium will increasingly favor the oil phase (*i.e.*, K_p increases) as the aqueous polysorbate 60 concentration decreases.

In Experiment 1, it was difficult to distinguish between the cholesterol retained on the filter and that taken up by the oil due to the large cholesterol–polysorbate 60 micelle size (Table I, Fig. 2). Consequently, the transport rate could not be measured.

In Experiments 2–4, transport rates increased as expected with a decrease in the mean droplet diameter, d_{vs}, due to the increase in interfacial area available for mass transfer. The relative increases in transport rate (1.0:2.6:6.5) were less than the corresponding decreases in d_{vs} (3.7:2.1:1.0), probably due to the corresponding decreases in polydispersity, d_m/d_n. For example, when an emulsion is highly polydisperse (*e.g.*, Experiment 2), a large volume of the dispersed phase exists in a relatively small number of large droplets. Consequently, the transport rate should be slower than that in a less polydisperse emulsion of equivalent d_{vs} due to the relatively slow penetration of diffusing solute into large, as opposed to small, droplets.

Equilibration half-lives for transport of both labeled and unlabeled cholesterol varied from 2.02 to 28.1 hr (Table I). These values are similar to those (*i.e.*, 1.4–4.2 hr) estimated from the data of Bikhazi and Higuchi (10) for cholesterol-4-^{14}C transport in liquid hexadecane oil-in-water emulsions stabilized by polysorbate 80. These rates are extremely slow when compared to equilibration half-lives of normal alcohols (C_3–C_8), $t(50) < 15$ sec, in a similar system (4, 8).

Although emulsion rates could not be predicted, it was possible to use emulsion data and the scale-up model to predict the uptake rate in the stirred cell. Initial experimental results qualitatively confirmed the prediction (Fig. 1).

The effect of surfactant on the uptake rate of cholesterol-4-^{14}C was studied by omitting polysorbate 60 from the dilution medium and using a concentration level in the range of its solubility[21] (*i.e.*, approximately 0.1 μg/ml). The results in Fig. 3 suggest that when polysorbate 60 is omitted, cholesterol transport occurs in a two-stage process, probably due to the following scheme of events:

1. When the emulsion is diluted with aqueous cholesterol, the excess polysorbate 60 in the aqueous phase of the emulsion competes with the oil droplets for the monomeric cholesterol. A fraction of monomeric cholesterol is taken up by the oil droplets very rapidly, as shown in Fig. 3.
2. The remaining cholesterol is rapidly taken up by the polysorbate 60 micelles, and the uptake of surfactant-associated cholesterol is a slow process occurring at approximately the same rate as that in the earlier experiments where polysorbate 60 was included

[21] Aqueous cholesterol solubility was reported as approximately 0.025 μg/ml (11). The approximation in this study is larger, presumably because 0.15% ethanol increases the aqueous solubility.

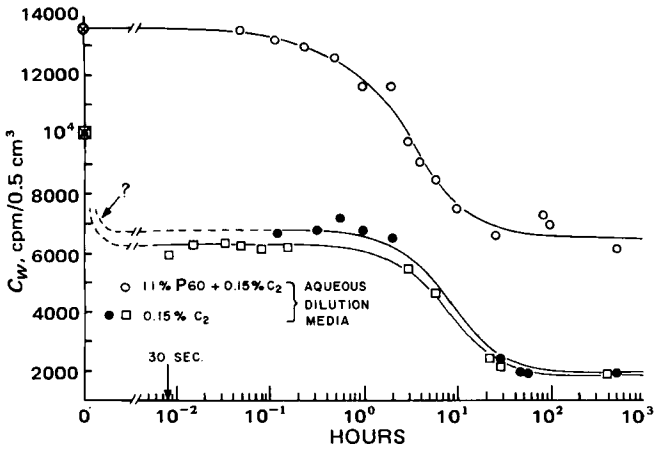

Figure 3—*Effect of the dilution medium composition on the transport rate of cholesterol-4-^{14}C in vegetable oil-in-water emulsions at 24° (Experiments 7 and 8; P60 = polysorbate 60; C_2 = ethanol).*

in the dilution medium. The slow transport is probably due to the opposing driving forces to which cholesterol is subjected, *i.e., (a)* an overall interphase gradient of chemical potential which drives cholesterol out of the micelle, through the water, and into the oil; and *(b)* a local gradient of chemical potential, which maintains the hydrophobic cholesterol in the micelle and out of the water.

It is suggested that, in ternary systems, cholesterol equilibration between oil and water should be rapid as indicated by the rapid monomeric cholesterol transport observed in this study. Furthermore, cholesterol equilibration between water and aqueous surfactant micelles should also be rapid. For example, Nakagawa (12) indicated that "solubilization is a phenomenon proceeding spontaneously." On the other hand, interphase cholesterol transport in quaternary (or multicomponent) emulsion systems proceeds very slowly.

As mentioned previously, the free drug delivery and micelle delivery models (1, 2) describe free drug transfer and micelle association or dissociation as the respective rate-determining steps in interphase cholesterol transport. In the absence of an opposing driving force, as in the described ternary systems, such equilibrations should be rapid. Therefore, the opposing driving force model proposed here appears to be more conceptually consistent.

It is interesting to speculate on the reported transport rates of related steroids (1). For example, in hexadecane oil-in-water emulsions stabilized by polysorbate 80, interfacial permeabilities for cholesterol, demosterol, β-sitosterol, and vitamin D_3 varied from 2.5 to 60 × 10^{-8} cm/sec. For 20α-hydroxycholesterol, progesterone, and testosterone, they varied from 2.0 to 30 × 10^{-5} cm/sec. No explanation was offered for the large difference in permeability between the two groups.

McNulty (4) observed that the steroids of lower permeability are sterols containing one hydroxy group. In contrast, the steroids of higher permeability possess an additional hydrophilic group on the opposite side of the steroid backbone; *i.e.*, 20α-hydroxycholesterol has two hydroxy groups, progesterone has two carbonyl groups, and testosterone has one carbonyl and one hydroxy group. The presence of an additional hydrophilic group will increase aqueous solubility and may produce a more randomly organized steroid–surfactant micelle, since both steroid hydrophilic groups will tend to orient themselves toward the hydrophilic exterior of the micelle. These two effects should reduce the magnitude of the opposing driving forces and thus increase permeability.

In conclusion, the equilibria and kinetics of steroid transport should be quantitatively established in steroid–oil–water and steroid–surfactant–water. [For the latter case, investigators should note the procedure of Short and Rhodes (13) who proposed a thermodynamic description of the effect of surfactants on diffusion of steroids across cellulose acetate membranes.] Characterization of such ternary systems should then permit a more quantitative interpretation of steroid transport in quaternary (or multicomponent) emulsion systems and also help to establish unambiguously the mechanism (or mechanisms) of interphase steroid transport.

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