The Intestinal Uptake of Phenol from Micellar Systems Does Not Conform to the Aqueous Transfer Model

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Purpose. To evaluate the aqueous transfer model as the mechanism for the micelle-mediated uptake of phenol in the rat *in situ* intestinal perfusion model.

Methods. Phenol in isotonic HEPES buffer was perfused through the jejunal segment at two flow rates and at various concentrations. Phenol was then dispersed in two, distinct mixed micelle systems composed of sodium taurocholate and phosphatidylcholine at 10 mM:2.5 mM (10:2.5 system) and at 10 mM: 10 mM (10:10 system) and its uptake studied in each case. Equilibrium dialysis was done to determine the aqueous fraction of phenol in each system.

Results. The P_{eff} of phenol in isotonic HEPES buffer at a low flow rate (n = 6) was $1.7 \pm 0.4 \times 10^{-4}$ cm/s and at a high flow rate (n = 13) was $1.8 \pm 0.5 \times 10^{-4}$ cm/s. The P_{eff} for the 10:2.5 system at the high flow rate (n = 3) was $1.5 \pm 0.4 \times 10^{-4}$ cm/s and at the low flow rate (n = 3) was $1.4 \pm 0.3 \times 10^{-4}$ cm/s. Uptake was membrane rate-limited in both the non-micellar and 10:2.5 systems. P_{eff} at a high flow rate (n = 3) in the 10:10 system was $1.3 \pm 0.1 \times 10^{-4}$ cm/s. Equilibrium dialysis (n = 4) revealed free fractions of 0.60 $\pm$ 0.05 and 0.50 $\pm$ 0.03 for the 10:2.5 and 10:10 systems.

Conclusions. The uptake of micellized phenol did not follow the aqueous transfer model of uptake.

KEY WORDS: Micelle-mediated uptake; equilibrium dialysis; aqueous transfer model; phenol.

INTRODUCTION

Micelles have dramatically increased the intestinal uptake of a number of poorly soluble, lipophilic substances (1–3). However, little information exists on the specific molecular mechanism at the enterocyte that is responsible for micelle-mediated uptake. Current knowledge in the area suggests three possible models to explain this phenomenon. The first is the collisional model (4,5), wherein the micelle collides by random Brownian motion against the wall of the enterocyte resulting in the fusion of some of the micellar components with the enterocyte wall, leading to uptake. The second model is that of active uptake (6–8) of the micellar components or the drug (or both). The last possibility is the aqueous transfer model (4,5). This proposes that the micellized drug partitions itself between the micelle and the surrounding aqueous milieu and only this 'free' drug is then taken up by the enterocyte.

The literature does not clearly endorse any one of these models. It is noteworthy, however, that most of the existing literature is derived from *in vitro* experiments (4–9). In order to develop more useful micellar systems for drug delivery, it is imperative to study micelle-mediated uptake in a more physiological model. Hence, the objective of this work was to characterize micelle-mediated uptake in an inherently more physiological model, the rat single-pass *in situ* intestinal perfusion model. The micelle system used was that of sodium taurocholate (NaTC) and egg phosphatidylcholine (PC), both of which are known to form mixed micelles in lumenally secreted bile in most mammals (10). The marker compound, phenol, was chosen for a number of reasons. At a physiological pH of 6.5, the net negative surface charge rendered by the NaTC on the NaTC-PC micelle is minimally perturbed by a weak acid such as phenol (pKa 10), which is largely unionized at this pH. This helps to retain the intrinsic shape and integrity of the micelle. The hydrophilicity of the compound also allows its characterization in a non-micellar environment, which provides a "baseline" for the uptake studies from micellar systems. Moreover, the literature does not show any evidence for the active uptake of phenol.

An initial characterization of the uptake of phenol in a non-micellar environment, i.e., isotonic HEPES buffer, was carried out. This was then compared to its uptake from two distinct, well-characterized (11) and physiologically realistic (1,6) mixed micelle systems of NaTC and PC at concentrations of 10 mM:2.5 mM (henceforth called the 10:2.5 system) and 10 mM: 10 mM (henceforth called the 10:10 system), respectively. The latter system is about ten-fold larger in diameter (12) and has a higher PC concentration. Both these factors could favor the partitioning of the phenol into the micelle and therefore, afford a smaller 'free' fraction of drug. The rate-limiting step in uptake was determined by conducting perfusions at two different flow rates. An attempt was also made to determine the concentration of non-micelle-associated or 'free' drug by equilibrium dialysis so that it might be correlated to the *in situ* results.

MATERIALS AND METHODS

Phenol, sodium pentobarbital, NaTC and the internal standard for the HPLC assay, *p*-cresol, were purchased from Sigma-Aldrich (St. Louis, MO). NaTC was recrystallized before use by the method of Pope (13) and the purity of the recrystallized product was evaluated by DSC. Egg PC purchased from Avanti Polar Lipids (Alabaster, AL), was stored at 4°C and protected from light. All other chemicals were reagent grade or better.

For the studies involving perfusion in a non-micellar environment, the phenol was dissolved in 10 mM HEPES buffer (pH 6.5) to yield the required concentration. The general method for micelle preparation has been previously detailed (1).

Animal Studies

Animal studies were carried out in accordance with "Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985)". Male Sprague-Dawley rats, 250–275 g, purchased from Harlan Bioproducts (Indianapolis, IN), were fasted overnight with free access to water. The surgical preparation of the intestinal segment has previously been described (1). The inlet to the jejunal cannula was connected to a glass syringe containing the drug solution and its flow was controlled by a

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Harvard infusion pump (Harvard Apparatus, Holliston, MA). Flow rates of either 0.1 ml/min or 0.3 ml/min were used. The drug solution was kept at 37°C during the course of the experiment by placing an isothermal pad around the glass syringe. The effluent perfusate was collected in pre-weighed glass vials in ten minute intervals for 120 minutes. Samples were stored at −20°C until analysis and were subsequently analyzed for phenol. At the end of the experiment the perfused segment was weighed and its length noted.

Equilibrium Dialysis

Dialysis bags were prepared by tying one end of the dialysis membrane (Spectropor® 6, MWCO 1000, Spectrum Medical Industries, Los Angeles, CA) with a 3-0 silk suture. A 1 ml volume of the donor solution consisting of phenol in a micelle system was pipetted into the bag and the other end of the bag was then tied. The bag was placed in the receiver chamber which was a glass scintillation vial containing 2 ml of NaTC at the intermicellar concentration (IMC) of each of the systems. The literature values of the IMC, 4.5 mM for the 10:10 system and 8 mM for the 10:2.5 system, were used (11,12). The vials were sealed after purging the headspace with N_2 gas and were incubated at 37°C in a shaking water bath. A pilot experiment was conducted to determine the time for equilibration (T_{eq}) with the 10:2.5 system (data not shown), and a T_{eq} of 20 h was chosen for the experiments to determine free fractions. Quality control samples indicated that phenol remained stable over the period of the experiment (data not shown). For the free fraction experiments, sample vials (n = 4) were set up as described above. At the end of the dialysis experiments, the donor and receiver chambers were analyzed for phenol. In addition, the integrity of the micelles was ascertained by assaying NaTC and PC concentrations in the receiver chamber.

Sample Preparation and Analysis

In case of phenol in HEPES buffer, the samples were directly injected onto the HPLC. For micellar samples, an aliquot of 500 μl was placed in a glass culture tube. To this, 50 μl of the internal standard, *p*-cresol (100 μg/ml) was added. The phospholipid was then precipitated by adding 1 ml of acetone. The tubes were subsequently vortex-mixed for 1 minute and centrifuged at 1300 × *g* (Clinicool, Damon/IEC, MA) for 15 minutes at 4°C.

The phenol was assayed by a modification of a previously published method (14). The HPLC set-up consisted of a Waters (Milford, MA) Model 6000A pump, a Waters WISP 710B autosampler, a Shimadzu (Shimadzu Corporation, Kyoto, Japan) RF 530 fluorescence detector and a Shimadzu CR501 integrator. A reversed-phase C_{18} Supelcosil (4.6 × 150 mm; 5 μ particle size; Supelco, Bellefonte, PA) column was used. The mobile phase consisted of water and acetonitrile (70:30 volume ratio) delivered at a flow rate of 1 ml/min with isocratic elution. The excitation and emission wavelengths were 260 and 305 nm, respectively. The retention times were 4.5 and 6.9 minutes for phenol and *p*-cresol, respectively.

PC and NaTC were analyzed in the equilibrium dialysis studies. PC was assayed spectrophotometrically by modification of the method of Chen (15). Taurocholate was assayed by HPLC with UV detection by a modification of the method of Nakayama (16). Sample preparation was similar to that for phenol except that testosterone in acetone was used as the internal standard. The acetone caused precipitation of the phospholipid. A reversed phase C_{18} (4.6 × 250 mm, 5 μ particle size) Supelcosil column and UV detection at 220 nm was used. The mobile phase consisted of methanol:phosphate buffer:acetonitrile in a volume ratio 55:35:11. The pH of the phosphate buffer (0.05 M KH_2PO_4) was adjusted with phosphoric acid to 3.4. The flow rate was 1.2 ml/min with isocratic elution. Retention times were 7.3 and 8.6 minutes for testosterone and NaTC, respectively.

Tissue Viability

Phenol is known to be fairly caustic and initial concerns of tissue necrosis were addressed by a histological comparison of segments perfused for 2 h with phenol in isotonic HEPES buffer at concentrations of 20 or 2 μg/ml. A control segment was perfused with blank isotonic HEPES buffer. These perfusions were conducted at a high flow rate so as to maximize tissue damage if it occurred. Histological analysis was conducted as previously described (1). The evaluation of the histological samples was conducted in a blinded manner.

Data Analysis

For the perfusion experiments, the effective permeability, P_{eff} was calculated based on the radial cup-mixing model which assumed that the intestine was a perfect right cylinder of length L and radius R and had uniform permeability along its length (17):

$$P_{eff} = \frac{-\ln(C_{out}/C_{in})Q}{2\pi RL} \quad (1)$$

where C_{in} is the concentration of the phenol in the inflow perfusate, C_{out} is the effluent phenol concentration corrected gravimetrically for water flux and Q is the flow rate. In order to allow gravimetric correction of net water flux, the samples were collected in pre-weighed vials. The volume collected in the time interval was then determined. If this was less than that theoretically expected (10 min × Q), the sample was deemed more 'concentrated' due to water efflux from the lumen and a correction factor was multiplied by the assayed phenol concentration. This correction factor was the ratio of volume collected/theoretical volume. In cases where the volume collected was greater than that theoretically expected, the ratio was reversed. Steady state was determined by observation of the C_{out}/C_{in} values. The values of C_{out}/C_{in} after steady state was reached were averaged and the mean used in equation (1) to calculate P_{eff}. The radius, R, was taken to be 0.18 cm (17). The absorptive clearance (CL_a) was obtained by rearrangement of equation (1):

$$CL_a = P_{eff} \times A = -\ln(C_{out}/C_{in})Q \quad (2)$$

where A is the surface area ($2\pi RL$). The uptake rate was then calculated as:

$$\text{Uptake rate} = (CL_a)(C_{in}) \quad (3)$$

These uptake rates were normalized to the length of the segment.

In case of the equilibrium dialysis experiments, the aqueous or free fraction of phenol (fu) was calculated as:

$$fu = \frac{C_{aq}}{C_{tot}} \quad (4)$$

where C_{aq} is the concentration of the free or aqueous phenol which is considered to be the equilibrating species and is measured in the receiver chamber. C_{tot} is the concentration of phenol in the donor chamber and consists of free and micelle-associated phenol.

Statistical analysis consisted of analysis of variance (ANOVA) which was conducted with Statview software (Version 4.01, SAS Institute, Cary, NC).

RESULTS

Tissue Viability

No morphological differences were seen between the phenol-perfused and control segments (data not shown). Furthermore, the eosin-hematoxylin stain was well taken up by the enterocyte nuclei indicating that phenol did not adversely affect the viability of the tissue at the concentrations chosen.

Perfusion in HEPES Buffer

Perfusions were initially done with phenol in isotonic HEPES buffer at flow rates of 0.1 and 0.3 ml/min. Two inflow concentrations of 2 μg/ml and 20 μg/ml were evaluated. Table I summarizes the results obtained from these studies. A two-way ANOVA indicated that neither the flow rate nor the concentration had a significant effect on the P_{eff} of phenol. Based on the two-resistance theory of intestinal uptake (17), the similarity in P_{eff} values at high and low flow rates indicated that the uptake of phenol was membrane rate-limited. In an attempt to characterize the mechanism of uptake of phenol, the length normalized uptake rate was plotted against inflow concentration (open circles in Fig. 1). These studies were done at the higher flow rate of 0.3 ml/min. The data afforded a reasonably good linear fit indicating that uptake of phenol was passive in the concentration range studied.

Perfusion in the Micellar Systems

The P_{eff} of phenol was then evaluated in the 10:2.5 system (Table II). Surprisingly, these values were not significantly different from the P_{eff} of phenol in buffer (Table I). Table II gives the P_{eff} values of phenol in the 10:2.5 system at a single inflow concentration of approximately 16 μg/ml and at two flow rates, 0.1 and 0.3 ml/min. One-way ANOVA showed that there was no significant difference in P_{eff} at high and low flow rates. Hence uptake was membrane rate-limited. An attempt was then made to increase the micelle-associated fraction by moving to the ten-fold larger 10:10 system (12). It is noteworthy that the 10:10 system also has a higher phospholipid content and could therefore favor the partitioning of the drug into the micelle. Table II also shows the uptake of phenol in the 10:10 system at an inflow concentration of 18 μg/ml. These studies were done at the higher flow rate of 0.3 ml/min so that the effect of the aqueous boundary layer, if any, would be minimized. A one-way ANOVA indicated that P_{eff} was not significantly different from that of the 10:2.5 system. Overall there was no significant difference in the P_{eff} of phenol from the non-micellar and micellar systems.

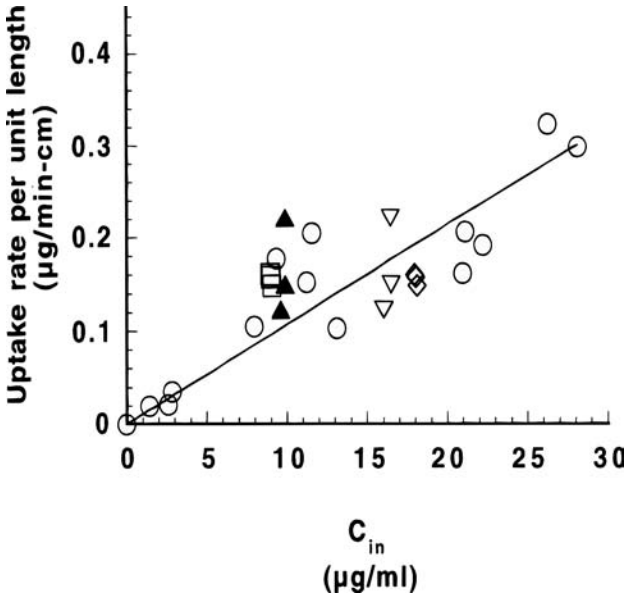

Fig. 1. Uptake rate (length normalized) vs. inflow concentration at high flow rate for phenol in: buffer (○), 10:2.5 system—total concentration (▽), 10:10 system—total concentration (◇), 10:2.5 system—free concentration (▲), 10:10 system—free concentration (□). It should be noted that the solid line represents fit of phenol *in buffer* to the equation $y = mx$ ($R^2 = 0.82$). The buffer data include the rats reported in Table I as well as others over the concentration range of 1.4–28 μg/ml (total n = 13)

Table I. Effective Permeability of Phenol in HEPES Buffer

Flow rate (ml/min)	Inflow concentration (μg/ml)	$P_{eff} \times 10^4$ (cm/s)	CL_a (ml/min)	Length of intestine (cm)
0.1	2.3 ± 0.5[a]	1.4 ± 0.5[a,b]	0.12 ± 0.06[a]	11 ± 2[a]
	20.7 ± 2.5	1.8 ± 0.1	0.13 ± 0.02	9 ± 1
0.3	2.3 ± 0.8	1.7 ± 0.4	0.12 ± 0.02	10 ± 2
	21.4 ± 0.7	1.6 ± 0.5	0.13 ± 0.07	11 ± 2

[a] Mean ± SD; n = 3.
[b] Two-way ANOVA on P_{eff} indicated that neither flow rate nor concentration had a significant effect ($p > 0.05$).

Table II. Effective Permeability of Phenol in the Two Micellar Systems

Micelle System	Flow rate (ml/min)	C_{in}[a] (μg/ml)	$P_{eff} \times 10^4$ (cm/s)	CL_a (ml/min)	Length of segment (cm)
10:2.5	0.1	15.5 ± 0.8	1.4 ± 0.3[b,c]	0.11 ± 0.01[b]	10 ± 1[b]
	0.3	16.4 ± 0.3	1.5 ± 0.4	0.12 ± 0.03	10 ± 1
10:10	0.3	18.1 ± 0.1	1.3 ± 0.1[d]	0.08 ± 0.01	9 ± 0

[a] Inflow concentration.
[b] Mean ± SD; n = 3.
[c] One-way ANOVA on P_{eff} indicated that flow rate had no significant effect, $p > 0.05$.
[d] One-way ANOVA on P_{eff} indicated that micelle type had no significant effect, $p > 0.05$.

Equilibrium Dialysis

In order to understand why the micellar systems did not appear to affect the uptake rate of phenol, an attempt was made to correlate the *in vitro* free fractions of phenol with the *in situ* P_{eff} values in each of the micellar systems. Equilibrium dialysis was used. Free fractions of 0.60 ± 0.05 and 0.50 ± 0.03 were found in the 10:2.5 and 10:10 systems, respectively, which were significantly different (Table III). Although minor PC leakage was found in the receiver chamber, the integrity of the micelles was assured since NaTC concentrations in the receiver chamber were at the IMC values.

To further elucidate the driving force of uptake from the micellar systems, total and free concentrations in each of the micellar systems were plotted against the length-normalized uptake rate (Fig. 1). The total concentrations in each case seemed to be closer to the fitted line from the buffer studies than did the free concentrations. This indicated that the buffer data predicted uptake based on total rather than free concentrations and showed that the aqueous phenol was not the driving force of uptake from micelles.

DISCUSSION

Currently accepted paradigms of micelle-mediated uptake have largely evolved from *in vitro* models (4–9). Whereas these models, due to their ease of handling and manipulation, are important in gaining a preliminary understanding of the system, it is imperative to eventually move to a more physiological system. *In vitro* models such as brush border membrane vesicles (BBMVs) and enterocytes lack an intact vasculature and disrupt the intestinal architecture. Perhaps more importantly, they lack a glycocalyx which is often implicated in ligand-receptor recognition. Thus, they are not truly representative of the complex nature of the intestine. Hence, our studies were conducted in the rat *in situ* single-pass intestinal perfusion model-an inherently more physiological setting.

The aqueous boundary layer, (ABL) has often been reported to be rate-limiting in the uptake of a number of substances, particularly those of lipophilic character (18–20). By increasing the bulk lumenal flow rate, the thickness of the ABL may be decreased, thus increasing the permeability of these type of drugs. Previous studies with the retinoids (1,21) have shown that when an ABL rate limitation exists, an increase in flow rate from 0.1 to 0.3 ml/min was adequate to increase the P_{eff} of these lipophilic compounds. Perfusions of phenol in buffer at high (0.3 ml/min) and low (0.1 ml/min) flow rates showed no significant difference in P_{eff} indicating that the uptake of phenol was not rate-limited by the ABL. If a rate-limitation in ABL was subsequently seen in the micellar systems, it could be attributed to the micelles.

A surprising outcome of the perfusion with the 10:2.5 system was that the P_{eff} was not significantly different from the non-micellar case. Furthermore, the rate-limiting step in the intestinal uptake remained unchanged. In other words, the micelles did not seem to influence the uptake of the drug. One possible explanation could be that phenol, being fairly hydrophilic, would not be housed in the hydrophobic interior of the micelle and in all likelihood was situated at the periphery of the micelle. Since the 10:10 system has been shown to be approximately ten-fold larger in size than the 10:2.5 system (12) and also has a higher PC content, it was thought that it might yield a smaller free fraction of phenol. Based on the aqueous transfer model, this should have translated to a difference in CL_a. However, no significant difference in CL_a was seen with the 10:10 system.

In contrast, work done previously in this lab (1), showed that the P_{eff} of three retinoid analogs, etretinate, acitretin and motretinid, from simple micelles of NaTC (10 mM) was about one-fifth to one-half that from the 10:10 system. Moreover, the more lipophilic analogs, etretinate and acitretin, revealed an ABL rate-limited uptake. Therefore, the lack of effect of different micellar systems on the uptake of phenol could be due to its more hydrophilic nature. It has also been suggested that the retinoids have specific carriers (1) which might influence the intrinsic uptake characteristics from the micellar systems.

In order to better understand the *in situ* results, equilibrium dialysis was done to determine the free fraction in each micellar system in order to correlate it with the *in situ* results. The dialysis membrane chosen had a molecular weight cut-off of 1000. The smallest aggregate possible in each of the micellar systems was a simple micelle of NaTC with an average aggregation number of 4 (10). The molecular weight of NaTC being approximately 520, this would yield an aggregate weight of a little more than 2000. Hence, if the donor solution was a micellar system, the dialysis bag would restrict all micellar aggregates within itself. The receiver chamber contained NaTC at the intermicellar concentration (IMC). The IMC is defined by the monomers of the bile salt which are in a dynamic equilibrium with the micelle and help to maintain its integrity. Each micellar system has a characteristic IMC, which has been previously determined (11,12). Therefore, in the present case, the two equilibrating species were rationalized to be the NaTC monomers and the aqueous phenol. From our equilibrium dialysis studies average free fractions of 0.6 and 0.5 for the 10:2.5 and 10:10 system were obtained. Although the receiver samples showed slight PC leakage, it was always less than 10% of the total PC concentration. This, taken together with the fact that the NaTC concentrations were at the IMC values, was indicative of micellar integrity.

The decrease in free fractions in the micellar systems was expected to be accompanied by a similar decrease in the CL_a.

Table III. Free Fraction, fu, of Phenol in the Two Micellar Systems from Equilibrium Dialysis

Micelle system	10:2.5	10:10
n	4	4
C_{aq} (μg/ml)	3.38 ± 0.07[a]	3.17 ± 0.04
C_{tot} (μg/ml)[b]	5.69 ± 0.51	6.38 ± 0.36
fu	0.60 ± 0.05	0.50 ± 0.03[c]
$[PC]_{rec}$ (mM)[d]	0.17 ± 0.10	0.14 ± 0.06
$[NaTC]_{rec}$ (mM)[e]	8.86 ± 0.53	4.45 ± 0.19

[a] Mean ± SD.
[b] Mean initial donor concentrations were 16 and 17 μg/ml for the 10:2.5 and 10:10 samples, respectively.
[c] One-way ANOVA indicated that free fraction, fu, was significantly different in the two systems, $p < 0.05$.
[d] $[PC]_{rec}$ the concentration of phosphatidylcholine in the receiver chamber.
[e] $[NaTC]_{rec}$ the concentration of taurocholate in the receiver chamber.

However, the CL_a remained unchanged from the non-micellar case. Hence, the micelle-mediated uptake of phenol does not conform to the aqueous transfer model. Moreover, the buffer clearance data seems to predict uptake based on total rather than free concentrations in case of the micellar predictions. Previous work with etretinate, a very lipophilic retinoid, also suggested that the aqueous transfer model could not explain its uptake from a 10:10 system (21).

In conclusion, it was shown that the uptake of phenol remained unchanged in two distinct micellar systems as compared to the non-micellar case. This behavior could not be explained by the aqueous transfer model of micelle-mediated uptake which is basically derived from *in vitro* intestinal models. Earlier work with the more lipophilic retinoids, in a similar experimental set-up, also indicated that micelle-mediated uptake could not be explained by the aqueous transfer model. Hence, further mechanistic studies with *in situ* models are warranted.

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