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# Protein dilution effect on thiouracil–seroalbumin interactions

Eduardo Ochoa de Aspuru *, Ma. Asunción Marquínez, Ana Ma. Lourdes Zatón

*Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of Basque Country, P.O. Box 450, Vitoria-Gasteiz, Spain*

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**Abstract**

As the spectra and binding parameters calculated for the thiouracil–albumin interaction change with the protein concentration, a human seroalbumin conformational change depending on protein concentration has been suggested. This protein-conformational change is tested by dilatometry and viscosimetry. At low concentrations, albumin showed a greater thiouracil binding capacity and a second positive peak in its interaction with the drug, detected by difference spectroscopy. Both effects are due to a monomerisation of protein dimers and not to a conformational change depending on protein concentration. This monomerisation would imply a major accessibility of thiouracil and propylthiouracil to other binding sites on HSA. © 1997 Elsevier Science B.V.

*Keywords:* Protein dilution effect; Protein concentration effect; Human serum albumin interactions; Thiouracil; Propylthiouracil

## 1. Introduction

Human serum albumin (HSA), a plasmatic protein dimer, is the principal drug-carrier in serum, thanks to its binding interactions with ligands. Drugs can be bound to, at least, four different sites on HSA. Two of them (sites I and II), are responsible for the binding of the majority of protein-carried drugs, while the remaining two sites—the digitoxin and tamoxifen ones—have a limited specificity [1,2]. Several thiouracils are carried by HSA in blood [3]. Thus, thiouracil binds to one low affinity binding site which is common with the benzodiazepines, 'site II' [4].

The binding parameters of the drugs to HSA are obtained from the degree of binding to the diluted protein, measured usually by equilibrium dialysis or by difference spectroscopy. Binding saturation experiments with propylthiouracil revealed that the association constant obtained by difference spectroscopy [5] is larger than the obtained one by equilibrium dialysis [4]. The cause of this disagreement could be the differences in the protein concentration used. All traditional methods of measuring ligand–protein interactions involve maintaining a fixed protein concentration whilst varying the ligand concentration. These approaches assume that the apparent association constant $K$ and the number of binding sites $n$ are independent of protein concentration. However, there is evidence that cortisol [6], digitoxin [7] and other drugs show albumin-concentration dependence on these parameters. When this concentration is re-

* Corresponding author.

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duced within the physiological range, the ratio bound:unbound drug decreased more than the expected value starting from predictions using the law of mass action.

Taking into account this evidence, both thiouracil and propylthiouracil were examined to ascertain its binding kinetics to HSA employing different protein concentrations.

## 2. Materials and methods

### 2.1. Chemicals

Human serum albumin (essentially fatty acid free), 2-thiouracil and 6-*n*-propyl-2-thiouracil, were purchased from Sigma Chemical (St. Louis, MO, USA). Solvents were spectral grade, and all other chemicals were reagent grade. Protein and drugs were dissolved in 0.067 M phosphate buffer pH 7.4 for all experiments.

### 2.2. Difference spectroscopy

In vitro protein binding of the thiouracils was measured by ultraviolet difference spectroscopy. Absorption spectra and difference absorbance measurements were made with a double-beam spectrophotometer model 3600 from Beckman Instruments (Fullerton, CA, USA) equipped with tandem cell holders.

All difference spectra were obtained using a pair of 4.375-mm light path split-compartment tandem cells and transformed into concentrations of ligand bound to HSA according to a procedure previously described [5].

### 2.3. Conformational changes measurement

Volume changes were measured by means of a dilatometer similar to the described one by Komiyama et al. [8], with lower and upper compartment volumes of 5.2 and 6.4 ml, respectively. An

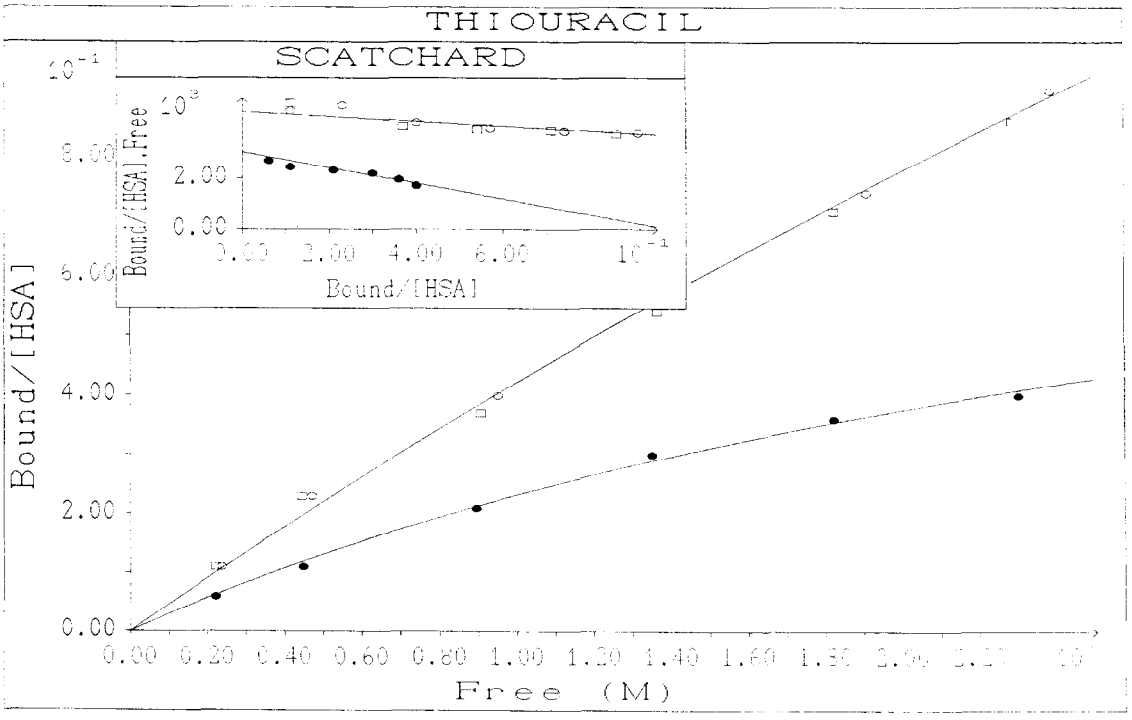


Fig. 1. PTU bound to HSA vs. concentrations of free PTU, and Scatchard plots for PTU–HSA binding. The HSA concentrations were $5 \times 10^{-5}$ M (●), $2.5 \times 10^{-5}$ M (□) and $1.25 \times 10^{-5}$ M (○).

additional inlet was provided with a Quickfit stopper. The capillary tube was 0.3 ± 0.01 mm i.d.

Volume changes at 37.0 ± 0.01°C were measured according to a procedure previously described [3]. Solution I, containing ureylene compound in the lower compartment, and solution II, containing 0.077 or 0.155 mM solutions of HSA in the upper compartment, were mixed by opening the internal stopper and stirring for 5 min. Volume changes observed in these experiments were expressed as $\delta V$ in ml $10^{-5}$ g of protein.

Viscometric measurements were made using an Ubbelohde suspended level microviscometer by means of a viscosity-measuring instrument, a Viscoboy from mgw-Lauda (Lauda-Konigshofen), thermostatically maintained at 37°C. The densities were obtained with a Gay-Lussac picnometer. The results were transformed into specific viscosities ($\eta_{sp}$) and expressed as $\eta_{sp}/c$ [3], where $c$ is the concentration of protein + bound ureylene in g $ml^{-1}$.

## 3. Results and discussion

For $5 \times 10^{-5}$ M, HSA binding parameters of $n = 0.99 \pm 0.11$ and $K_d = 3.26 \pm 0.54 \times 10^{-4}$ M ($n/K_d = 3029$ $M^{-1}$) were estimated, whereas, for lower concentrations of protein, 2.5 and $1.25 \times 10^{-5}$ M, values of $n = 4.25 \pm 1.21$, $K_d = 9.11 \pm 3.1 \times 10^{-4}$ M ($n/K_d = 4670.5$ $M^{-1}$), and $n = 4.98 \pm 1.07$, $K_d = 1.08 \pm 0.27 \times 10^{-3}$ M ($n/K_d = 4610$ $M^{-1}$), respectively, were obtained. Then, the binding kinetics for the interaction between thiouracil and HSA are protein concentration dependent (Fig. 1).

The binding of propylthiouracil to HSA is shown in Fig. 2. For HSA concentrations between 2.5–5.0 $\times 10^{-5}$ M, values of $n = 2$ and $K_d = 9.85 \times 10^{-5}$ M ($n/K_d = 20304.57$ $M^{-1}$) were estimated, on the assumption that there was only one type of affinity binding site. The standard deviation was in all the cases less than 15%. At concentrations of $7.25 \times 10^{-5}$ and $1.45 \times 10^{-4}$ M, no difference in the bind-

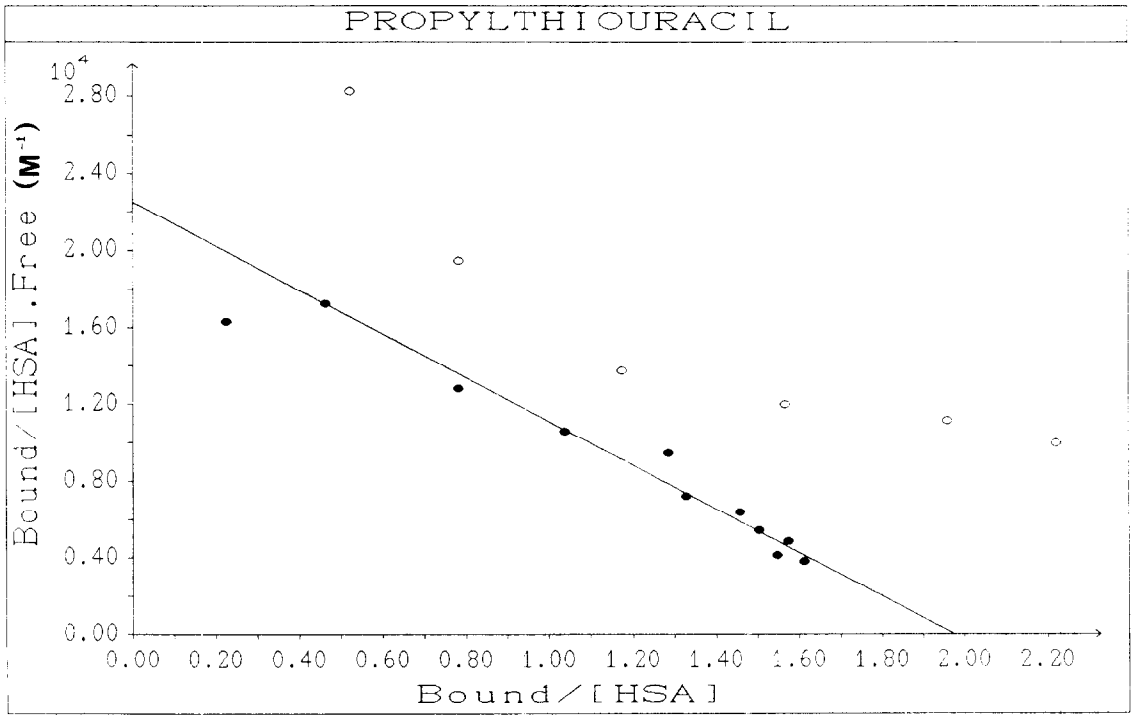


Fig. 2. Scatchard plots for propylthiouracil–HSA binding. The HSA concentrations were $5 \times 10^{-5}$ M (●) and $1.25 \times 10^{-5}$ M (○).

ing parameters was observed. However, for $1.25 \times 10^{-5}$ M HSA, Scatchard plots are non-linear, showing additional binding sites. In this case, values of $n_1 = 1$ and $K_{d1} = 1.056 \times 10^{-4}$ ($n_1/K_{d1} = 9469.70$ $M^{-1}$) were obtained for the moderate affinity binding sites and $n_2 = 4.13$ and $K_{d2} = 7.86 \times 10^{-4}$ M ($n_2/K_{d2} = 5251.10$ $M^{-1}$) for the low affinity binding sites. The standard deviation was in all the cases less than 15%. The dilution effect observed for both drugs may be due to conformational changes in HSA.

The ability of propylthiouracil to induce conformational changes in HSA [9,10] persuaded us to test whether the different binding kinetics obtained could be due to a conformational change produced by the thiouracil–HSA interaction, instead of protein dilution. In order to elucidate that, an evaluation of intrinsic viscosities ($\eta_{sp}$) and volume changes ($\delta V$) were made. At increasing thiouracil concentrations, the specific viscosity, expressed as $\eta_{sp}/c$, increases, whereas the protein volume, measured as $\delta V$, decreases (Table 1), suggesting a gradual dissociation of the globular protein dimer. The rise of $\eta_{sp}/c$ values may be due to the elongation inherent in the HSA monomerisation. Moreover, the effect of thiouracil concentration on $\delta V$ is also a consequence of the protein dissociation, because the monomer occupies a minor volume than the dimer. The results obtained at thiouracil concentrations between $0.5–5 \times 10^{-4}$ (Table 1) were fitted to exponential curves, and no change in the slope sign of resultant plots was shown, signifying that HSA does not undergoes structural changes caused by thiouracil in concentrations between $0.5–5 \times 10^{-4}$ M. However, its hydrodynamic volumes, $\eta_{sp}/c$, at several HSA concentrations were between 4.6 and 10 ml $g^{-1}$. The higher viscosity value was obtained at protein concentrations lower than $2.78 \times 10^{-5}$ M. Nevertheless, the difference spectra for HSA–thiouracil interaction shows a second positive peak (Fig. 3) at 268 nm, with a decreasing HSA concentration under $5.0 \times 10^{-5}$ M. This peak is characteristic of the red shift of phenylalanine spectrum [11] that was agreed with the transfer from polar to apolar environments. As a result of the protein monomerisation, this amino acid would appear on the molecule surface.

Both effects—the greater thiouracil binding capacity and the second positive peak appearing at low

Table 1
Values obtained for thiouracil–HSA system from viscometry ($\eta_{sp}/c$) and dilatometry ($\delta V$) at 37°C

| [Thiouracil] (mmole/l) | $\eta_{sp}/c$ (ml $g^{-1}$) | $\delta V$ (ml/$10^5$ g protein) |
|---|---|---|
| 0.050 | 2.400 | 880 |
| 0.100 | 2.695 | 690 |
| 0.150 | 2.942 | 512 |
| 0.200 | 3.108 | 351 |
| 0.250 | 3.338 | 227 |
| 0.300 | 3.477 | 121 |
| 0.350 | 3.572 | 40 |
| 0.400 | 3.651 | −50 |
| 0.450 | 3.702 | −112 |

The data were fitted to the following exponential curves: [Thiouracil] = $0.002 \times e^{1.45(\eta sp/c)}$ and $\delta V = 1650.17 \times e^{-9.11[\text{Thiouracil}]}$.
[HSA] = $7.75 \times 10^{-5}$ M.

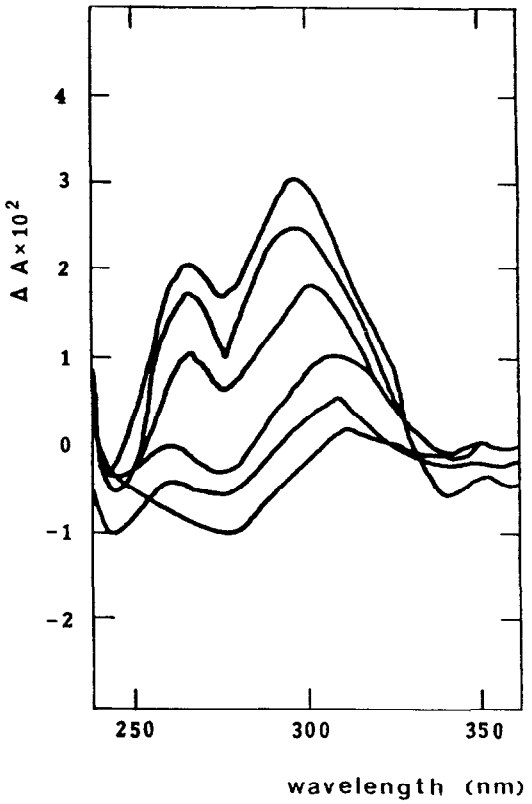


Fig. 3. Difference absorption spectra of HSA ($2.00 \times 10^{-5}$ M) with successive ($2.00 \times 10^{-5}$ M) increments of thiouracil in 0.067 M phosphate buffer pH 7.4. Absorbance difference increased with increasing thiouracil concentration.

albumin concentrations—would be due to a monomerisation of protein dimers and not to a conformational change depending on HSA concentration, according to the reported results of $\delta V$ and $\eta_{sp}$. This monomerization provides a major accessibility of thiouracil to other binding sites on the albumin and also implies that phenylalanine stay at the periphery of the protein in contact with the medium. At low HSA concentrations, the high affinity sites are in the surface of the albumin molecule and the drug affinity is higher.

For TU, the monomerization makes the protein more accessible to the drug, showing new binding sites on its molecule. The fact that the HSA concentration dependent effect was more evident in the thiouracil–albumin interaction may be explained by the localization of the drug binding sites on the interaction zone between both monomers, where TU, according to its lower molecular size, may have a better access. However, when HSA concentration decreases so that the protein monomerizates, different types of binding sites for PTU appear in the albumin molecule (Fig. 2).

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