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Journal of Photochemistry and Photobiology B: Biology 90 (2008) 41–46
Journal of Photochemistry and Photobiology B:Biology
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Distribution of urocanic acid isomers between aqueous solutions and *n*-octanol, liposomes or bovine serum albumin

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Received 28 June 2007; received in revised form 10 October 2007; accepted 28 October 2007
Available online 1 November 2007

Abstract

The distribution of urocanic acid (UCA) isomers between aqueous solutions and *n*-octanol, egg yolk phosphatidylcholine (eggPC) liposomes or bovine serum albumin (BSA) has been evaluated. Regarding its partitioning between water and *n*-octanol, the behaviour of both isomers is very similar, and the amount incorporated to the organic solvent is mostly determined by the fraction of the compound that, in the aqueous phase, is present as uncharged species. This implies that the highest hydrophobicity occurs near the isoelectric point.

cis- and *trans*-UCA are readily incorporated into eggPC unilamellar liposomes. A simple pseudophase treatment of ultrafiltration data renders a binding constant of 0.20 ± 0.04 mL/mg for the trans isomer at pH 7.4. The binding constant decreases, by a factor two, at pH 5.0, suggesting that the negatively charged species is more favourably bound to the liposomes than the neutral species, which is mostly present as zwitterions. The *cis*-isomer, at both pHs, is less incorporated to the bilayers.

trans-UCA and *cis*-UCA readily bind to BSA at pH 7.4, with binding constants of 3400 M^{-1} and 6900 M^{-1}, respectively. This result suggests that, as in the octanol/water partitioning, hydrophobic interactions predominate and the degree of binding is determined by the fraction present as uncharged species. A smaller binding constant at pH 5.0 indicates that the charge of the protein is also plying a relevant role.

Keywords: Urocanic acid; Octanol/water distribution; Liposomes; Bovine serum albumin

1. Introduction

Urocanic acid (UCA) is present in the stratum corneum of the epidermis as the *E*-isomer [1]. Upon exposure to UVB radiation, it converts to *Z*-UCA, a compound recognized as immunosuppressor [2] (see Scheme 1). UCA is stable in the skin, but *E*-UCA is metabolized in the liver by the enzyme urocanase, an enzyme that does not recognizes *Z*-UCA. Accumulation of this isomer is mainly controlled by its excretion in sweat. The transport of UCA through the skin and from the skin to the liver is determined by its capacity to penetrate and accumulate in cell membranes and its plasma solubility. This solubility could be modulated by the capacity of UCA isomers to be associated to drug carrying serum albumin. However, very few studies have been addressed to evaluate these properties for both isomers. The protonated and deprotonated forms are extremely soluble in water, while *E*-UCA is not very soluble at its isoelectric point (pH 5). The *cis* (*Z*) isomer is an order of magnitude more soluble that the *trans* (*E*) isomer, both in organic and aqueous solvents [1]. However, no comprehensive study of their partitioning between different solvents has been reported.

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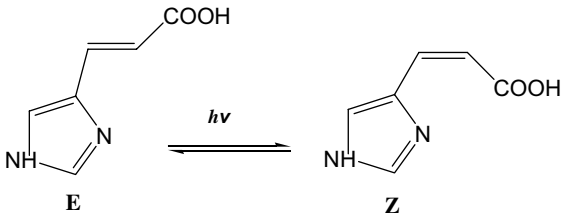
doi:10.1016/j.jphotobiol.2007.10.004



Scheme 1.

Other relevant properties of urocanic acids that can modulate their photoisomerization, distribution and transport are their association to biomolecules and lipidic bilayers. This non-covalent pre-association can also notably increase UCA photobinding to biomolecules *in vivo* [1]. Although evidence of UCA–DNA interaction has been provided [3], no indication of association to native or heat denatured DNA has been detected employing radiolabeled UCA [4]. Similarly, no significant non covalent association between *E*-UCA and BSA has been detected in dialysis experiments [5]. On the other hand, Schwazinger and Falk have reported that the presence of HSA significantly affects the photostationary *trans–cis* distribution [6], and it has been suggested that interaction of UCA with epidermal proteins contribute to a 10 nm bathochromic shift in stratum corneum preparations, increasing so the photoisomerization rate elicited by solar-like irradiations [7].

Data regarding the incorporation of UCA to membrane mimetic systems is also very scarce. This type of data is also of relevance since it has been shown that the efficiency of *trans*-UCA photoisomerization linearly correlates with solvent polarity [8]. Furthermore, transport of UCA is modulated by its capacity to penetrate membranes [9,10]. Barclay et al. have shown that 0.5 M SDS allows to obtain UCA concentrations up to 0.1 M in buffer, pH 7.4, at 37 °C [11]. Sirieix-Plénet et al. have reported the incorporation of *E*- and *Z*-UCA amphiphiles to monomolecular films of DPPC and cholesterol [12]. In the present communication, we report data bearing on the distribution of UCAs between aqueous solutions and *n*-octanol and on their capacity to interact with eggPC liposomes and BSA.

2. Materials and methods

E-UCA was a Sigma product employed as received. No significant impurities were detected by HPLC. Photolysis of *E*-UCA solutions, kept under nitrogen, afforded a mixture of *E*- and *Z*-UCA isomers without significant formation of secondary products. Kept in the dark at low temperature, these solutions remained stable for several days.

2.1. n-Octanol/water partition coefficient

Distribution of UCA between aqueous and octanolic solutions was evaluated by a hand-shaking procedure at 20 ± 1 °C. Solutions containing 0.1–1 mM UCA (mixture of *E*- and *Z*-isomers) were shaken with *n*-octanol for several minutes. Citrate (pH 3.7), citrate–phosphate (pH 5), phosphate (pH 6.8–7.4), or sodium tetraborate, (pH 8.33–9.18), were employed as buffers. The results were independent of the buffer concentration or NaCl (100 mM) addition. After centrifugation, aliquots of both phases were injected to the HPLC and measured the area corresponding to each isomer. The analysis was carried out in a Waters 600 system, equipped with a Waters PDA detector set at 276 nm. A Cyclobond column was employed with acetonitrile: water: phosphate buffer, pH 5.0 (85:10:5 V/V) as mobile phase. The octanol/water partition constant of each isomer was obtained from

$$K_p = [UCA]_{octanol}/[UCA]_{water} \quad (1)$$

2.2. Incorporation to egg yolk phosphatidylcholine (egg-PC) large unilamellar liposomes

Large unilamellar liposomes of nominal diameter 400 nm were prepared by extrusion of multilamellar egg-PC liposomes through polycarbonate membranes (Nucleopore), following the procedure previously described [13]. The multilamellar liposomes were prepared by re-suspending in buffer phosphate (10 mM, pH 5.0 or 7.4) the lipid deposited in the bottom of 50 mL test glass tubes. Deposition was achieved by evaporation in a nitrogen gas stream a solution of the lipids in chloroform (100 mg in 800 μL).

Liposome suspensions (0.5 and 1.0 mg/mL) and mixtures of *E*- and *Z*-UCA (from 5 to 40 μM) were incubated at 37 °C during 30 min. After equilibration, the solutions were ultrafiltered through membranes (cut off at PM 10000; Milipore PM-10). The concentration of UCA was determined by HPLC in the ultrafiltrated and in the original solution. Blanks without liposomes were carried out in order to disregard significant adsorption of UCA to the membranes. Partition constants for a given isomer were evaluated as

$$K_p = ([UCA]_{total} - [UCA]_{free})/([UCA]_{free}[lipids]) \quad (2)$$

that considers the liposome ensemble as a pseudophase. The use of this equation is justified by the fact that, in the above described experimental conditions, the amount of lipids is considerably larger than that of UCA.

Mixtures of isomers were obtained by irradiation of *E*-UCA solutions with a UV-B lamp under nitrogen. The concentrations of each isomer was determined by HPLC. A C-18 μBondapak Waters (3.9 × 300 mm) column and acetonitrile:buffer phosphate 10 mM, pH 7.4 (5:95 V:V) as mobile phase (1 mL/min) were employed in the analysis.

2.3. Association of UCA to bovine serum albumin

The extent of association of *E*- and *Z*-UCA to BSA was evaluated in micro ultrafiltration experiments. A mixture of *E*- and *Z*-isomers was passed through a Microcom membrane and the free acids determined by HPLC in the ultra-

filtrate. Solutions (BSA 5 mM; UCA between 0.5 and 0.05 mM, pH 7.4) in phosphate buffer were prepared and put in a vortex mixer during 45 s. After this, were centrifugued during 30 min in a 3 mL Eppendorff bearing the ultrafiltration membrane. Experiments were carried out in pure *E*-UCA and in mixtures of isomers obtained by a previous photolysis of *E*-UCA solutions kept under nitrogen. Experiments with the *E*-isomer were also carried out in citrate buffer, pH 5.0. All measurements were carried out at 20 ± 1 °C.

Displacement of bound dansylsarcosine (DS) and 5-dimethylaminonaphthalene-1-sulfonamide (DNSA) was assessed by measuring the change in the probes fluorescence intensity (Excitation 350 nm; emission 470 nm) elicited by *E*-UCA addition to a solution of BSA-fluorophore complex. Measurements were carried out at 20 ± 1 °C in an Aminco–Bowman spectrofluorimeter with 2.5 nm excitation and emission slits.

3. Results and discussion

In the pH range considered, a protic equilibria between cationic, uncharged + zwitterionic, and anionic forms must be taken into account. Relevant pK_a are 4.0 and 6.1 for the *trans*-isomer, and 3.3 and 7.0 for the *cis*-isomer. The isoelectric point is nearly 5.0. This value is close to the stratum corneum pH, which is mainly regulated by UCA itself [10].

3.1. Octanol/water partitioning

Partition constants of both UCA isomers between buffered aqueous solutions and *n*-octanol were measured in the 3.7–9.07 pH range. Values of log *K*, plotted as a function of the aqueous phase pH, are shown in Fig. 1. These data indicate that

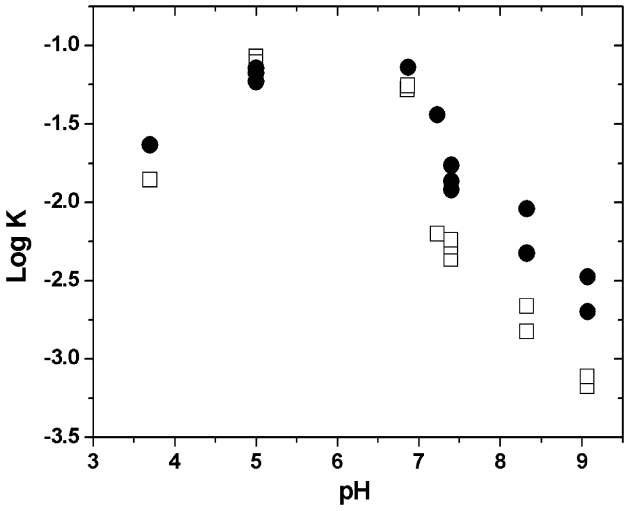


Fig. 1. Partition constant of UCA isomers between an aqueous and *n*-octanol solution. Temperature: 20 ± 1 °C. Data are presented as log *K* vs. pH. (●) *cis*-isomer; (□) *trans*-isomer.

(i) At pH 5, where the neutral species predominated, the *trans*-isomer is more incorporated to the organic solvent than the *cis*-compound (Table 1). This difference is independent of the buffer (phosphate–citrate) concentration.

(ii) At pH 3.7 (were predominates the cationic form) or over 7.0 (where the anionic form dominates) the solubility in the organic phase is strongly diminished. In both branches, the decrease seems to be smaller for the *Z* compound, a result compatible with the larger range of pH in which predominate the uncharged/zwitterionic forms of this compound.

At pH 5.0 most of the UCAs are present as neutral (zwitterionic plus uncharged) species. The data of Table 1 would indicate that even under these conditions the solubility in aqueous solution is considerably higher than in the organic solvent. If this partition is considered as a measure of the molecule hidrophobicity, we must conclude that, even as uncharged species, urocanic acids are very hydrophilic. In part, this low hydrophobicity can be due to the fact that, as a neutral species, both urocanic acids are mostly present as zwitterions, reducing notably the concentration of the uncharged species. In fact, it has been estimated that, even at pH 5.0, only ca. 5% of the acids are present as uncharged species [6]. This would strongly increase the stability of the molecule in the aqueous phase. It is interesting to note that the data given in Fig. 1 and Table 1 would indicate that at the isoelectric pH, the *Z* isomer is more hydrophilic than the *E*-isomer, while as anion and cation, the opposite happens. This last result can be related to the wider pH range in which the *cis*-isomer is mostly present as neutral (zwitterionic plus uncharged) species.

At pH 5.0, the percentage of uncharged species of the *trans*-isomer (6.5%) is slightly higher than that of the *cis*-isomer (5.3%) [6]. This difference can partly explain the higher partition constant of the *trans*-isomer reported in Table 1. This would imply that there are not significant differences between the hydrophobicity of the uncharged molecules. In fact, if the partition constants reported in Table 1 are corrected by the fraction of uncharged species, the value obtained is 1.2 for both isomers. This value is very close to that calculated (1.4) by an atom/fragments contribution method [14,15].

3.2. Incorporation to liposomes

Data obtained with the *E*-isomer at two lipid concentrations are shown in Fig. 2. The concentration measured by

Table 1
Partition constants measured at pHs 5.0 and 7.4

pH	K_{cis} [a]	K_{trans} [a]
5.0 ($n = 30$)	0.068 ± 0.001	0.081 ± 0.001
7.4 ($n = 18$)	0.014 ± 0.0001	0.0050 ± 0.0003

[a] Errors given correspond to estimated error of the mean values.

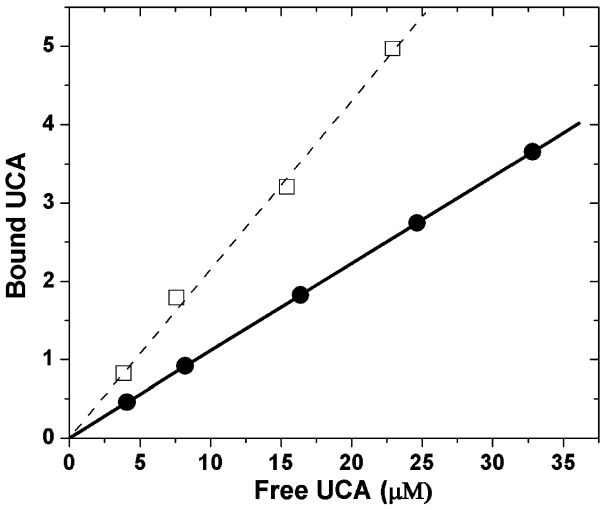


Fig. 2. Association to liposomes of *t*-UCA. The analytical concentration of the bound compound, obtained from Eq. (3), is plotted against the concentration measured in the ultrafiltrate. Lipid concentration: (●) 0.5 mg/mL; (□) 1.0 mg/mL. pH 7.4.

HPLC in the ultrafiltrate was equated to the free substrate concentration. The amount bound to the liposomes was calculate from

$$[\mathrm{UCA}]_{\mathrm{bound}} = [\mathrm{UCA}]_{\mathrm{total}} - [\mathrm{UCA}]_{\mathrm{free}} \quad (3)$$

and plotted as a function of the free UCA. Lineal plots were obtained, with slopes proportional to the lipid concentration. The slopes of these plots, divided by the lipid concentration, provides the value of K_{p} as 0.22 ± 0.01 mL/mg at pH 7.4. A similar procedure, applied to the mixtures (Fig. 3), affords the values collected in Table 2. These data show that the partition of UCA depends both on the pH and the isomer considered. In particular, the data indicate that, at both tested pHs, the *trans*-isomer is better incorporated to the liposomes (by a factor two) than the *Z*-isomer.

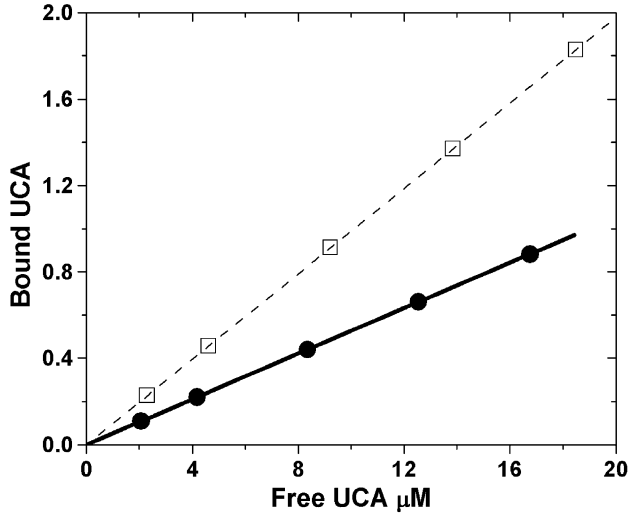


Fig. 3. Association to liposomes of *t*-UCA and *cis*-UCA from mixtures comprising similar amounts of both isomers. Data plotted as indicated in Fig. 2. Lipid concentration 0.5 mg/mL, pH 7.4. (□) *trans*-isomer; (●) *cis*-isomer.

Table 2
Incorporation of *E*- and *Z*-UCA to eggPC LUVs and BSA

pH	Isomer	K_{P} (mL/mg)[a]	K_{BSA} (M^{-1})[a]
5.0	*E*	0.128 ± 0.001	280 ± 110
	Z	0.062 ± 0.001	–
7.4	*E*	0.200 ± 0.04	3400
	Z	0.104 ± 0.02	6900

[a] Error indicated are the errors in the slopes of the $[\mathrm{UCA}]_{\mathrm{bound}}$ vs. $[\mathrm{UCA}]_{\mathrm{free}}$ plots comprising data from three experiments.

The data shown in Table 2 indicate that

(i) at both pHs, the *E*-isomer better associates (by a factor two) than the *Z*- isomer,
(ii) UCAs associate better to the liposomes at pH 7.4.

The first point can be qualitatively explained in terms of the predominant conformations of both isomers. In fact, the more globular conformation of the *cis*-isomer [16–18] could hinder its incorporation to the liposomes [19].

The fact that more UCA is incorporated to pH 7.4 implies that its solubilization into the liposomes cannot be described in terms of the neutral species. Most probably, the UCA is associated to the bilayer as a single charged anion. A larger proportion of this species in the *trans*-isomer ($\mathrm{p}K = 6.1$) than in the *cis*-isomer ($\mathrm{p}K = 7.0$), and a more favourable conformation (see supra) would explain the increased solubilization of the *E*-isomer.

3.3. Association to BSA

In order to simplify data treatment, experiments were carried out at a BSA concentration (5 mM) considerably larger than UCA concentrations. Under these conditions, it can be assumed that the association constant (K_{BSA}) of each UCA isomer can be evaluated by

$$K_{\mathrm{BSA}} = [\mathrm{UCA}]_{\mathrm{bound}}/([\mathrm{UCA}]_{\mathrm{free}}[\mathrm{BSA}]) \quad (4)$$

where $[\mathrm{UCA}]_{\mathrm{bound}}$ is calculated with Eq (3) and [BSA] is the analytical protein concentration. A plot of $[\mathrm{UCA}]_{\mathrm{bound}}/[\mathrm{BSA}]$ against $[\mathrm{UCA}]_{\mathrm{free}}$ allows to obtain K_{BSA} values from the slope. These type of data are shown in Fig. 4 and the derived K_{BSA} values included in Table 2. Furthermore, at low occupation numbers (condition achieved by keeping $[\mathrm{BSA}] \gg [\mathrm{UCA}]$) there is no competition between the isomers and the K_{BSA} value of a given isomer is not affected by the presence of the other isomer. This is confirmed by the data given in Fig. 4.

K_{BSA} values obtained in the present work imply that, for the *E*-isomer at pH 7.4, 50% of the UCA should be incorporated to BSA when the protein concentration is ca. 0.3 mM. An extensive adsorption to albumins in vivo conditions could promote covalent photobinding [5].

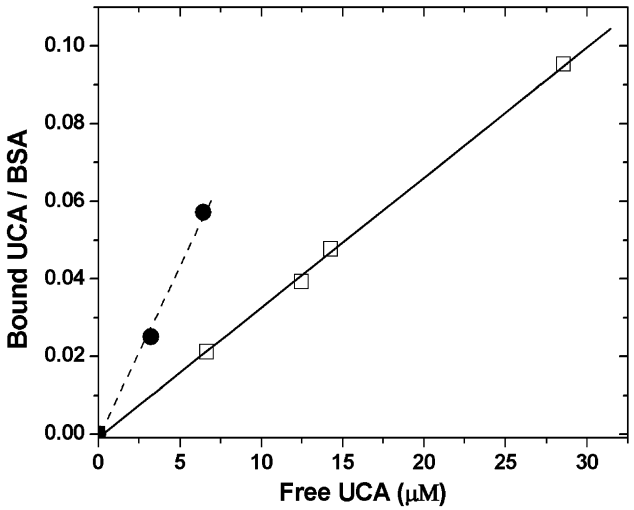


Fig. 4. Association of UCA isomers to BSA. Results plotted according to Eq. (4). Data obtained at pH 7.4 in presence of 5 mM BSA. (●) *cis*-isomer; (□) *trans*-isomer. Data of the *trans*-isomer comprise values for the pure compound and mixtures of the *trans* and *cis*-isomers.

From HSA intrinsic fluorescence quenching it has been derived, at pH 7.0, a binding constant of $4.1 \times 10^4\ M^{-1}$ for the *E*-isomer [6]. A comparison between both sets of data is difficult since the methodology employed in the evaluation of the binding constant from fluorescence quenching assumes a one-to-one complex and that a single bound UCA molecule completely quenches the intrinsic protein fluorescence. We have applied the same approach to estimate a binding constant from intrinsic fluorescence quenching measurements. The quenching efficiency of the *trans*-isomer, after correcting the data for the UCA absorption at the exciting wavelength, plotted according to the Stern–Volmer equation is shown in Fig. 5. These data provide a Stern–Volmer quenching constant (K_{SV}) of

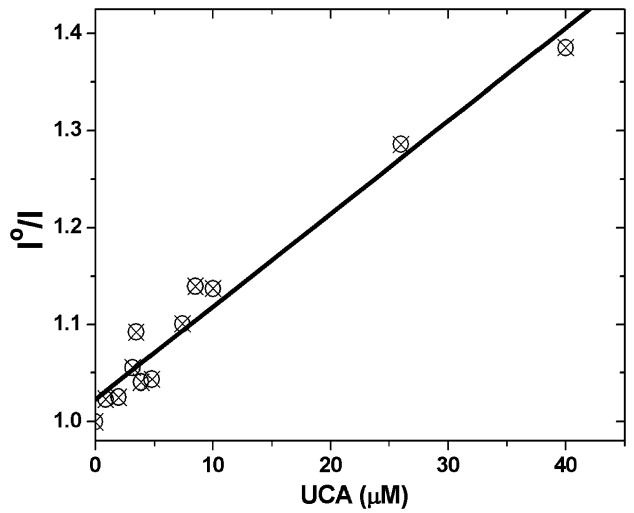


Fig. 5. Quenching of BSA intrinsic fluorescence by *trans*-UCA. Excitation: 295 nm; emission: 340 nm; BSA concentration: 1.5 μM. Data are plotted as I^0/I as a function of the added UCA concentration. Values of I have been corrected to take into account UCA absorption at the exciting wavelength.

$9.6 \times 10^3\ M^{-1}$. Even if it is assumed for BSA tryptophan residues an average lifetime of 10 ns, a dynamic quenching requires a quenching constant of $1 \times 10^{12}\ M^{-1}\ s^1$. This value is considerably higher than that expected for a diffusion controlled process in water ($\approx 5 \times 10^9\ M^{-1}\ s^{-1}$), and suggests a strong association in the ground state. In fact, from these data and the above mentioned considerations it can be estimated that $K_{BSA} = 6.7 \times 10^3\ M^{-1}$. This value, although bound to a large uncertainty, due to the assumptions involved in its evaluation and the magnitude of the correction required to take into account the UCA filter effect, is of the same order of magnitude as that obtained from ultrafiltration experiments, and confirms a strong interaction between UCAs and BSA.

The data given in Table 2 would indicate that, at pH 7.4, the *Z*-isomer interacts more strongly with BSA than the *E*-isomer. Since the IP of BSA is ca. 4.7, the protein and UCA isomers are mostly negatively charged at the working pH. It can be speculated that the fraction of neutral UCA present at this pH is the dominant species that associate to the protein. A considerably larger fraction of the *Z*-isomer ($pK = 7.0$) than that of the *E*-isomer ($pK = 6.1$) present as uncharged species at pH 7.4 could explain the preference of the protein for the *Z*-isomer. Other factors, such as hydrophobic interactions and topological factors could also influence the selectivity of the binding. In any case, it can be speculated that this preferential binding of the *Z*-isomer could increase the photobinding to the protein and affect the photoisomerization process [6,20].

Ultracentrifugation measurements carried out employing the *E* isomer at pH 5.0 (citrate buffer) indicate a much weaker binding at this pH. In fact, the amount bound to the protein was barely measurable, rendering large errors in the evaluation of the binding constant. In spite of the large error involved (see Table 2) the data clearly indicate that binding is reduced by ca. a factor 10 when pH drops from 7.4 to 5.0. In agreement with a small amount of binding at pH 5.0, quenching of the protein fluorescence by UCA was undetectable in all the additive concentration range considered. At pH 5.0, UCA is mainly zwitterionic, with the indol moiety positively charged. Repulsion between this group and the positively charged protein could explain the low level of binding.

In order to asses the main site of *trans*-UCA binding to BSA, displacement experiments were attempted employing DS (selective binding to site II in subdomain IIIA of the protein) and DNSA (selective binding to site I in subdomain IIA of the protein) [21,22]. The results obtained at pH 7.4 are shown in Fig. 6. These data show that UCA addition modifies the fluorescence intensity emitted from the protein-bound chromophores, confirming its interaction with BSA. Furthermore, the decrease in fluorescence intensity from DS, explainable in terms of its displacement by UCA, would indicate a significant adsorption of *trans*-UCA onto subdomain IIIA of the protein. This would reduce the probe fluorescence by its displacement by UCA and/or by fluorescence quenching. On the other hand, the

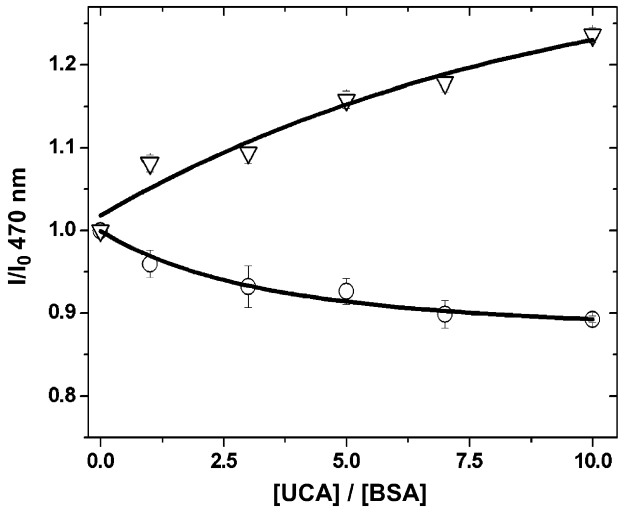


Fig. 6. Changes in DS and DNSA fluorescence elicited by *trans*-UCA addition to previously formed chromophore-BSA complexes. Experiments were carried out at pH 7.4 and complexes were preformed employing BSA (2 μM) and DS (○) or DNSA (Δ) (0.2 μM). Fluorescence measured at 470 nm.

enhancement of DNSA fluorescence elicited by UCA addition would indicate that this compound is not being associated to the protein subdomain IIA. Similar increases in fluorescence have been explained in terms of conformational changes of the protein elicited by the solute binding [21].

4. Conclusions

Partition of *cis*- and *trans*-UCA between an aqueous solution and octanol strongly depends upon the pH and the isomer considered. Although both isomers are relatively hydrophilic, they readily bind to egg PC liposomes and proteins (BSA), particularly at pH 7.4. Differences in binding of the *Z*- and *E*-isomers to BSA at pH 7.4 can be explained in terms of the relative amounts of neutral species in equilibrium with the anionic forms of UCA. The data obtained in the present work regarding the binding affinity of UCA isomers to lipidic bilayers and transport proteins can contribute to our understanding of the distribution and transport of urocanic acids from the skin to other tissues.

Acknowledgement

This work has been financed by FONDECYT (Project 1030033).

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