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The ionization behavior of retinoic acid in aqueous environments and bound to serum albumin

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The ionization behavior of retinoic acid (RA) in an aqueous phase and when bound to bovine serum albumin was studied. Titrations of RA in the various phases were followed by monitoring the red shift in the absorption maximum of RA that occurred upon deprotonation. The apparent pK of RA was dependent on the concentration of this compound. At the concentration range 6–20 μM, the pK of RA in water had a value of approximately 8.0. As the concentration was decreased in the range 1–6 μM, the value of the pK decreased continuously. The lowest pK observed was approximately 6.0. It was concluded that RA in an aqueous phase at concentrations in the μM range, forms micelles, and that the values of the pK of RA monomers and micelles in water are < 6.0 and 8.0, respectively. The presence of 0.15 M NaCl caused a decrease in the pK of RA micelles and lowered the value of the CMC. Titration of RA in the presence of bovine serum albumin revealed the presence of a heterogeneous population comprised of three distinct microenvironments for RA associated with this protein. Two populations of RA were found to undergo complete titration in the pH range 4–8. A third population became apparent at pH > 9.5.

Introduction

Excessive vitamin A intake produces toxic manifestations, the basis of which has not been clarified as yet [1]. It has been repeatedly suggested that at least some of the toxic effects of this vitamin are due to its surface-active, 'membranolytic' properties. These suggestions are based on studies that have shown that the presence of retinoids affect various aspects of structure and function of membranes [2–10], and on the chemical structure of retinoids which are hydrophobic compounds with one polar end group, so that they have 'detergent like' characteristics. Of the three most prevalent retinoids: retinol, retinal, and retinoic acid, the last compound is the only one that may carry an actual net charge at its end group. Thus, two important questions regarding the effects of retinoic acid (RA) on membranes, and possible differences between the effects of RA vs. other retinoids [11,12] should be considered. (1) Does RA self-associate to form micelles in an aqueous environment? (2) Is the carboxyl group of retinoic acid protonated or is it negatively charged at physiological pH?

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All-*trans*-RA is present in plasma at a concentration of about 12 nM [13]. Unlike the quantitatively predominant vitamin A derivative, all-*trans*-retinol, which circulates, at a concentration of about 2 μM, complexed with a specific serum retinol-binding protein [14], RA in blood is bound to serum albumin [15]. The grounds for the apparent differentiation in the mode of transport of the two retinoids in the circulation have not been clarified as yet. This question is particularly intriguing since it was shown that at least in vitro, serum albumin binds retinol [16] and retinol-binding protein binds RA (e.g. Ref. 17). Regarding the mode of binding of RA to albumin, it has been suggested [18] that this ligand associates with the high-affinity sites that albumin is known to have for long-chain fatty acids [19]. Long-chain fatty acids associate with serum albumin in heterogeneous binding sites that can be grouped into several classes based on their relative affinities [19–21]. Up to a fatty acid/albumin mole ratio of 3:1, three distinct high affinity binding sites exist and the distribution of fatty acids between them is dependent on the fatty acid/albumin mole ratio [22,23]. Fatty acids bind at these sites in the ionized form and can not be titrated in the pH range 3–8 [23,24]. Additional, weaker sites for long-chain fatty acids on albumin become apparent at high fatty acid/albumin mole ratios. Fatty acids associated with one of the weaker

sites can be titrated and has been shown to display an apparent pK similar to that of fatty acids monomers in an aqueous phase [24].

In the present study, the ionization behavior of RA in an aqueous environment and RA bound to serum albumin was investigated.

Materials and Methods

All-*trans*-retinoic acid was from Kodak. Bovine serum albumin (fatty-acid free) was from Calbiochem. All other chemicals were from Sigma Chemical Co.

Titration of retinoic acid. RA was added to assay mixtures from a concentrated solution in ethanol to a final concentration of 1–20 μM. The final concentration of ethanol in assay mixtures never exceeded 1% (v/v). Titration was carried out by addition of dilute solutions of either HCl or NaOH, and the pH was measured by a Sigma Tris electrode using a Radiometer Research Grade pH meter. The absorption of the mixture at various pH values was measured using a computer driven Cary-14 spectrophotometer (On-Line Instruments). To ensure that equilibrium was reached between all phases of the system (RA monomers, micelles, and RA precipitates), the following procedure was used. A 100 ml solution of the appropriate mixture was prepared and adjusted to designated pH values by the addition of either HCl or NaOH. As each pH point was achieved, a 1 ml aliquot was removed to a separate test tube. The samples were then incubated at room temperature in the dark, and pH values and the absorption at 340 nm and at 380 nm were measured at 15-min intervals until constant values were observed. For the titration of RA bound to serum albumin, albumin was dissolved in 0.15 M NaCl and RA was added from a concentrated solution in ethanol. Titrations were carried out as outlined above. Buffers and assay mixtures were purged with argon before use to minimize oxidation of RA, and mixtures were kept in the dark whenever possible. Titrations and absorption measurements were carried out in dim light.

Results

Effect of pH on the absorption spectrum of RA

The absorption spectra of 3 μM RA in water at pH 4 and pH 10.5 are shown in Fig. 1. The absorption maxima were at 340 nm and at 390 nm at pH 10 and at pH 4, respectively. The shift in λ_{max} was completely reversible when the pH of the solution was adjusted from 10.5 to 4 and back. In addition to the shift in the absorption maximum, the spectra in Fig. 1 show an apparent decrease in the absorption of RA at λ_{max} upon lowering the pH of the solution. This may be partially due to the lower solubility of protonated vs. anionic RA which resulted in formation of RA precipitates and an apparent decrease in absorption caused by scattering. Precipitation of RA was, in fact, observed at low pH when solutions containing RA at a concentration of 3 μM or higher were used. To examine whether the shift in λ_{max} resulted from precipitation of RA rather than from the protonation/deprotonation process, the absorption spectra of RA were examined at various RA concentrations in the range 1–20 μM. These measurements were used to study the origin of the shift in λ_{max} because it can be expected that a larger fraction of RA will precipitate, and larger aggregates will be present in solutions comprised of higher concentrations of RA [25]. To ensure that all phases in the system were at equilibrium, the pH values of the samples were adjusted to each designated value, pH values and the absorptions at 340 nm and at 380 nm were measured at intervals until constant values were observed (see Methods). It was found that incubation for 30 min at room temperature was sufficient to equilibrate samples containing 1–5 μM RA. A 2-h incubation period was required to achieve equilibrium at higher concentrations of RA. All samples were subsequently incubated for 2 h at room temperature before final measurements were taken. The data in Fig. 2 show that the absorption ratio $A_{340\ nm}/A_{380\ nm}$ was constant both at pH 10.5 and pH 4 throughout the concentration range studied. It was thus concluded that the shift in λ_{max} was not caused by the precipitation but by deprotonation of RA that occurred upon decrease in pH.

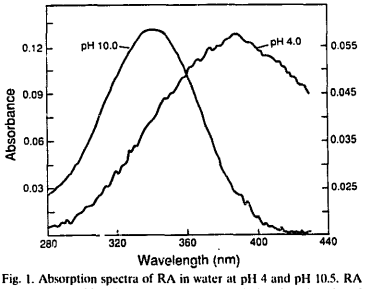

Fig. 1. Absorption spectra of RA in water at pH 4 and pH 10.5. RA (3 μM) was added from a concentrated solution in ethanol and incubated for 30 min The pH of the mixture was adjusted by the addition of either NaOH or HCl.

The ionization behavior of RA in an aqueous environment as a function of concentration

The progression of the shift in λ_{max} as a function of pH was followed by monitoring the absorption ratio

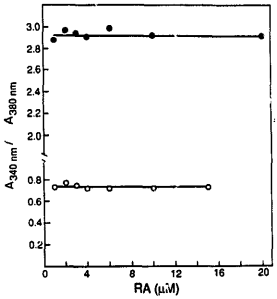

Fig. 2. Absorption ratio $A_{340\ nm}/A_{380\ nm}$ as a function of RA concentration at pH 4 (○) and at pH 10 (●). Absorptions were measured following a 2-h incubation, as described in Methods. Each point represents the mean of two measurements.

$A_{340\ nm}/A_{380\ nm}$. This ratio as a function of pH was a sigmoidal function that reached constant values at both low and high pH values (Fig. 3). At these extremes of pH, it was assumed that RA existed as the fully protonated form or as the anion, respectively. An apparent pK for RA could thus be obtained at half the maximal change. Shown in Fig. 3 are representative titration curves obtained with 1 μM and with 10 μM RA in water.

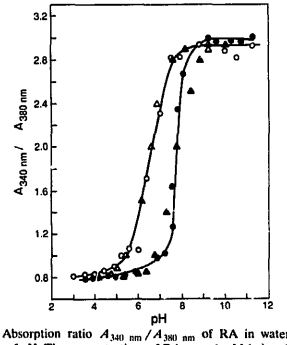

Fig. 3. Absorption ratio $A_{340\ nm}/A_{380\ nm}$ of RA in water as a function of pH. The concentrations of RA were 1 μM (○) or 10 μM (●). Titrations were carried out as described in Methods. Calculated points of the theoretical Henderson-Hasselbach curve for 1 μM (△) and 10 μM (▲) are also shown.

Theoretical Henderson-Hasselbach titration curves for the two concentrations are also shown in Fig. 3. To calculate the Henderson-Hasselbach curve, the limiting ratios of 340 nm/380 nm (R_{max} and R_{min}) were used to express the fractions of ionized RA.

$$[RA^-]/([RA^-]+[RAH]) = (R - R_{min})/(R_{max} - R_{min}) \quad (1)$$

RA^- and RAH in Expression 1 represent the concentrations of the ionized and the protonated RA, respectively. Expression 1 was solved for the ratio $[RA^-]/[RAH]$. This ratio was substituted into the Henderson-Hasselbach equation and Eqn. 2 was obtained.

$$pH = pK + \log[(R - R_{min})/(R_{max} - R)] \quad (2)$$

The experimentally obtained pK value was used in Eqn. 2 to calculate pH values for given R values.

The titration curve with 10 μM RA deviated slightly from the theoretical curve. The apparent pK values at 10 μM and at 1 μM RA were 8.04 ± 0.15 and 6.51 ± 0.2 (n = 4), respectively. The shift in the apparent pK of RA to a lower value can be explained by self-association of RA at the higher concentration. In non-polar hydrocarbon solvents, RA self-associates by forming tail-to-tail dimers that are stabilized by hydrogen bonding between the carboxyl groups of two RA molecules [26]. Such dimers do not form in polar media where the -OH part of the carboxyl groups of RA is involved in hydrogen bonds with the solvent [26]. Self-association of RA in water thus most likely originated from hydrophobic interactions between the rings of several molecules resulting in the formation of micelles. In a solution comprised of the lower concentration of RA

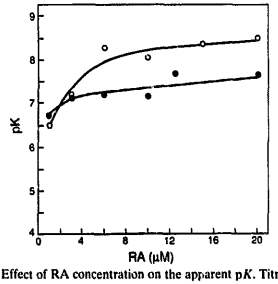

Fig. 4. Effect of RA concentration on the apparent pK. Titrations of RA in water (○) or in 0.15 M NaCl (●) were carried out as described in Methods. S.E. values for the values of pK of RA in water were ±0.27 pH units (n = 4 for each concentration). The given values of pK for RA in 0.15 M NaCl are means of two measurements for each concentration.

(1 μM), the fraction of RA monomers increases and a mixed population of micelles (displaying a high pK) and monomers (displaying a lower pK) exists. Fig. 4 shows the apparent pK as a function of RA concentration. The ionization behavior of RA as a function of concentration is similar to the well documented behavior of the bile acids cholic acid, deoxycholic acid, and chenodeoxycholic acid (for review, see Ref. 25). At high concentrations, all of the added acid molecules are bound in micelles and the pK remains fairly constant. Over a concentration range where some added acid is bound to micelles and some is present as monomers, the pK decreases. pK should again reach a constant value at concentrations below the critical micellar concentration of the acid (CMC) [25]. Since the absorption coefficient of RA in water is about 37600 M^{-1} [27], it was not possible to measure the pK at concentrations below 1 μM by this method. However, if the above interpretation is correct, the observations indicate that the critical micellar concentration of RA in water (CMC) is lower than 1 μM.

The apparent pK of RA as a function of concentration in a medium containing 0.15 M NaCl is also shown in Fig. 4. Similarly to the ionization behavior in water, the pK of RA in the presence of salt is fairly constant at high RA concentration. However, two major differences were observed between the ionization behavior of RA in water vs. the behavior in the presence of salt. It was found that the presence of NaCl decreased the pK of RA in micelles by about 1 pH unit. In addition, the shift in pK to lower values, indicating the presence of monomers, occurred at a lower RA concentration when salt was present (at 1 μM) vs. in water (at 3–4 μM). Consequently, the pK values in water and in the presence of NaCl were similar at the low range of concentrations studied (1–3 μM). These data suggest that the presence of salt lowered the CMC of RA (see Discussion).

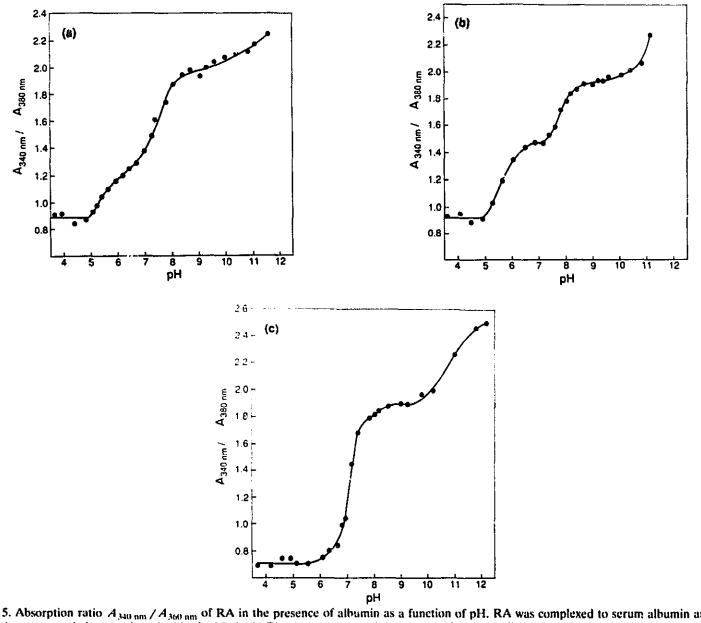

Fig. 5. Absorption ratio $A_{340\,nm}$ / $A_{360\,nm}$ of RA in the presence of albumin as a function of pH. RA was complexed to serum albumin and the titrations was carried out as described in the Methods. The concentration of albumin was 30 μM in all the assay mixtures. The concentrations of RA were: (a) 5 μM; (b) 20 μM; (c) 50 μM.

The ionization behavior of RA bound to serum albumin

Serum albumin serves as a carrier protein for RA in blood [15] and it was suggested [18] that albumin binds RA at the high-affinity binding sites that this protein possesses for long-chain fatty acids. It is of interest, therefore, to investigate the ionization behavior of RA bound to serum albumin In Fig. 5 are shown representative titration curves of RA bound to bovine serum albumin in a medium containing 0.15 M NaCl. In these experiments, the mole ratio of RA/albumin was varied in the range 0.17:1 to 1.67:1. The titration curves obtained at RA/albumin mole ratios of 0.17:1 (Fig. 5a) and 0.67:1 (Fig. 5b) indicate that RA was apparently distributed between three distinct microenvironments. At the pH range 4–9, a composite of two complete titrations curves exhibiting apparent pK values of approximately 5.6 and 7.8 were observed. At pH > 9.5 another change in the ratio of absorption of RA at 340 nm/380 nm became apparent. This part of the titration curve could not be completed and it was not clear whether the third RA population was released from albumin which may have been denatured at this extreme pH, whether this population showed a very high pK within its binding site on albumin, or whether this observation reflects changes within the protein affecting the spectral properties of RA (see Discussion). The data in Fig. 5 indicate that the fractional distribution of RA between the first two sites is approximately equal, suggesting similar affinities for RA.

The observations suggest that RA was bound to BSA under the conditions of the experiments and that two or three distinct binding sites for RA exist on serum albumin That RA was bound to albumin under the experimental conditions was indicated by the lack of precipitation of RA at concentrations of RA of 5 and 20 μM, while in the absence of protein, precipitation of RA at low pH values could be observed at concentrations as low as 1–2 μM. The observations indicating different microenvironments for RA within the protein confirm the conclusion that RA was bound to albumin in the above experiments. This follows from the apparent pK values of RA bound in site I and in site II which were 5.6 and 7.8, respectively. These values are, respectively, lower and higher than the pK of RA in micelles (Fig. 4). The total concentrations of RA in the experiments depicted in Figs. 5a and 5b were 5 and 20 μM, respectively. These concentrations are well above the CMC of RA in 0.15 M NaCl, so that if RA was unbound, it would have displayed a pK of approx. 7.2 (see Fig. 4).

Upon increasing the concentration of RA in the assay mixture to 50 μM and the mole ratio RA/albumin to 1.67:1 (Fig. 5c), a new population of RA displaying a pK of approx. 7.2 appeared. The existence of this population obscured the titration of RA in sites I and II. This observation can be explained if titration of RA in these sites could not be observed because of the higher concentration of RA in the population displaying a pK of 7.2. Alternatively, redistribution of RA from the sites observed at low ratios into the RA population observed at the higher ratio could have occurred. The apparent pK of the new population corresponded to the pK of RA in micelles, and the steep slope of this part of the titration curve had the characteristics of the titration process of RA in micelles (Fig. 4). The data thus suggest that at the high RA/albumin ratio, the concentration of unbound RA was higher than the CMC, resulting in micelle formation that could be readily observed by its ionization behavior. This interpretation of the data was supported further by the observation that when a high concentration of RA was used, precipitation of RA occurred upon lowering the pH of the medium, indicating that a fraction of RA was not bound to the protein under these conditions.

Discussion

It was found in the present study, that the absorption maximum of RA in water undergoes a red shift of about 40 nm upon protonation of the carboxyl end group (Fig. 1). This phenomenon was used to follow the titration of RA in aqueous environments. The apparent pK of RA was found to be affected by the concentration of the acid. The pK was fairly constant at the concentration range 5–20 μM, and continuously decreased as the concentration of RA was decreased from 6 to 1 μM. The behavior of RA in respect to concentration in water was thus similar to the behavior of bile acids [25,28,29] and unlike the behavior of long-chain fatty acids that can exist in several physical states (oil droplets, crystals, lamellar phases, micelles etc.) and display a complex ionization behavior in water [30]. The observations most likely reflect the formation of RA micelles which was complete at about 6 μM. Since the extinction coefficient of RA in water is 37600 M^{-1} [27], titration at concentrations lower than 1 μM could not be followed by the method used. However, it can be concluded from the data (Fig. 4) that the critical micellar concentration of RA was lower than 1 μM. This upper limit for the value of the CMC of RA is more than three orders of magnitude lower than reported CMC values for bile acids [25] or five orders of magnitude lower than the CMC for potassium decanoate (Ref. 31, cited in Ref. 30). Fluorescence polarization studies of all-*trans*-retinol in an aqueous environment indicated that the CMC of that retinoid was lower than 2 μM [32,33]. It seems then that retinoids self-associate to form micellar suspensions at very low concentrations as compared to other small amphipathic ligands.

The data indicate that the pK of RA monomers in water was lower than 6.5. The pK of RA in micelles was about 2 pH units higher, suggesting that the protonated form of RA is dramatically stabilized by micelle formation. A shift to higher pK values was reported to occur upon self-association of fatty acids and of bile acids in an aqueous environment [25,28–30]. These shifts could originate from partial shielding of the carboxyl group from the bulk aqueous phase by the less polar environment of the micelle. Another factor that could contribute to the apparent stabilization of the protonated form of carboxylic acids is the negative surface charge of the micelles which will create a proton concentration gradient with proton concentrations that are higher at the micelle/water interface vs. in the bulk water. Consequently, the apparent pK of RA will be higher in micelles as compared to RA monomers in the bulk aqueous phase. The data showing that the presence of counter-ions decreased the pK of RA in micelles support the idea that micellar surface charge plays a role in shifting the pK of RA in micelles to higher values. The data (Fig. 4) suggest further that the presence of NaCl lowered the CMC of RA, which also corresponds to the known effects of salts on the CMC of micelles [25].

The data reported above, taken together, indicate that RA in an aqueous environment forms micelles at concentrations that are at the μM range. The ionization behavior of RA, and the details of the response of the ionization state to changes in the concentration of RA and to the presence of counter-ions concur with the well documented behavior of other micelle forming, amphipathic, carboxylic acids. It can thus be concluded from the observations reported above that at physiological pH and at concentration of RA that are higher than the CMC, about 50% of RA will be negatively charged. At RA concentrations which are lower than the CMC, a predominant fraction of RA will be ionized. The anionic form of RA is soluble at concentrations of 20 μM or higher, as was evidenced by the clarity of the solutions and lack of any observed scattering at pH > 7.2. The solubility of RA under physiological conditions is thus high enough to allow this compound to diffuse across aqueous phases in vivo over distances comparable to the size of cells without the aid of soluble binding proteins. Specific binding proteins for RA, cellular RA-binding proteins (CRABP's), are known to exist in the cytosol of various cells [34,35]. The roles of these proteins for RA function and metabolism have not been clarified as yet. It has been suggested, however, that CRABP's serve as carriers for the hydrophobic ligand across cytosol (e.g. REf. 36). The results of the present study suggest that diffusion of RA may not require such facilitation.

Titrations of RA in the presence of serum albumin (Fig. 5) suggested the existence of three distinct microenvironments for RA which could be observed at a RA/albumin mole ratios of < 1. The results indicated that RA observed in these assays was bound to albumin and not dissolved in the aqueous phase. This conclusion is based on the observations that the values of the apparent pK values in the presence of albumin did not coincide with the pK of RA in micelles, even though the concentration of RA in these experiments exceeded the CMC of RA in 0.15 M NaCl. In addition, no precipitation of RA could be observed upon lowering the pH in the presence of albumin, in contrast to distinct precipitation at low pH of free RA. RA in two of the albumin sites could be completely titrated in the pH range 4–9 showing apparent pK values of about 5.6 (site I) and 7.8 (site II). The fractional distribution of RA between the sites was approximately equal, indicating similar affinities for RA.

RA bound in site I showed an apparent pK of 5.6. This value closely corresponds to the pK of RA monomers in an aqueous environment, suggesting that RA is bound in this site in a manner that allows it to freely exchange protons with the bulk aqueous phase. A second fraction of RA was bound to albumin at a site in which it showed an apparent pK of about 7.8. This value for the pK is about 2 pH units higher than the pK of RA monomers in an aqueous phase. The stabilization of the protonated form of RA associated with site II vs. RA monomers suggests that the carboxylic group of RA in this site is shielded from the bulk aqueous phase presumably by a non-polar environment.

When the concentration of RA was increased to 50 μM (Fig. 5c), sites I and II could not be observed and a population of RA displaying a pK of about 7.2 became apparent. The concentration of RA in the bulk phase in this experiment most likely increased beyond the CMC and micelles formed. The apparent pK of the 'new' RA fraction corresponded well with the pK of RA micelles in 0.15 M NaCl. The failure to observe RA in sites I and II under these conditions may thus be understood by a higher affinity for RA of micelles vs. these sites and the consequent extraction of RA from these sites into micelles. Alternatively, titration of RA in sites I and II could be obscured by the higher concentration of RA in micelles undergoing titration in a close pH range.

A third fraction was found to bind to albumin in a site that did not become apparent up to a pH of about 9.5. An incomplete spectral change representing this population occurred at the pH range 9.5–12.0. The spectral properties of RA associated with site III corresponded to the spectral properties of neutral RA, i.e. it showed a low $A_{340\ nm}/A_{3480\ nm}$ absorption ratio which increased as the pH was increased in the range 10.0–12.0. One possible explanation for this observation is that RA in site III was released from albumin at

pH > 10.5 because of conformational changes or denaturation of the protein occurring at this extreme pH. However, it has been reported that spectral changes characteristic of protein unfolding or peptide cleavage in bovine serum albumin do not occur at pH values lower than 11.5 [24], while the changes in ionization of RA discussed here were observed at a pH as low as 9.5. Another possible explanation is that the observations reflect binding of the protonated form of RA at site III. This explanation is not very likely, however, because the pK values of carboxyl groups are usually much lower than the pH range being considered here. A third possibility is that RA binds in this site in an ionized form and is involved in electrostatic interactions with a basic amino acid residue within site III. Not enough information is currently available regarding the spectral properties of RA associated with a positive charge in a system of this type. However, it is possible, given the sensitivity of the absorption spectrum of RA to the environment of the carboxyl group, that negatively charged RA which is involved is an electrostatic interaction with a basic amino acid residue will show a lower 340 nm/380 nm ratio than negatively charged RA with a free carboxyl group. If this is so, the apparent 'titration' of RA in site III at pH > 9.5 may be explained by neutralization of the basic amino acid residue at this pH range resulting in release of ionized RA with a free carboxyl group and an increase in 340 nm/380 nm absorption ratio. It was reported that the binding of long-chain fatty acids at one of the albumin high-affinity sites involves interaction of the ionized carboxyl group of the fatty acid with a ε-ammonium group of a lysine residue [24]. The ionization of such groups in bovine serum albumin have a pH midpoint of approximately 10.8 [37]. RA in site III may thus bind to the same site as long chain fatty acids and the observed change in spectral properties of RA in site III at pH > 9.5 may be due to titration of a lysine ε-ammonium group in this binding site.

Further studies are needed to correlate the albumin binding sites for long-chain fatty acids and for RA. However, the data reported here suggest that of the three binding sites for RA on serum albumin observed, two can be correlated to known binding sites for long-chain fatty acids on this protein: site I and site III. Site I: it was found from NMR studies of the binding of fatty acids to albumin, that a fraction of bound fatty acids exhibits an ionization behavior similar to that of fatty acids. This fraction of fatty acids was only observed when the mole ratio of fatty acid/albumin was higher than 3 [19,20]. Bile acids bound to albumin exhibit pK values that are similar to the pK values of bile acid monomers [28]. The data shown above suggest that site I for RA may correspond with the fatty acids binding regions, which is likely to also be the binding domain for bile acids. Unlike fatty acids, RA occupied this site at a ligand/albumin ratio as low as 0.17, which implies a higher affinity of this site for RA vs. long-chain fatty acids. Site III: as discussed above, the carboxyl group of fatty acids occupying one of the 'high affinity' sites on albumin exhibits a decrease in NMR chemical shift upon increasing the pH above pH 10.0. This was correlated with the ionization of a lysine residue at this pH range [19,20]. The incomplete titration of RA in site III occurring at pH > 9.5 may correspond with such a lysine ionization process. RA bound in site II was found to have an apparent pK of approx. 7.8. This observation implies that binding of RA at this site involves hydrophobic interactions resulting in partial shielding of the carboxyl group from the bulk aqueous phase. The ionization behavior of RA at this site does not correspond to any known site for long-chain fatty acids, and it is possible that site II is a unique RA site. This conclusion was supported by preliminary studies of competition between palmitic acid and RA on binding to albumin which showed that even a large molar excess of palmitic acid did not compete RA out of site II (data not shown). Notably, the studies reported here did not detect RA occupation in two of the three 'high affinity' binding sites of albumin for fatty acids. This may be due to electrostatic binding of ionized RA which would not have been detected unless the amino acid residue involved in the interaction is titrated in the pH range employed in this study, as was the case for RA bound in site III. However, it is clear that at least one of the microenvironments in which RA is associated with albumin involves a binding site that is distinct from the fatty acids sites, and that the affinity of at least one other site is higher for RA vs. long-chain fatty acids.

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