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THE BINDING OF THIOUREYLENE COMPOUNDS TO HUMAN SERUM ALBUMIN

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Abstract—The binding interactions of some thioureylene compounds to human serum albumin were studied *in vitro* by ultraviolet spectroscopy and equilibrium dialysis. Binding of 6-*n*-propyl-2-thiouracil, 6-*n*-benzyl-2-thiouracil and 2-thiouracil to human serum albumin results in a red shift of the ultraviolet absorption maximum, suggesting that the binding site is a hydrophobic area of the protein. Bindings of 6-*n*-propyl-2-thiouracil and 6-*n*-benzyl-2-thiouracil to human serum albumin are characterized by two classes of sites while 6-*n*-propyl-uracil and 2-thiouracil bind to one low-affinity binding site. In addition, an identification of those sites was performed by measuring the displacement of these drugs. The data show that the moderate-affinity site is common with the warfarin site while the low-affinity site is likely to be shared by benzodiazepines.

It is concluded that the binding is enhanced by the hydrophobicity of the substituent in the thioureylene compounds, and it is further shown that thiol-group substitutions in the thioureylene ring will weaken the binding.

After introduction into the circulation most drugs are bound to different blood constituents (cells, proteins). Many xenobiotic compounds have been shown to bind in blood to human serum albumin (HSA).† The binding properties of this protein are at present explained by the presence of several binding regions [1, 2] but only a few studies [3, 4] are available on the possible relationships between the structure of the ligands and their affinity to HSA. Zia and Price [5] in agreement with Bird and Marshall [6], suggest that hydrophobicity is an important determinant of ligand binding to albumin, while Bickel and Bovet [7] say that all albumin-interacting amines studied contain one or more tertiary amino group. However, for these latter authors the relationship between structure and albumin interaction is less clear in the case of compounds containing nitrogen in a cyclic system.

Since thioureylene antithyroid drugs are sulfhydryl compounds containing nitrogen in a cyclic system, the present studies were conducted to define further in detail the binding between HSA and several thioureylene compounds (2-thiouracil, 6-*n*-propyl-2-thiouracil (PTU) and 6-*n*-benzyl-2-thiouracil (BTU)) differing in their substituents on the thiouracil ring. Moreover it has long been known that sulfhydryl (—SH) groups and disulfide (—S—S—) bonds may play a role in the regulation of binding processes for several ligands. Thus studies of uracil and 6-*n*-propyluracil binding to HSA were also performed to determine whether the thiol group is responsible for the thioureylene binding to HSA.

In addition, structure-binding studies of several ureylene compounds were performed to define which parts of the thioureylene molecule are essential for the binding to HSA, and what is the nature of the binding sites in HSA for the thioureyelene molecules.

MATERIALS AND METHODS

Chemicals

HSA (essentially fatty acid-free, prepared from fraction V human albumin), uracil, 2-thiouracil, PTU, BTU and 3-(α-acetonylbenzyl) 4-hydroxycoumarin (warfarin) were bought from Sigma Chemical Co. (St. Louis, MO), and diazepam from Roche (Madrid, Spain). 6-*n*-Propyluracil was synthesized according to the method of Lindsay *et al.* [8]. All other chemicals were of reagent grade. HSA, antithyroid drugs and derivatives were dissolved in M/15 phosphate buffer pH 7.4 for all experiments.

Ultraviolet difference spectra

Difference spectra were obtained by the Herskovits' double-cell compensation technique [9]. The spectra were recorded with a Beckman 3600 UV–vis. spectrophotometer.

(i) *The drugs in aqueous and ethanol media.* A solution of 1 mM of drug in M/15 phosphate buffer pH 7.4 was diluted to 6.25×10^{-6} M (1:160) with the same buffer or with absolute ethanol. The baseline was set to zero with the ethanol blank (buffer/ethanol, 1:160) in the sample beam and the buffer blank in the reference beam. The difference spectrum was recorded with the buffered drug solution in the reference beam and with the ethanol solution in the sample beam.

(ii) *The drugs, free and HSA-bound.* The baseline was set to zero with buffer solution and HSA (7.25×10^{-5} M) in double cells in both reference and

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† Abbreviations used: HSA, human serum albumin; PTU, 6-*n*-propyl-2-thiouracil; BTU, 6-*n*-benzyl-2-thiouracil; UV, ultraviolet; vis., visible.

Table 1. Extinction coefficients for the spectra of thiouracil, PTU and BTU in phosphate buffer pH 7.4 alone and in excess of HSA or in 50% ethanol. The concentration of thioureylene was 6.25×10^{-6} M; that of HSA 5.00×10^{-5} M

	Extinction coefficients ($mM^{-1} cm^{-1}$)	
	At minimum of difference spectra	At maximum of difference spectra
Thiouracil	12.5 (at 275 nm)	3.28 (at 313 nm)
Thiouracil in HSA	12.0 (at 275 nm)	3.88 (at 313 nm)
Thiouracil in ethanol	11.9 (at 275 nm)	4.00 (at 313 nm)
PTU	15.7 (at 275 nm)	2.24 (at 315 nm)
PTU in HSA	13.0 (at 275 nm)	5.04 (at 315 nm)
PTU in ethanol	12.5 (at 275 nm)	5.34 (at 315 nm)
BTU	21.1 (at 267 nm)	2.40 (at 325 nm)
BTU in HSA	18.7 (at 267 nm)	4.80 (at 325 nm)
BTU in ethanol	17.4 (at 267 nm)	6.10 (at 325 nm)

sample beams. Difference spectra were recorded after addition of the same amount of drug to the buffer solution of the reference compartment and to the HSA solution of the sample compartment.

Binding experiments

In vitro protein binding of the drugs was measured by equilibrium dialysis. A Dianorm apparatus and Diachema dialysis membranes type 10.17 (Diachema, Zurich, Switzerland) were used. Dialysis chambers had a volume of 1.2 ml and contained 1 ml of solution. One compartment contained phosphate buffer in which the drug was dissolved and the other contained 7.25×10^{-5} M HSA dissolved in the same buffer. The chambers were rotated in a water thermostat at 37° for 1.5 hr which was found to be sufficient for the equilibrium. Post dialysis concentrations of free drug were determined by UV spectrophotometry in the compartment without HSA. For the study of interaction between thiouracil derivatives and other substances, the data were corrected for the possible presence of warfarin or diazepam in the protein free compartment. In this way, after each dialysis the warfarin or diazepam concentration in the protein-free solution was determined by spectrophotometry at 310 nm, the wavelength at which the thioureylene compounds do not exhibit absorbance. Its absorbance at the wavelength of the thiouracil derivative absorbance (270 nm for PTU, 273 nm for thiouracil and BTU and 260 nm for propyluracil) was obtained by means of calibration plots and this absorbance was subtracted from the respective absorbance measured for the determination of free drug concentrations. Results were corrected for possible adsorption on cells and membrane by comparison with an experiment carried out without protein. Other sources of error, such as leakage of albumin through the membrane, albumin decay, and osmosis of water were negligible. In some experiments, drug-binding concentration was measured by gel filtration chromatography as has been previously described [10]. The Pharmacia K 16/40 glass column was packed with Sephadex gel G-25 fine grade. The minimum detectable concentration for the drug was 0.02 μM after injection of 1 ml of the sample. The results obtained by both methods, equilibrium dialysis and chromatography were in good agreement.

The results of the dialysis experiments were processed according to Scatchard's method [11]. Each point is a mean of three experiments. For PTU and BTU, the theoretical curves were calculated by means of the non-linear least-squares method using a Hewlett-Packard 91 computer. The binding parameters were estimated by means of the expressions derived by Klotz and Hunston [12] for the slopes and intercepts of tangents to the upward-curved Scatchard plot.

RESULTS

Ultraviolet difference spectra

Interaction of thiouracil with HSA leads to the formation of complexes that exhibit an absorption band with the maximum at 273 nm. This band is shifted about 3 nm towards the red region in comparison to the spectrum of free thouracil (λ_{max} = 270 nm). As a result of this the difference adsorption spectra appear which are characterized by a positive extreme at 313 nm, a negative extreme at 275 nm and an isosbestic point at 300 nm. Dissolving thiouracil in ethanol produced a similar difference spectrum

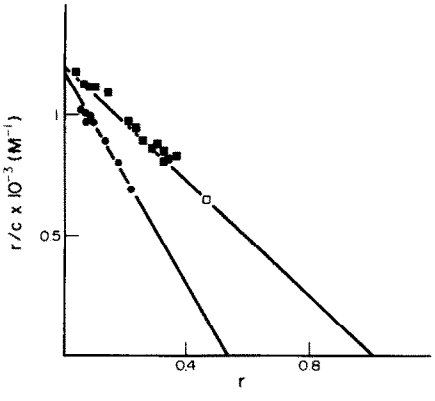

Fig. 1. Scatchard plots for equilibrium binding of thiouracil (●) and propyluracil (■) to HSA 7.25×10^{-5} M. r, Number of moles of ligand bound per mole of albumin; c, molar concentration of free ligand.

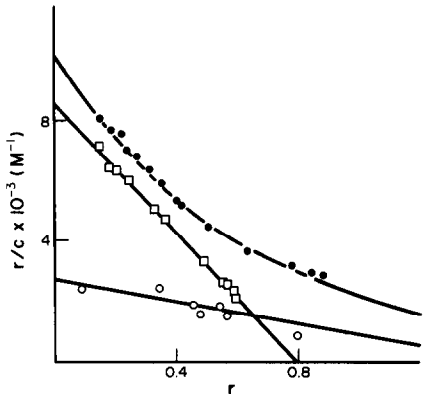

Fig. 2. Scatchard plots of the binding of PTU to HSA (●) and to HSA in presence of 1 mole of warfarin (○) or benzodiazepine (□) per mole of HSA. Protein concentration was 7.25×10^{-5} M. r, Number of moles of PTU bound per mole of albumin; c, molar concentration of free PTU.

(Table 1). The binding of PTU to HSA gave rise to the same shift of the PTU absorbance maximum as in the case of thiouracil. A small difference spectrum was generated with a maximum at 315 nm and a minimum at 275 nm. Dissolving PTU in ethanol produced a similar difference spectrum (Table 1). Using Bell and Dalziel's method [13], a binding constant of $8.4 \times 10^3\,M^{-1}$ was determined. Dissolving propyluracil or uracil in ethanol did not produce a difference spectrum.

The binding of BTU to HSA leads to a shifting of the BTU absorbance maximum to longer wavelengths. The difference absorption spectrum is characterized by a maximum at 325 nm and a minimum at 267 nm. Dissolving BTU in ethanol produces a greater difference spectrum (Table 1).

Characteristics of the binding to HSA

Determination of binding parameters. The binding constants and stoichiometry for binding of thiouracil, propyluracil, PTU and BTU to HSA were determined by equilibrium dialysis, over a range of drug concentrations (10^{-6}–5×10^{-4} M). The Scatchard plots were linear for propyluracil and thiouracil (Fig. 1) with an interception of about 1 and 0.5 for propyluracil and thiouracil, respectively, indicating the presence of a single binding site, with an association constant of $1.11 \pm 0.05 \times 10^3\,M^{-1}$ for propyluracil, and $2.15 \pm 0.10 \times 10^3\,M^{-1}$ for thiouracil. Scatchard plots of the data for PTU are presented in Fig. 2. Two successive saturable processes occurred, which are the association constants for each binding site of $K_1 = 8.92 \pm 0.89 \times 10^3\,M^{-1}$ and $K_2 = 1.08 \pm 0.05 \times 10^3\,M^{-1}$, estimated from the slopes of the Scatchard plot according to the method outlined under Materials and Methods. Thus, PTU was found to have an affinity about four times stronger for HSA than propyluracil, and than thiouracil.

As shown in Fig. 3 the two classes of binding sites in the HSA for BTU exhibit association constants several times greater than those for the PTU, which are $K_1 = 4.08 \pm 0.40 \times 10^4\,M^{-1}$ and $K_2 = 2.23 \pm 0.11 \times 10^3\,M^{-1}$ for BTU. These binding constants were calculated on the assumption that there were two independent binding sites, one moderate affinity site and three weaker equal sites. The association constant for thiouracil is about the same as K_2 for BTU. However, no binding of uracil to HSA could be detected.

Interactions between thioureylene compounds and other substances. The binding of thiouracil, propyluracil, PTU and BTU were studied at a HSA concentration of 7.25×10^{-5} M, in presence of warfarin (7.25×10^{-5} M) or diazepam (7.25×10^{-5} M). As shown in Table 2, the binding of propyluracil, PTU and BTU was significantly inhibited by warfarin. The presence of 1 mole of warfarin per mole of HSA increases the free fraction of PTU and BTU by 15.3 and 37.4%, respectively, the HSA concentration being 7.25×10^{-5} M. The warfarin displacement of these drugs at low drug-to-protein ratios indicates a competition for the moderate-affinity binding site, usually called the warfarin binding site [14, 15] or site I [16].

The diazepam showed no influence on propyluracil binding but inhibited the thiouracil binding and the PTU binding only for high concentrations of PTU. The presence of 1 mole of diazepam per mole of HSA has almost no influence on the moderate-affinity binding of PTU, although the binding to secondary binding sites might be decreased as can be seen from the shape of the Scatchard plot (Fig. 2).

DISCUSSION

The binding of thiouracil, PTU and BTU to HSA results in a UV difference spectrum very similar to the one obtained when the drugs are dissolved in ethanol. As ethanol has a lower dielectric constant and is less polar than water, it can be suggested that the spectrum results from the movement of the ligand molecule from an aqueous to a more hydrophobic region in the albumin molecule. Moreover the binding constant for the PTU obtained by the difference spectra was similar to that obtained by equilibrium dialysis for the primary binding site in HSA. Thus it

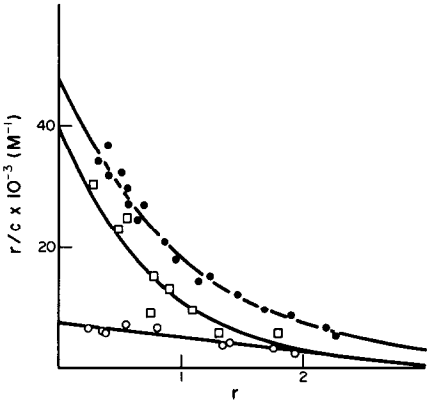

Fig. 3. Scatchard plots of the binding of BTU to HSA (●) and to HSA in the presence of 1 mole of warfarin (○) or benzodiazepine (□) per mole of HSA. Protein concentration was 7.25×10^{-5} M. r, Number of moles of BTU bound per mole of albumin; c, molar concentration of free BTU.

Table 2. Binding parameters of the interaction of thiouracil, propyluracil, PTU and BTU with HSA in the presence of 1 mole of warfarin or benzodiazepine per mole of HSA. The HSA concentration was 7.25×10^{-5} M

Ligand	Moderate-affinity n	Moderate-affinity K (M^{-1})	Low-affinity n	Low-affinity K (M^{-1})
Thiouracil alone	—	—	0.5	2150 ± 100
Thiouracil + warfarin	—	—	0.6	2050 ± 100
Thiouracil + benzodiazepine	—	—	—	—
Propyluracil alone	—	—	1.0	1115 ± 50
Propyluracil + warfarin	—	—	—	—
Propyluracil + benzodiazepine	—	—	1.3	850 ± 40
PTU alone	1.0	8925 ± 892	1.0	1085 ± 50
PTU + warfarin	—	—	1.4	1820 ± 90
PTU + benzodiazepine	0.8	10000 ± 1000	—	—
BTU alone	1.0	40800 ± 4080	3.0	2230 ± 110
BTU + warfarin	—	—	3.1	2330 ± 115
BTU + benzodiazepine	1.0	34730 ± 3473	2.0	2015 ± 100

follows that hydrophobicity is responsible for the primary binding of PTU to HSA.

The UV difference spectra resulting from the drugs–HSA interaction are in agreement with the association constants obtained by equilibrium dialysis for the drugs–HSA binding. Both the UV difference spectrum and the association constant for the BTU–HSA interaction are the highest of those studied. This agrees with the fact that apolar compounds show a stronger binding to HSA.

Since uracil does not bind to HSA, and the binding of thiouracil and propyluracil is very weak, the thiol and propyl groups are needed for the binding of PTU to HSA.

PTU binding to HSA follows two saturable processes. The binding constant for the primary binding site is moderate ($8.9 \times 10^3\ M^{-1}$) while that for the second site is eight times lower ($1.1 \times 10^3\ M^{-1}$). In the class of sites of moderate affinity, as discussed above, the interaction seems to take place close to the apolar group, but the sulfhydryl group is also necessary, as is shown by the fact that propyluracil presents a low association constant ($1.1 \times 10^3\ M^{-1}$).

The primary binding site for PTU binding is influenced by the occupation of the primary binding center for warfarin since the decreased PTU binding occurs at about PTU/warfarin molar ratio of 1. Therefore, this binding site is of an apolar nature and might be identical with the site for the azapropazone or for the warfarin, since the basic assumption is that this zone consists of the overlapping, high-affinity binding sites of azapropazone and warfarin. Similarly the secondary binding site for PTU binding might be identical with the primary binding site for the benzodiazepine, since large amounts of free PTU occur in the solution of diazepam–HSA complexes at molar ratios PTU/diazepam–HSA above 1.

Similarly, two binding sites exist for the binding of BTU to HSA, the primary with the binding constant value of $4.1 \times 10^4\ M^{-1}$, the secondary with the binding constant of $2.2 \times 10^3\ M^{-1}$. The nature of these centers is the same as that of the PTU binding centers. On the other hand, the association constant for thiouracil ($2.1 \times 10^3\ M^{-1}$) is of the order of K_2 for BTU, and no binding of uracil to HSA could be detected. These results seem to indicate that the sulfhydryl group in BTU is the principle contributor to the low-affinity binding sites on HSA. However the benzyl group helps the binding of BTU to more equal binding sites (N = 3).

Although it has been shown that binding constants of lesser than $1 \times 10^4\ M^{-1}$ have weak effect on drug distribution [17] the binding of compounds with sulfhydryl groups can displace drugs, as diazepam, with a binding constant greater than 1×10^4 from its binding sites.

We have demonstrated that all the ureylene compounds with an apolar radical which have been studied, share one primary binding site on HSA; that is, the azapropazone/warfarin site. This could indicate that the apolar groups (propyl, benzyl) are answerable for the interaction of these drugs with the warfarin binding site on HSA.

Since these ligands share one common binding site on HSA, we examined the relationships between the structures of these ligands and their binding parameters to HSA. What is most clearly shown in this study is the increase in the association constant with the increasing bulk of the apolar radical. Our results agree with the structural features defined by Sudlow *et al.* [16] or the drugs which bind to the azapropazone/warfarin binding site.

On the other hand, thiouracil, PTU and BTU are not displaced from the low-affinity binding sites by warfarin, but are displaced by diazepam, while ureylene compounds without a sulfhydryl group (propyluracil) are not displaced by diazepam. Consequently, the sulfhydryl group could be the main ligand group that contributes to the low-affinity binding to site II (benzodiazepine binding site) on HSA. This shows that the free cysteine (34) on HSA is present in the benzodiazepine binding site of HSA. In addition the apolar nature of the azapropazone/warfarin binding site and the sulfhydryl group interaction with the benzodiazepine binding site might be interesting for future ligand-binding studies.

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