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# CONFORMATIONAL CHANGES OF PEROXIDASE AND ALBUMIN IN SOLUTIONS OF PROPYLTHIOURACIL

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**Abstract**—Conformational changes of peroxidase and albumin in buffered solutions of propylthiouracil, an antithyroid drug, were evaluated by dilatometry and viscometry, showing that the structural alteration of peroxidase is related to the decoupling of the reactions which it catalyses. Thus, propylthiouracil probably inactivates the peroxidase by altering its structure. Equilibrium dialysis showed that albumin is the principle propylthiouracil-transporting protein in human serum. Propylthiouracil induces a conformational change in albumin when 1 mole of drug per mole of protein is bound, a structural alteration that can change the binding capability of other ligands.

In the last 30 years propylthiouracil (PTU†), which is a reducing agent, has been used as an antithyroid drug [1–6]. It is injected into the blood stream, where it can interact with several proteins, such as albumin, which transport it to the thyroid gland. There, the PTU inhibits the iodide organization by the decoupling of the reactions catalysed by thyroid iodoperoxidase, which is responsible for iodide oxidation and thyroid hormone synthesis. However, the mechanism by which the PTU decouples the reaction catalysed by the peroxidase is not clear. Mahoney and Igo [1] relate this decoupling to the peroxidase inhibition by PTU, and other authors have shown that this inhibition is accompanied by a change in the heme structure [7]. This change seems to be due to a reaction between the PTU and the oxidized heme group produced by interaction between the peroxidase and hydrogen peroxide [8].

On the other hand, this reducer can also change the activity of certain proteins [9], especially those that can be modulated by a thiol-disulphide exchange of some of their cysteine residues [10–12]. Among these proteins is human serum albumin (HSA), which contains 17 intra-chain disulphide bindings and a unique free cysteine [13–16]. This cysteine is related to the conformational changes in HSA [17, 18] and the latter have been considered important for the binding capability of a variety of ligands [17–19].

Therefore, the effect described of PTU on the protein conformation and activity could likewise cause inactivation of HRP. Our object is the determination of conformational alterations which occur in peroxidase as signs of peroxidase inhibition. These changes can be determined by the specific volumes ($\bar{V}_{3P}$) and viscosities ($\eta$ sp/c) of the protein in several PTU concentrations [20, 21], such as albumin in the HSA model, whose conformational changes will be studied too.

## MATERIALS AND METHODS

Horseradish peroxidase (Type I, essentially salt free powder), human serum albumin (from Fraction V, essentially fatty acid free), human globulins (predominantly $\alpha$-globulins) and 6-*n*-propyl-2-thiouracil were purchased from the Sigma Chemical Co. (St Louis, MO). The serum binding of 6-*n*-propyl-2-thiouracil was determined in a 1:4 dilution of a serum pool from normal subjects. It had the following characteristics: total proteins 17.5 g/L, $\alpha$-globulins 5.6 g/L; HSA 0.145 mM. $\alpha$-Globulins and HSA were estimated by electrophoretic analysis on 12% polyacrylamide gels. Proteins and drug were dissolved in 67.0 mM phosphate buffer pH 7.4 for all experiments.

Volume changes at 37° were measured by means of a dilatometer similar to that described by Komiyama *et al.* [22] of which respective volumes of the lower and upper compartments were 5.2 and 6.4 mL. The capillary tube was 0.3 ± 0.01 mm i.d. Solutions containing PTU in the lower compartment, and solutions containing a 0.077 or 0.155 mM solution of HSA in the upper compartment, were mixed by opening the internal stopper and stirring for 5 min. The capillary miniscus height was read 5–15 min after mixing. The volume change, in microliters, produced by mixing both compartments is referred to as $\Delta V$. Blank experiments were run in which the PTU solutions were mixed with phosphate buffer. The procedure used for analysing the data is practically identical to that of Abad *et al.* [23]. Values reported here are averages of two or three experiments and could be reproduced to ±0.07 $\mu$L, which leads to an error of 5 mL/$10^5$ g of protein.

An Ubbelohde suspend level microviscometer was used thermostatically maintained at 37°, together with a Viscoboy (from mgw-Lauda, Konigshofen, F.R.G.), to measure the viscosity. The results were transformed in specific viscosities ($\eta$ sp) taking into

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† Abbreviations: PTU, 6-*n*-propyl-2-thiouracil; HSA, human serum albumin.

account the observations of Reynolds *et al.* [24]. Densities were determined by a pyknometer. The pyknometer was of standard form with a 25-mL capacity bulb provided with a capillary neck (0.1 cm bore) and calibrated frequently against distilled water. The values obtained are the mean results of 10 experiments. Relative errors calculated for $\eta$ sp/c values are about 1%.

*In vitro* serum and protein binding of PTU 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. 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 absorbances at 273 nm in the compartment without protein. Results were corrected for possible adsorption on cells and membrane by comparison with an experiment carried out without protein.

Assay of peroxidase activity was carried out by the measurement of guaiacol peroxidation [25]. The assay medium contained 0.012 mmoles of guaiacol, 0.074 mmoles of phosphate buffer (pH 7.4), $5 \times 10^{-10}$ mmoles of enzyme and various concentrations of propylthiouracil in a final volume of 0.95 mL.

## RESULTS

The evaluation of the partial molar volume of protein gives an idea of the macromolecular volume for the different PTU concentrations. This parameter can be determined from the respective volume changes.

Volume changes produced by addition of PTU to proteins are the sum of two major processes: PTU–protein and PTU–buffer interactions. The volume effects due to PTU–protein interaction are defined as follows:

$$\delta V = \frac{\Delta V_3 - \Delta V_2 - \Delta V_1}{W_3}$$

where $\Delta V_3$ is the volume change produced when a protein solution of defined volume and concentration is mixed with an equally defined PTU solution; $\Delta V_2$ is the volume change determined for PTU-buffer interactions; $\Delta V_1$ is the volume change determined for the protein–buffer interaction; $W_3$ is the protein weight in grams. From the $\delta V$, the partial specific volumes of the protein solutions, $\bar{V}_{3P}$, can be calculated [26] using the expression:

$$\bar{V}_{3P} = \bar{V}_3 + \delta V$$

where $\bar{V}_3$ is the partial specific volume of the protein in the buffer. $\bar{V}_3$ is obtained from the ordinate intercepts to the plot of $\delta V$ expressed in mL/g against the inverse of protein concentrations.

Volume effects for peroxidase–PTU interactions at protein concentration 0.050 mM are shown in Fig. 1. The $\bar{V}_3$ determined by peroxidase was 0.880 mL/g. Volume effects are always positive and are a function of the PTU concentration. It can be observed that $\bar{V}_{3P}$ increases with increasing PTU concentration until a maximum is attained at about 0.15 mM PTU. For higher PTU concentrations $\bar{V}_{3P}$ decreases with the increasing PTU concentration. Since the dilatometric isotherms provide information about conformational changes of protein in solution [20, 21], the changes in the sign of the slope of the $\bar{V}_{3P}$ isotherm at greater concentrations of 0.15 mM PTU signify that peroxidase undergoes a structural change. Table 1 shows an insignificant variation in the PTU binding on peroxidase, where PTU binds to 19–23% of peroxidase molecules. Therefore, the conformational change in peroxidase is not directly related to its binding to PTU. The dilatometric values agree well with the results obtained by viscometry (Fig. 1). As in the case of $\bar{V}_{3P}$, $\eta$ sp/c increases with increasing PTU concentration until about 0.15 mM, due to the peroxidase binding of PTU. That both parameters decrease with higher PTU concentrations signifies that peroxidase undergoes a structural change at PTU/protein ratios higher than 3. These results are in concordance with the fact that the peroxidase is inactivated only with 3 or more PTU moles per mole of enzyme (Fig. 1). Thus, the PTU as well as changing the heme structure [7] changes the protein conformation, probably by reduction of disulphide binding on the HRP.

Since HSA is a well characterized protein with disulphide binding, which can interact with PTU, the HSA–PTU interaction was studied in order to see the possible effect of PTU on the HSA-conformation, in comparison with the effect on the HRP-conformation. The binding of PTU to 0.145 mM HSA followed a saturable process [27] and was much greater than the binding to globulins (Table 1). These results clearly show the much greater affinity of HSA for PTU and therefore the fact that HSA is the main protein-transporter of PTU in serum. In addition the PTU effect on the HSA-conformation could be an indication of the pharmacokinetic and therapeutic effect of this drug.

Volume effects determined at 0.072 mM HSA concentration are shown in Fig. 2. It can be seen that for HSA–PTU there is a steady increase in $\bar{V}_{3P}$ up to 0.20 mM, which is in agreement with the high percentages of PTU-binding to HSA (Table 1). However, for greater PTU concentrations, $\bar{V}_{3P}$ decreases and reaches a minimum at about 0.30 mM. This change in the $\bar{V}_{3P}$ isotherm between 0.20 and 0.30 mM PTU (PTU/protein ratios between 2.8 to 4.2) signifies that these concentrations of PTU cause a structural alteration in HSA. Table 1 shows that at a PTU/HSA ratio of 2.8, the HSA is saturated with one PTU molecule. Therefore, the maximum conformational change in HSA occurs at a stoichiometric ratio of 1 mole of PTU per mole of HSA.

Dilatometric results were compared with those obtained by the viscometric technique (Fig. 2). Intrinsic viscosities of macromolecules ($\eta$) are proportional to their corresponding hydrodynamic volumes, therefore, an evaluation of their viscosity gives a qualitative idea of their macromolecular volume for different PTU concentrations such as have been used in this study. From a qualitative point of view, ($\eta$ sp/c) is practically coincident with the variation of $\bar{V}_{3P}$ from the dilatometric measurement. Both

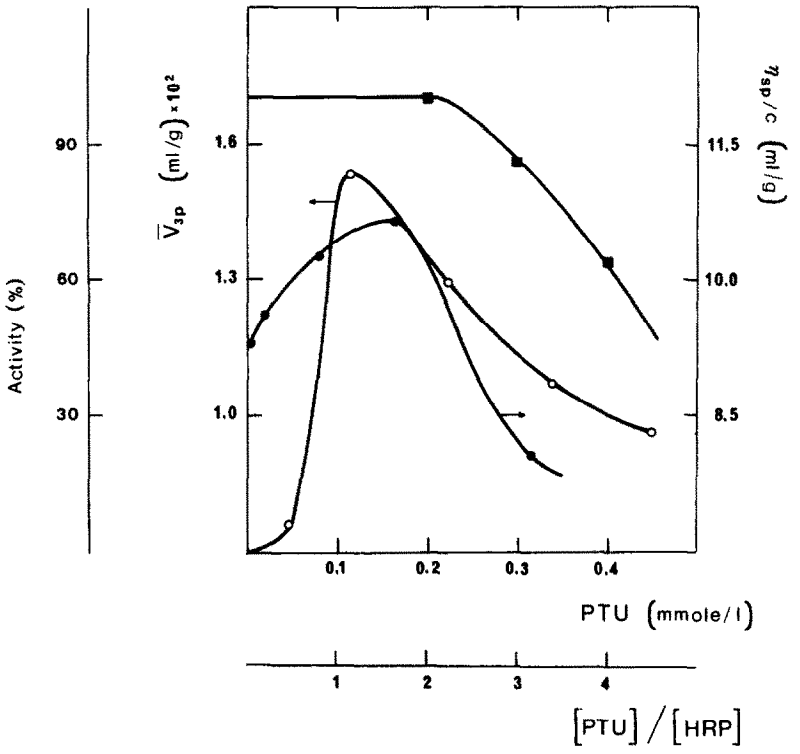


Fig. 1. Plot of $\bar{V}_{3P}$, $\eta$ sp/c and activity against PTU concentrations by the peroxidase–PTU system. (○), $\bar{V}_{3P}$ is the partial specific volume of peroxidase obtained by dilatometry; (●), $\eta$ sp/c, (■) peroxidase activity in the assay conditions described in Materials and Methods. Protein concentration is 0.050 mM.

Table 1. Binding of PTU to peroxidase (0.050 mM), HSA (0.145 mM) and to globulins (5.6 g/L)

| [PTU] free (mM) | [PTU] binding (mM) | | |
|---|---|---|---|
| | Peroxidase | HSA | Globulins |
| 0.04 | 0.0111 | 0.0380 | 0.0061 |
| 0.05 | 0.0112 | 0.0459 | 0.0081 |
| 0.10 | 0.0115 | 0.0720 | 0.0120 |
| 0.15 | 0.0110 | 0.0845 | 0.0202 |
| 0.20 | 0.0103 | 0.0955 | 0.0271 |
| 0.25 | 0.0095 | 0.1005 | 0.0272 |

methods show that 0.20 mM and greater PTU concentrations originate conformational changes in HSA.

The structural alteration described of the HSA coincides with a drastic decrease in the digitoxin binding to HSA (digitoxin/HSA ratio of 1), from 100 to 15% by a PTU/HSA ratio of 3. The warfarin binding and the benzodiazepine binding on HSA also decreases by a PTU/HSA ratio of 3, but meanwhile benzodiazepine binding decreases progressively with increasing concentrations of PTU (Fig. 2), PTU seems to have no effect on warfarin binding, causing only a 13% decrease in the warfarin binding.

## DISCUSSION

Intrinsic viscosities of proteins are proportional to the corresponding hydrodynamic volumes, and volume changes can be used to determine the partial molar volume of protein [26–28]. Consequently an evaluation of them will give a qualitative idea of the macromolecular volume for the different PTU concentrations.

The reaction of PTU with the proteins studied always produces positive volume effects (Figs 1 and 2). The slope and sign of the $\bar{V}_{3P}$ and $\eta$ sp/c isotherms change as a function of PTU concentration and the most pronounced effects are produced in the range 0.10 to 0.30 mM.

The protein contribution in the volume change is basically determined by both the release of water from the binding site upon complex formation and the structural changes of the protein. In principle the volume rise can be explained in terms of PTU bound to the protein with displacement of hydration water initially located around the ionic groups [26, 29]. Meanwhile the change of sign in the slope of the $\bar{V}_{3P}$ and $\eta$ sp/c isotherms could be due to a structural alteration of the protein [26, 30].

For the PTU–peroxidase systems, both $\bar{V}_{3P}$ and $\eta$ sp/c parameters decrease with higher PTU concentrations than 0-15 mM. This means that peroxidase undergoes a structural change at PTU/protein ratios higher than 3. On the other hand, since PTU does not directly inhibit peroxidase at low concentrations [2]—inactivating it only with 3 or more PTU moles per mole of peroxidase (Fig. 1)—the structural alteration observed in the peroxidase can be related to the decoupling of reactions catalysed by this enzyme in the presence of PTU. Thus,

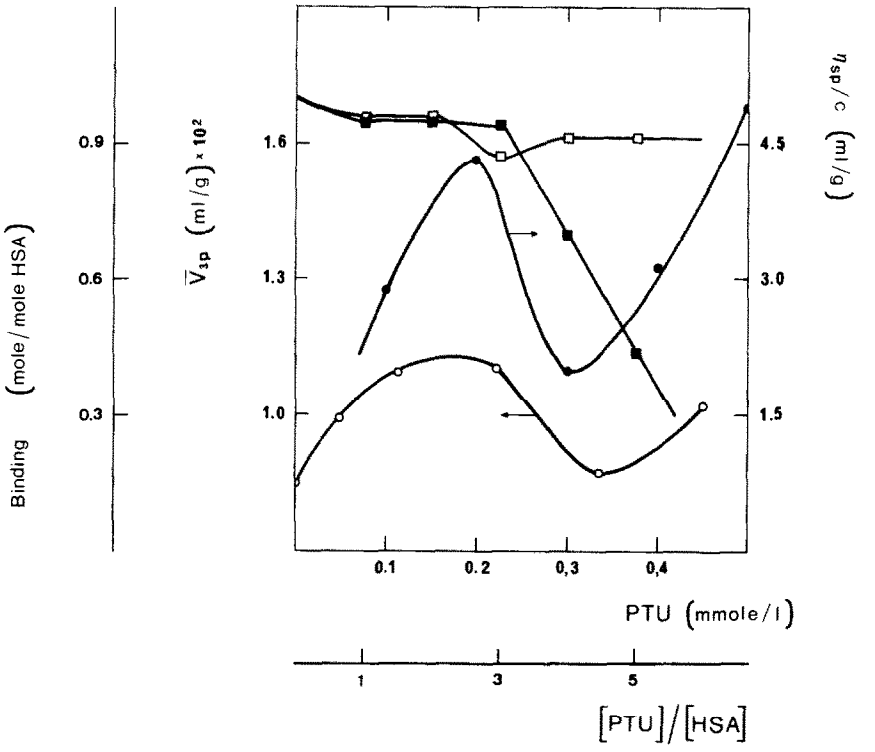


Fig. 2. Plot of $\bar{V}_{3P}$, $\eta$ sp/c and warfarin and benzodiazepine–HSA binding against PTU concentration by the HSA–PTU system. (○), $\bar{V}_{3P}$ is the partial specific volume of HSA; (●), $\eta$ sp/c; (□), rate of warfarin binding to HSA, the initial concentration of warfarin being 1 mole/mole of HSA, (■), rate of benzodiazepine binding to HSA, the initial concentration of benzodiazepine being 1 mole/mole of HSA. Protein concentration is 0.072 mM.

the antithyroid drug PTU inactivates the peroxidase not only by a change in the heme structure as is described by other authors [7, 8] but by means of a structural alteration on the protein.

The low binding of PTU to globulins in relation to the binding to HSA shows that this latter protein is the main protein-transporter of PTU in serum. So the HSA–PTU interaction was studied in order to see the possible effect of PTU on the HSA conformation and on the consequent binding capability of this protein.

The $\bar{V}_{3P}$ and $\eta$ sp/c isotherms for the HSA–PTU system do not change below a PTU/HSA ratio of 2.8. At this PTU/HSA ratio the protein is saturated with one PTU molecule (Table 1) which results in loss of the binding capacity of HSA (Fig. 2). The loss in the warfarin binding capacity is due to the PTU competition by the primary binding center for warfarin on HSA [27]. However, the loss in the binding capacity for the other binding centers on HSA can be explained by a conformational change on the protein. Thus, the PTU induces a conformational change in HSA at PTU/HSA ratio of 2.8 when 1 mole of PTU is bound to 1 mole of HSA.

Therefore, in therapy for hyperthyroidism, PTU/HSA ratios of less than 2.8 should be used, in order to avoid conformational changes in HSA which can change the capability of binding of a variety of ligands.

However, HSA can transport PTU without undergoing a structural alteration at PTU concentrations which disturb the peroxidase conformation (0.15–0.20 mM PTU per 0.050 mM peroxidase).

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