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Spectral properties and ionization behavior of retinoids, II. Bile salt solutions

Chien-Hsuan Han [1], Cheryl L. Zimmerman, Timothy S. Wiedmann *

Department of Pharmaceutics, College of Pharmacy, University of Minnesota, 308 Harvard St. SE, Minneapolis MN 55455, USA

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Abstract

Purpose: The spectral properties and ionization behavior of retinoic acid (RA) and three structurally related arotinoids, MTTO, TTNPB, and TTNN, have been determined in simple micellar and mixed micellar solutions to provide a better understanding of the intestinal absorption of retinoids. Methods: Spectrophotometric and pH measurements have been made to determine the ionization constant in bile salt solutions. The fluorescent intensity and polarization of TTNN was determined. The extent of solubilization of retinoic acid in 10 mM NaTC/10 mM egg PC solutions was determined as a function of pH. Results: The rank order of wavelengths of maximum absorption, λ_{max}, was as follows: aqueous solution > dihydroxy bile salt > trihydroxy bile salt > ethanol > mixed micelles. The interaction as reflected in the λ_{max} of arotinoids with the bile salts at low pH depended on the mixing order, but this was not the case for retinoic acid. The rank order of the observed negative logarithm of the ionization constants, $pK_{a_{obs}}$, was aqueous solution > mixed micelles > dihydroxy bile salt > trihydroxy bile salt which reflects both the polarity as well as the electrostatic charge of the aggregates. The calculated electrostatic and dielectric effects on the ionization constant were comparable in bile salt micelles suggesting that TTNN is repositioned with a change in the ionic strength and that the size distribution of bile salt simple micelles appears to be perturbed by the presence of TTNN. In addition, the size of the bile salt-TTNN aggregate was independent of ionic strength and the type of bile salt. The solubilization of retinoic acid in bile salt/egg PC mixed micelle does not follow the expected dependence of pH indicating the presence of specific interactions. Conclusions: Retinoic acid and its derivatives exist almost exclusively in aggregated forms with RA forming a distinct type of aggregate in comparison to the arotinoids. The values of the $pK_{a_{obs}}$ of the retinoids ranged from near 5 in simple bile salt aggregates to > 7 in self-associated aggregates and mixed micelles which reflects the sensitivity of the ionization to the environment. This sensitivity has direct implications for the extent solubilized in the intestine and thereby complicates efforts at understanding the mechanism of the oral absorption of retinoids.

Keywords: Retinoids; Ionization; Spectral properties; Aggregation

* Corresponding author. Tel.: +1 612 6245457; fax: +1 612 6242974; e-mail: wiedm001@maroon.tc.umn.edu

[1] Present address: Oread, Palo Alto, 94303 CA.

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1. Introduction

All *trans*-retinoic acid is effective in the treatment of acute promyelocytic leukemia (APL) and other disorders of cellular differentiation (Weigand et al., 1993). Despite its efficacy, significant problems remain regarding its use, particularly because patients quickly become resistant to its effects. The mechanism of resistance appears, at least in part, to have a pharmacokinetic basis, and there is evidence to indicate that there is some change in the absorption of retinoic acid from the intestine upon chronic administration (Weigand et al., 1993). Since retinoic acid is poorly water soluble, it is administered as a suspension in oil. However, intestinal solubilization of retinoic acid by the bile salt micelles is likely to play a role in its absorption, as for retinol (Weigand et al., 1993; Regazzi et al., 1997). Thus, a clear understanding of intestinal luminal events is required in order to determine the mechanism for retinoic acid absorption, and further, changes in that mechanism during chronic administration.

In addition to retinoic acid, the arotinoids (third-generation retinoids), MTTO, TTNPB, and TTNN have also been shown to have promise in cancer chemoprevention and chemotherapy. While structurally related, little information about the absorption of these compounds is available (Pithavala et al., 1995). The ionization and spectral properties of retinoic acid and three arotinoids have been determined in aqueous solution (Han and Wiedmann, 1998), but for oral absorption, the interaction between these compounds and the surface active agents present in the intestine is of greater importance. With solubilization, the total amount of retinoid in solution is increased by the amount associated with the micelle. However, little is known of the dependence of micellar solubilization on the chemical structure of the retinoid (Li et al., 1996a). Moreover, many of the retinoids have a carboxylic acid moiety that may be ionized at the pH of the intestinal lumen. Therefore, the amount in solution will be the sum of the ionized and nonionized retinoid in the aqueous phase plus the sum of the ionized and nonionized retinoid associated with the micelles (Rippie et al., 1964). It becomes evident that the pH of the solution may have a dramatic influence on the amount of retinoid in solution.

The apparent ionization constant of the drug solubilized in the micelle will in general differ from that observed in the aqueous solution (Fernandez and Fromherz, 1977; Drummond et al., 1989). Aside from specific molecular interactions, the main energetic contributions giving rise to the observed pK_a are divided into an electrostatic effect and a nonelectrostatic effect. For the electrostatic effect, Gouy–Chapman theory provides a quantitative relationship between the surface charge density of the micelle and the ratio between the bulk and surface hydrogen ion activity (Fernandez and Fromherz, 1977; Drummond et al., 1989), and it is the hydrogen ion activity that determines the extent of ionization. The nonelectrostatic contribution arises from the environment of the micelle being less polar than that of the bulk aqueous solution. Depending on the location of the solubilizate in the micelle, the effective dielectric constant can range from a high bulk aqueous value near 80 to a value as low as 2 (Fernandez and Fromherz, 1977; Drummond et al., 1989). For the intestinal lipid aggregates, both the electrostatic and nonelectrostatic contributions are expected to be unfavorable for ionization of the carboxylic acid (Small et al., 1984; Cabral et al., 1986; Wiedmann et al., 1997). The magnitude of these contributions has not yet been determined for retinoids.

Evidence has been presented that retinoic acid and some of its derivatives undergo self-association in the aqueous phase (Noy, 1992a,b; Li et al., 1996a; Han and Wiedmann, 1998). In addition, the aggregation was shown to modulate the ionization behavior of the retinoid (Noy, 1992a,b; Han and Wiedmann, 1998). Thus, the ionization and aggregation behavior of retinoids when present in aqueous and micellar solutions will have a direct impact on the amount of retinoid in solution in the intestine. In this study, the spectral properties and ionization of structurally-related retinoids, all *trans*-retinoic acid, MTTO, TTNN, and TTNPB, have been determined as a function of pH in micellar solutions.

2. Experimental

2.1. Materials

The retinoids and structures are provided in the preceeding paper (Han and Wiedmann, 1998). Sodium taurocholate (NaTC) was purchased from Sigma and was recrystallized from ethanol/ethyl acetate (Pope, 1967). Sodium taurodeoxycholate (NaTDC) and sodium taurochenodeoxycholate (NaTCDC) were purchased from Sigma and used as received. Egg phosphatidylcholine (egg PC) was obtained from Avanti Polar Lipids (Alabaster, AL) and was stored at −4°C. The water for preparing solutions was double-distilled and deoxygenated by repeatedly exposing the solutions to reduced vacuum followed by bubbling with argon. All other chemicals were reagent grade or better.

2.2. Methods

2.2.1. UV spectra, stability, and titrations

The UV absorbance spectra were obtained with a Beckman DU series 70 spectrophotometer (Fullerton, CA). Measurement of the stability to UV irradiation and $pK_{a_{obs}}$ follows the method described previously (Han and Wiedmann, 1998). The concentration was 1.83 μM for RA, 1.41 μM for MTTO, 1.55 μM for TTNPB, and 1.04 μM for TTNN. Simple micellar solutions consisted of 10 mM sodium taurocholate (NaTC), sodium taurodeoxycholate (NaTDC), or sodium taurochenodeoxycholate (NaTCDC) in 150 mM NaCl, and the mixed micellar solutions consisted of 10 mM NaTC and 10 mM egg PC also in 150 mM NaCl. The NaTC/egg PC mixture were prepared by first lyophilizing egg PC from an ethanol–cyclohexane mixture (Li et al., 1996a). From the measured dry weight, the appropriate weight of 10 mM NaTC was added to achieve the 10:10 molar ratio of NaTC:egg PC. The mixed lipid solutions were allowed to equilibrate in excess of 24 h at room temperature before use.

2.2.2. Fluorescence

The fluorescence method has been described (Han and Wiedmann, 1998). The concentration of TTNN was 1.045 μM and contained either 5 or 150 mM NaCl and the concentration of bile salt in solution was 10 mM. A standard solution was prepared by diluting a 95% ethanol stock solution of TTNN into 229 mM SDS in water to yield a final concentration of 1.045 μM. The temperature of the sample chamber was maintained at 20.0 ± 0.5°C.

The fluorescence polarization values were obtained as previously described (Canter and Schimmel, 1980; Lakowicz, 1986; Han and Wiedmann, 1998). The molar volume of the aggregate, V, was estimated from the polarization as follows: the Perrin equation is defined as (Canter and Schimmel, 1980; Lakowicz, 1986):

$$(1/P - 1/3) = (1/Po - 1/3)(1 + RT)\tau/V\eta \quad (1)$$

where Po is the intrinsic polarization which was taken to be equal to 0.367 (Schachter and Shinizky, 1977), R is the gas law constant, T is the absolute temperature, τ is the fluorescent lifetime, and η is the viscosity. By applying this equation each to the standard SDS and the unknown bile salt aggregate and then taking the ratio, the following expression is obtained:

$$(1/P_{BS} - Po)/(1/P_{SDS} - Po) = (\tau_{BS}/V_{BS})/(\tau_{SDS}/V_{SDS}) \quad (2)$$

The ratio of the lifetimes was estimated from the fluorescent intensity, F, and absorptivity, ε_{max}, as follows:

$$(\tau_{BS}/\tau_{SDS}) = (F/\varepsilon_{max}C)_{BS}/(F/\varepsilon_{max}C)_{SDS} \quad (3)$$

where C is the molar concentration. The aggregation number was estimated from the molar volume by assuming a spherical shape for the aggregate and a hydration number of 20 for the bile salt.

2.3. Solubilization

In determining the solubilization in bile salt and bile salt/egg PC solutions, excess solid all *trans*-retinoic acid was added directly to the pyrex test tubes along with several milliliters of the micellar solution. Two buffers, 10 mM citric acid and 10 mM HEPES, were used in combination to control the pH which ranged from 3 to 8. The dispersions

Table 1
Wavelengths of maximum absorbances in nm

	Nonionized				Ionized			
Solvent (mM)	RA	MTTO	TTNPB	TTNN	RA	MTTO	TTNPB	TTNN
Saline (150)	380.0	370.0	309.0	261.2, 321	340.0	325.0	285.2	258.3, 295
Ethanol	355	342	301	261, 308	—	—	—	—
NaTC (10)	354.3	347.0	308.0	261.3, 309	337.8	330.1	296.1	259.5, 295
NaTDC (10)	359.0	351.5	308.5	263.5, 313	338.8	330.0	300.1	259.5, 309
NaTCDC (10)	359.0	351.2	307.5	263.5, 314	340.0	332.5	298.5	259.5, 312
NaTC (10)/egg PC (10)	350.2	331.0	304.0	ND	338.5	325.3	268.0	ND

ND, not determined.

were equilibrated for 48 h at 37 ± 0.5°C. The solid was removed by centrifugation for 5 min in a table top centrifuge, and the retinoic acid was quantified by HPLC as previously described (Pithavala et al., 1995; Han and Wiedmann, 1998).

3. Results

Table 1 contains the wavelengths of maximum absorbance of the retinoids in aqueous and bile salt solutions at low (3.03 ± 0.02) and high pH (10.73 ± 0.03). In both solutions, an increase in pH resulted in a reduction in the wavelength of maximum absorbance. The magnitude of this hypsochromic shift was smaller as the number of benzene rings in the structure was increased, where a > 15 nm shift was observed for retinoic acid in NaTC while there was almost no difference in λ_{max} for TTNN.

The values of the λ_{max} for retinoids in 10 mM sodium taurodeoxycholate and 10 mM sodium taurochenodeoxycholate occurred at longer wavelengths in comparison to that obtained in 10 mM NaTC solution, and the difference at low pH was larger than that observed at high pH. There was little or no difference in the λ_{max} obtained with the two different dihydroxy bile salts, NaTDC and NaTCDC. The rank order of λ_{max} of retinoids in different environments was as follows: bile salt/egg PC mixed micelles < trihydroxy bile salt simple micelles < dihydroxy bile salt simple micelles < water. The rank order of the magnitude of the absorptivities was reversed in comparison to the rank order of the λ_{max} (data not shown).

The absorption spectra of RA and MTTO are shown in Fig. 1A and B. In Fig. 1A, the spectra of RA in water at low pH is given as well as the spectra of RA in a low pH solution containing sodium taurodeoxycholate. The order of the addition of bile salt and RA resulted in the same hypsochromic shift relative to the aqueous solution. The high pH absorption spectrum obtained in the presence of NaTC is also provided.

For MTTO, a hypsochromic shift, relative to the spectrum obtained in an aqueous solution, was observed when MTTO was added to NaTC at low pH (Fig. 1B). As above, the spectrum was reversibly altered by increasing the pH. However, when sodium taurocholate was added to an aqueous solution of MTTO at low pH, little or no change was observed in the spectrum. When the solution was titrated to high pH, the spectrum underwent an irreversible change to the high pH spectrum. With titration to low pH, the spectrum was superimposable to that obtained by the addition of MTTO to NaTC. Thus, reversible changes with pH could be obtained if MTTO was added to a NaTC solution at either low or high pH or if NaTC was added to MTTO solution at high pH. However, if NaTC was added to MTTO at low pH, the spectrum could not be recovered after the solution was titrated to high pH. Moreover, no change in the absorption spectrum was observed if the solution was equilibrated at low pH for 3 h. Analogous observations to MTTO were found for TTNPB and TTNN. These results were also cor-

roborated for TTNN by measurement of the change in fluorescent intensity with pH. Thus, at low pH, the spectra of the arotinoids, MTTO, TTNPB, and TTNN, were sensitive to the mixing order.

In determining the apparent $pK_{a_{obs}}$ in the presence of bile salts, the retinoids were added to the bile salt solutions, since reversible spectral shifts with titration were obtained. The titration curves of the RA and MTTO in 10 mM NaTC, NaTDC, and NaTCDC are given in Fig. 2A and B. The solid lines in the figures represent the titration curves obtained from nonlinear regression of the pooled data from individual titrations. The data

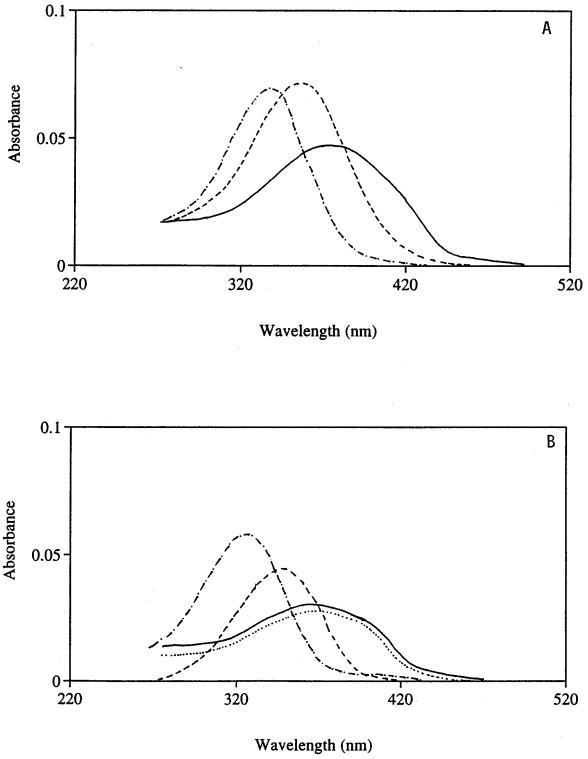

Fig. 1. Absorbance as a function of wavelength for (A) retinoic acid in aqueous solution at low pH (–), at high pH in presence of 10 mM sodium taurocholate (– · – · – ·), and low pH in the presence of 10 mM sodium taurocholate (– – – –); and (B) MTTO in aqueous solution at low pH (–), at low pH when MTTO was added to 10 mM sodium taurocholate (– – – –), at low pH when sodium taurocholate was added to MTTO (· · · · · · ·), and at high pH in the presence of NaTC (– · – · – ·).

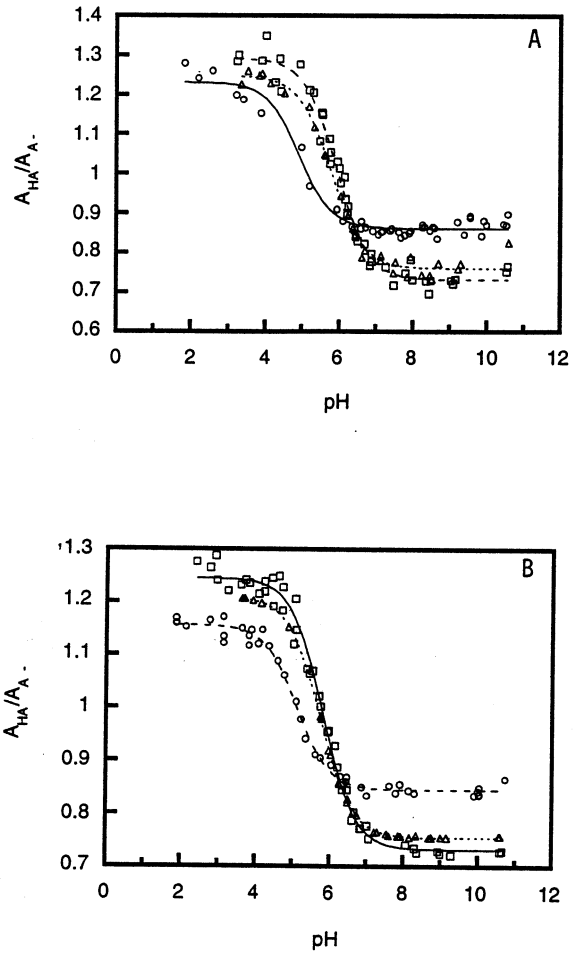

Fig. 2. Absorbance ratio plotted as a function of pH for (A) retinoic acid, (B) MTTO, in (○) 10 mM sodium taurocholate, (□) 10 mM sodium taurodeoxycholate, and (△) 10 mM sodium taurochenodeoxycholate in 0.15 M NaCl. The data were derived from three individual determinations and the solid line represents the best fit of the pooled data to Eq. (1).

were well fit by the Henderson–Hasselbalch relationship (Han and Wiedmann, 1998). The difference between the maximum and minimum absorbance ratio was smaller in the trihydroxy bile salt solution than that observed in dihydroxy bile salts, which is in agreement with the measured absorptivities.

The results of the $pK_{a_{obs}}$ of the retinoids plotted as a function of sodium taurocholate concentration are shown in Fig. 3. For retinoic acid, no significant changes were seen in the value of the

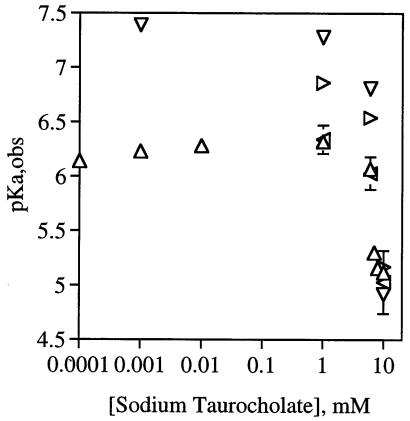

Fig. 3. Observed pK_a given as a function sodium taurocholate concentration for (◁) retinoic acid, (◁) MTTO, (▽) TTNBP, and (▷) TTNN.

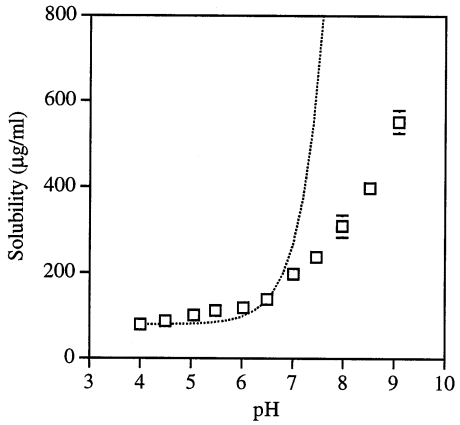

Fig. 4. Solubilization of RA in 10 mM sodium taurodeoxycholate/10 mM egg phosphatidylcholine as a function of pH. Dotted line represents theoretical prediction.

$pK_{a_{obs}}$, over the concentration range of 0.1 μM to 1 mM. At sodium taurocholate concentrations >1 mM, the pK_a values decreased with further increases in sodium taurocholate concentration. Between 4 and 6 mM NaTC, the pK_a values were dramatically decreased. At 10 mM sodium taurocholate, the pK_a was 5.12. Analogous observations were seen for the arotinoids.

The $pK_{a_{obs}}$ (Table 2) for all the retinoids in 10 mM NaTDC and NaTCDC solutions were higher by about 0.5 units than those obtained in NaTC solutions. However, the difference between the two dihydroxy bile salt solutions was relatively small. For the retinoids in the mixed micellar solution of 10 mM NaTC/10 mM egg PC, the values of the $pK_{a_{obs}}$ were relatively large.

The solubilization of retinoic acid by 10 mM NaTC/10 mM egg phosphatidylcholine dispersion as a function of pH is shown in Fig. 4. The total amount of retinoic acid in solution was 79 ± 5.9 μM at pH 4.00, which was higher than that obtained in aqueous solution by about a factor of 250 (Han and Wiedmann, 1998). With an increase in pH, the amount of retinoic acid in solution increased. The amount in solution at pH 9.09 was 552 ± 27 μg/ml, which was about a factor of seven higher than that obtained at pH 4.00 (unpublished data).

For the fluorescent measurements, the wavelength of maximum intensity of TTNN in each bile salt solutions was about 420 nm. The intensity and polarization of TTNN in bile salt solutions with NaCl concentrations of 5 and 150 mM

Table 2
Values of the pK_a (mean $\pm$ S.D., $n=3$) in 0.15 M NaCl, simple micelles and mixed micelles

Solvent (mM)	RA	MTTO	TTNPB	TTNN
Saline	6.05 ± 0.06	7.18 ± 0.07	6.49 ± 0.08	6.54 ± 0.18
NaTC (1)	6.32 ± 0.03	7.28 ± 0.02	6.86 ± 0.04	6.34 ± 0.13
NaTC (10)	5.12 ± 0.04	4.92 ± 0.07	5.17 ± 0.8	5.03 ± 0.29
NaTDC (10)	5.82 ± 0.03	5.94 ± 0.03	5.64 ± 0.08	5.29 ± 0.18
NaTCDC (10)	5.74 ± 0.01	5.79 ± 0.04	5.84 ± 0.14	5.34 ± 0.17
Egg PC/NaTC	6.63 ± 0.07	7.01 ± 0.09	6.38 ± 0.29	ND
Model compounds	4.76[a]	4.76[a]	4.19[b]	4.17[c]

Model compounds, [a] acetic acid; [b] benzoic acid; [c] naphthoic acid.

Table 3
Fluorescence intensity and polarization values of TTNN

	Fluorescence intensity ($\times 10^6$)[a]		Polarization		Hydrated molar volume (m^3/mol)	
	5 mM NaCl	150 mM NaCl	5 mM NaCl	150 mM NaCl	5 mM NaCl	150 mM NaCl
NaTC	2.90 ± 0.01	2.84 ± 0.02	0.021 ± 0.005	0.027 ± 0.002	0.009 ± 0.002	0.011 ± 0.001
NaTDC	2.58 ± 0.03	2.43 ± 0.01	0.028 ± 0.009	0.036 ± 0.005	0.012 ± 0.004	0.015 ± 0.002
NaTCDC	2.88 ± 0.02	2.58 ± 0.02	0.023 ± 0.005	0.030 ± 0.008	0.010 ± 0.002	0.013 ± 0.003
SDS[b]	3.78 ± 0.02		0.071 ± 0.005			

[a] TTNN at emission wavelength of 420 nm in 10 mM micellar solutions at basic pH. Values represent the mean $\pm$ S.D. with $n = 3$.
[b] A concentration of 229 mM SDS in water.

NaCl are given in Table 3. The polarization values were always higher in the 150 mM NaCl than that obtained at 5 mM NaCl, although the differences were not statistically significant ($p = 0.05$). At both low and high ionic strengths, the polarization values of TTNN in dihydroxy bile salt micellar solutions were also higher than those determined in trihydroxy bile salt micellar solutions, but again the differences were not statistically significant. The polarization value of TTNN in 229 mM SDS micellar solution was 0.071 ± 0.005 which is higher than those obtained in the bile salt micellar solutions.

The apparent molar volume of the TTNN aggregate was estimated relative to the standard SDS micelles (Li et al., 1996b). The results are summarized in Table 3. There was no significant difference between the apparent molecular volumes of bile salt–TTNN aggregate in 150 and 5 mM NaCl ($p \leq 0.05$). In addition, there were no significant differences ($p \leq 0.05$) between the apparent molecular volumes of dihydroxy bile salt–TTNN aggregates and trihydroxy bile salt–TTNN aggregates.

4. Discussion

Retinoic acid and its derivatives have potential as chemopreventive agents (Weigand et al., 1993). For this purpose, however, an oral dosage form would be preferred. The absorption process of retinoic acid and its derivatives is complicated because of their inherently low water solubility and strong tendency to self-associate (Pithavala et al., 1995; Regazzi et al., 1997). Pharmacokinetic studies have revealed that the extent of absorption is variable and may depend on an active process and/or is limited in absorption due to its poor solubility (Regazzi et al., 1997). In this study, the spectral properties and ionization behavior of the retinoids in the presence of bile salt micelles were examined for the purpose of laying the foundation for investigating the mechanism of their intestinal absorption.

4.1. Spectral properties

Dilution of the retinoids into a bile salt solution resulted in a lower λ_{max} in comparison to that obtained in an aqueous solution, especially at low pH. This indicates that the retinoids associate with the bile salts in solution. Since the retinoid molecule is largely hydrophobic, it presumably forms mixed aggregates with the bile salt micelle. The polar carboxyl group of the retinoid is expected to be preferentially located at the micelle–water interface.

In comparing the λ_{max} in the presence and absence of bile salt micelles, the difference in wavelength is comparable to that observed between aqueous to ethanol solutions (Han and Wiedmann, 1998). This indicates that the change in the λ_{max} is dominated by the disruption of the hydrogen bonds between carboxyl groups of the self-aggregated retinoids as solubilization in bile salt micelles occurs. At high pH, the difference between the λ_{max} of the retinoids obtained in aqueous and bile salt micellar solutions was relatively small. Since the retinoids are ionized, there

would be no intermolecular hydrogen bonding when self-associated which is consistent with the lack of a major change in the wavelength.

Since intermolecular hydrogen bonding among the retinoids does not occur within the bile salt micelle as suggested above, the observed λ_{max} should be largely a result of solvent–solute dipole interactions. As such, a hypsochromic shift reflects an increase in the polarity. Thus, the relative values of the λ_{max} indicate that the dihydroxy bile salt micelles provide a less polar environment for retinoids than the trihydroxy bile salt micelles. This is consistent with the differences in the expected hydrophilic–hydrophobic balance of the two bile salts (Armstrong and Carey, 1982).

The lowest λ_{max} was observed for the retinoids in the bile salt/egg PC solution. This may indicate that the mixed micelle provides the most polar environment. This would also be consistent with previous estimates of the polarity of NaTC simple micelles and the NaTC/egg PC mixed micelles (Matsuzaki et al., 1989). Overall, the rank order of the observed λ_{max} at low pH suggests that the rank order of polarity is as follows: bile salt/egg PC mixed micelle > trihydroxy bile salt simple micelle > dihydroxy bile salt simple micelle.

The absorption spectra of retinoic acid in sodium taurocholate solutions were independent of mixing order. In contrast, the absorption spectra and presumably the nature of the interaction between arotinoids and sodium taurocholate at low pH were sensitive to mixing order. These observations indicate that the nature of the retinoic acid aggregate in aqueous solution is distinct from the arotinoid aggregates. Moreover, even with 3 h of equilibration after the bile salt was added to the solution of arotinoid at low pH, there was no significant change in the observed absorption spectra. Thus, it also appears that at low pH the bile salts do not readily disrupt the self-associated arotinoids even though the bile salt molecules are in a micellar phase. It is plausible that the rigid structure of the arotinoids results in an aggregate that is dynamically distinct from aggregates that arise from amphiphiles with flexible alkyl chains.

The effect of mixing order of TTNN at low pH was also assessed by measurement of the fluorescence polarization. The fluorescent intensity was increased with the addition of bile salts which indicates a certain degree of interaction between TTNN and sodium taurocholate. However, there was no significant change in the polarization. One reasonable possibility is that the bile salt monomers may adsorb onto the surface of the TTNN aggregates. This would provide a less polar environment for TTNN, consistent with the increase in the fluorescent intensity that was observed, yet not appreciably changing the polarization.

4.2. Analysis of the acid dissociation constants

Before considering the ionization constants, several factors are noteworthy. Decomposition can be considered negligible in the analysis of the ionization behavior. Second, the retinoids are incorporated into the bile salt aggregate as was evident by the measurement of the $pK_{a_{obs}}$ as a function of bile salt concentration. Finally, the dramatic drop in the $pK_{a_{obs}}$ in the concentration range corresponding to the CMC of NaTC is a consequence of the disruption of the retinoic acid aggregate and the formation of a mixed micelle with NaTC.

The observed values of the pK_a in micellar solution were all higher than that of the model compounds (Table 2). These model compounds were selected such that the pK_a value would be equal to the estimated value of the retinoids in an infinitely dilute aqueous solution as described previously (Han and Wiedmann, 1998). The change in the pK_a is a consequence of the change in the environment of the carboxylic acid moiety of retinoids. The higher values of the $pK_{a_{obs}}$ indicate that the bile salt micelles provide not only a less polar environment but also an unfavorable surface charge. If the chromophoric portion of the retinoid and carboxylic acid were in the same environment, the wavelength shifts may be used to estimate the polarity. However, this does not seem to be the case. Therefore, the approach that was followed was to estimate the size of the aggregate from fluorescent measurements of TTNN from which the surface charge was calculated. From the surface charge, the shift in the

$pK_{a_{obs}}$ due to the electrostatic charge was determined, and then the remaining difference was assigned to the polarity.

For a comparison of the micelle size with that reported in the literature (Kratohvil and Dellicolli, 1968; Small, 1968; Mazer et al., 1979; Armstrong and Carey, 1982; Barnes, 1984; Small, 1985; Matsuzaki et al., 1989), the fluorescence results must be converted to aggregation numbers. The apparent molar volume of bile salt–TTNN aggregate was corrected by the hydration of 20 water molecules per cholate ion (Barnes, 1984), and the densities were assumed to be equal to the nonconjugated bile salts as follows, where sodium cholate has a value of 1.33, sodium deoxycholate has a value of 1.31, and sodium chenodeoxycholate has a value of 1.28 g/cm^3 (Small, 1985). Previous investigations (Mazer et al., 1979; Zana and Guveli, 1985; Li and McGown, 1994) indicated that NaTC micelles at pH 8–9 at 20°C have an average aggregation number of 3.6 at a NaCl concentration of 10 mM. When the ionic strength was increased to 150 mM, the aggregation number only increased to 4.6. On the other hand, the ionic strength had a profound effect on the size of the dihydroxy bile salt micelles (Kratohvil and Dellicolli, 1968; Small, 1968; Mazer et al., 1979). As the NaCl concentration was increased from 5 to 150 mM, the aggregation number of NaTDC and NaTCDC increased from 6.2 to 21.8 and 9.5 to 19.5, respectively. This was explained by the dehydration of the polar groups and/or the salting out effect on the hydrocarbon portion.

The apparent aggregation number of NaTDC–TTNN aggregates at low ionic strength was larger than the literature value by a factor of two, while at high ionic strength the value was slightly smaller than that found in the literature. Although the apparent aggregation number of NaTCDC/TTNN aggregates at low ionic strength was comparable to the literature values, at high ionic strength, the experimental value was slightly smaller than the literature value.

There are a number of possibilities that may account for these discrepancies in addition to the fact that this approach only provides information about the micelles containing the TTNN molecule. First, the reliability of the technique depends on the lifetime which has only been estimated. Second, the likely presence of polydispersity of the TTNN/bile salt aggregates also presents a complication. Two other factors that have only a minor effect on the calculations are that the aggregates may not be spherical as assumed and the value used for intrinsic polarization was taken to be 0.367. The latter is the value of retinol in propylene glycol at -50°C (Schachter and Shinizky, 1977).

In addition to the error inherent in the fluorescent technique, an error may result from the estimation of hydration number of bile salt micelles. The number used was based on the aggregate of sodium cholate (Small, 1985). Since the charge distribution of the conjugated and unconjugated bile salts may differ, the hydration number may not be valid. In spite of the assumptions, the apparent aggregation number of NaTC micelles was 12.6 ± 1.1 in 150 mM NaCl, which is reasonable given the literature value of the aggregation number of 10 (Mazer et al., 1979).

The other interpretation for the discrepancy of the effect of ionic strength on the aggregation number between experimental results and literature is that the size of bile salt aggregates were altered by TTNN. There are two possibilities: firstly, that bile salt aggregates are polydispersed and TTNN is preferentially solubilized in the larger aggregates as observed with planar fluorescent probes (Zana and Guveli, 1985; Li and McGown, 1994), and secondly, TTNN alters the size distribution of bile salt aggregates. Since the observed aggregation number was higher than the literature value for NaTC, but with dihydroxy bile salt micelles, the aggregation number was smaller than the literature values, the second alternative may be more reasonable. That is, TTNN alters the size distribution of the bile salt micelles.

The suggestion of a perturbation of the aggregation number agrees well with the findings obtained by the technique of excimer formation (Matsuzaki et al., 1989). The aggregation number of NaDC was reported to be 12 ± 2, which was independent of bile salt concentration and NaCl concentration. Furthermore, the aggregation number for NaTCDC was 13 ± 2, which was not much different from that of NaDC.

Table 4
Dielectric and electrostatic effects on the $pK_{a_{obs}}$ of TTNN

	10 mM NaTC		10 mM NaTDC		10 mM NaTCDC	
	5 mM NaCl	150 mM NaCl	5 mM NaCl	150 mM NaCl	5 mMNaCl	150 mM NaCl
Aggregation number	9.7 ± 2.5	12.6 ± 1.1	13.3 ± 4.3	17.5 ± 2.2	11.0 ± 2.2	14.1 ± 3.5
$F\Phi/2.303\ RT$[a]	1.45 ± 0.92	0.39 ± 0.17	1.41 ± 0.96	0.42 ± 0.21	1.42 ± 0.83	0.40 ± 0.25
$F\ (\varepsilon)$[b]	1.23	0.47	1.39	0.70	1.44	0.77
Surface potential (mV)	86 ± 54	23 ± 10	83 ± 57	25 ± 12	84 ± 49	24 ± 15

[a] Change in the pK_a due to the surface potential (electrostatic effect).
[b] Change in the pK_a due to the polarity contribution.

With the size estimate of the micelles containing TTNN, the $pK_{a_{obs}}$ may be analyzed. The apparent surface charge density, σ, was calculated as follows:

$$\sigma = A_g p\ e/4\pi r^2$$

where A_g is the apparent aggregation number of the bile salt–TTNN aggregate, p is the fraction of charged groups that are ionized which for NaTDC micelle was 0.29 in 150 mM NaCl and 0.77 in water (Kratohvil and Dellicolli, 1968; Small, 1985), e is the unit charge, and r is the radius of the aggregate. With the surface charge, linearized Gouy–Chapman theory was used to calculate the associated Debye screening length, and the values of apparent surface potential of bile salt micelles in 150 and 5 mM NaCl were 23 and 86 mV, respectively. The values are in reasonable agreement with the literature where the surface potentials of the NaTDC micelle in 150 mM NaCl and water were reported to be 39 or 89 mV, respectively (Kratohvil and Dellicolli, 1968; Small, 1985).

With the estimated surface potential, the electrostatic effect on the ionization constant was calculated and is given in Table 4. In 150 mM NaCl, an increase of about 0.40 in the pK_a (or equivalently, $F\Phi/2.3\ RT$) for both dihydroxy and trihydroxy bile salt micelles was obtained. After correcting for the electrostatic effect, the shift in the pK_a from the dielectric effect was estimated to be between 0.47 and 0.77. The magnitude of the electrostatic effect was about 1.4 in 5 mM sodium chloride. The dielectric effect also induced a lower shift at the low salt concentration being between 1.2 and 1.5. Thus, the electrostatic and dielectric effects were both higher in 5 mM NaCl. Moreover, the electrostatic effect was comparable to the dielectric effect at both low and high ionic strengths.

The magnitude of the electrostatic effect is expected to be lower at the higher ionic strength because the high counterion concentration would swamp out the partial charges on the surface of the micelle. Since the size of the bile salt micelles was largely independent of ionic strength, the surface charge density is only sensitive to the counterion bonding. Thus, a relatively large change was found. In contrast, the magnitude of the dielectric effect is expected to be similar at both low and high ionic strengths. However, this was not the case which may suggest that the retinoid is repositioned with a change in the ionic strength. This is also consistent with the results obtained with 4-alkyl benzoic acid derivatives (Wiedmann et al., 1997).

4.3. Mixed micelles

The values of the $pK_{a_{obs}}$ obtained in the mixed micelles were higher than those observed in bile salt simple micelles but comparable to those obtained in aqueous solution. The rank order of the pK_a of the retinoids was: aqueous solution and mixed micelles > dihydroxy bile salt > trihydroxy bile salt. This suggests a different polarity than indicated by consideration of the λ_{max} and the study using pyrene. One explanation of this discrepancy is that the carboxylic group in the bile salt/egg PC mixed micelle experiences a less polar

environment and the chromophore of retinoid experiences a more polar environment than in bile salt simple micelle. From the structural point of view, the curvature of the mixed micelle from the rod-shape model may provide more space for water molecules.

Noy, (1992b) has examined the ionization behavior of retinoic acid in aqueous dispersions composed of neutral and charged phospholipids. For five different neutral phospholipid dispersions, the observed ionization constants were between 6.8 and 7.1. With the use of charged phospholipids, the observed ionization constants were >8. The calculated surface charge of the mixed micelles would lie between the surface charge of the neutral and charged phospholipid dispersions, yet the ionization constants fell between 6.5 and 7.0. This suggests that the mixed micelle provides a more polar environment for the carboxylic group in comparison to the phospholipid dispersions. This higher polarity may arise from a perpendicular orientation of the retinoid to the surface of the rod. The carboxylic acid moiety at the curved surface would thereby be expected to be have a more polar environment relative to that provided by the relatively flat phospholipid surface.

4.4. Solubilization

Finally, the solubilization results of RA in mixed micelles is considered. Rippie et al. (1964) were the first to investigate quantitatively the equilibrium of an ionizable solute in a micellar solution. The total amount of a weak acid in solution is dependent on the concentration of nonionized and ionized forms in the aqueous phase and the concentration of nonionized and ionized forms in micellar phase. As such, the amount of retinoic acid in solution is affected by the extent of ionization of retinoic acid in solution and the other is the solubilization of retinoic acid by bile salt micelles. At low pH, the experimental data were reasonably well fit by the calculated values which involves assuming a simple equilibrium between the ionized and nonionized form, infinite solubility of the ionized form, and a value of the pK_a of 6.63 (Han and Wiedmann, 1998). Above pH 6.5, the amount of retinoic acid in solution deviated from the calculated curve in that smaller values were obtained.

One possible interpretation for the deviation is that a solubilization limit was reached in the mixed micelles. Thus, the data obtained experimentally are lower than expected. At pH values of 8.5 and 9, the molar percentages were 0.1 and 0.2, respectively. Thus, at low pH, the amount of retinoic acid in mixed micelles may not be sufficiently high to perturb the structure of the micelle, but as the pH was increased, the amount of retinoic acid in the mixed micelles is significantly increased. In this case, perturbation in the electrostatic and structural interactions are more likely.

4.5. Implications for bioavailability

In considering the intestinal absorption, it is important to recognize the rather complex interactions of retinoids with bile salt micelles. A recent summary of the appropriate pharmacokinetic studies has led to the suggestion that active transport is involved with the absorption of retinoic acid and the limit in solubility is less critical (Regazzi et al., 1997). In these studies, the doses ranged from 17000 to 200000 $\mu g/m^2$. The amount of retinoic acid that can be in aqueous solution at pH 6.5 is 0.56 $\mu g/ml$ which would mean that the intestinal volume would need to be on the order of 70 l for complete dissolution of retinoic acid. The solubility of retinoic acid in mixed micelles was 100 $\mu g/ml$ which is significantly higher than that observed in aqueous solution. Moreover, if a comparable micelle composition was present in the intestine, only 2 l would be required for complete dissolution. Therefore, minimizing the importance of solubilization in the mechanism of absorption of retinoic acid is unwarranted. In addition, since the absolute bioavailability can only be estimated due to the difficulty in obtaining intravenous results, the alternative approach of this study provides a means to examine the effect of food and active transport on the extent of absorption of retinoids. Finally, the results also provide direction for the development of a suitable oral formulation of the retinoids which depends on the inherent bile salt

solubilization capacity of the intestine so that greater and possibly more reproducible intestinal absorption may be obtained.

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