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COMMUNICATIONS

Thermodynamics of the binding of salicylate to human serum albumin: evidence of non-competition with imidazole

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Abstract—The thermodynamic characteristics of the binding of salicylate to human serum albumin have been studied using a technique based on the variation of the quantum yield of fluorescence of salicylate when it binds to the protein. The binding constants, number of sites and the values of ΔG°, ΔH° and ΔS° were determined. The results are consistent with a model that proposes two equal and independent types of binding site with a predominantly ionic interaction and an important hydrophobic contribution in one of the sites. The technique was also used to demonstrate that imidazole and salicylate (that can be found simultaneously in plasma following administration of imidazole-2-hydroxybenzoate) do not compete for the same binding sites on the protein.

Analysis of numerous experimental data published on the binding of salicylate to human serum albumin (HSA) shows that the conclusions drawn concerning the thermodynamic characteristics of this kind of binding are scanty and contradictory. Behm et al (1981) and Zarolinski et al (1974) disagree even about the linearity of the Van't Hoff plot (ln K versus 1/T) when studying the variation in the binding constants with temperature. The former authors throw doubt on the possibility of obtaining thermodynamic parameters other than ΔG° from the binding constants. In the work just mentioned, the techniques of equilibrium dialysis and Sephadex gel filtration were used to study the process.

In the present work we developed a spectrofluorimetric technique which, together with its known advantages of speed and simplicity, affords better precision and, in particular, does not require devices such as filters, membranes etc., which may alter the equilibrium characteristics. The technique, based on the fact that the salicylate quantum yield of fluorescence increases when the drug binds to HSA, and allows the possible displacements of salicylate bound to HSA by other ligands to be examined. In this work we show that imidazole does not compete with salicylate for the same binding sites in HSA, a matter of great interest because both substances may be present simultaneously in plasma after the administration of drugs containing imidazole-2-hydroxybenzoate.

Materials and methods

Chemicals. Human serum albumin (essentially fatty acid-free) was obtained from Behring as lyophilized powder. Salicylic acid (Fluka) and imidazole (Merck) were used without further purification. All solutions were prepared in 0·01 M phosphate buffer (Merck) at pH 7·4. The water used to prepare the solutions was purified by a Milli Q (Millipore) system. Chemicals in the buffer solution were of reagent grade.

Apparatus. Fluorescence measurements were made on a Shimadzu RF-540 spectrofluorimeter coupled to a SELECTA 389/0·1°C thermostat and a HETOFRIG CB4 cryostat. This permitted a precision in the control of temperature of $\pm 0{\cdot}1$°C. Titrations were performed with a Gilson 20 μL micropipette.

Methods. When the quantum yield of fluorescence of a ligand is modified significantly on binding to a macromolecule, this modification can be used to study the binding process quantitatively. Salicylate is a substance with an elevated degree of fluorescence in aqueous solution and a quantum yield of 0·28 at room temperature (ca 20°C) (Ceballos et al 1979). The emission spectra of salicylate in the absence and presence of HSA did not reveal any modifications in the shape of the band nor in the position of the maximum, although in the second case a notable increase was observed in the intensity of emission. Fig. 1 shows the variation in the fluorescence intensity of salicylate with the HSA/salicylate molar ratio. Measurements were carried out at 30°C, exciting the samples at 320 nm, with an excitation slit of 5 nm and recording emission at 410 nm with a slit of 10 nm. Under these conditions, the interference of the fluorescence of HSA is negligible. The concentration of salicylate was 5·10–6 M. The quantum yield of fluorescence of salicylate may be seen to be increased by approximately two-fold when the drug binds to the protein. This variation allows one to determine the fraction of the ligand bound to the HSA.

The procedure employed was as follows: Two solutions of HSA were prepared, one of 'high concentration', $8{\cdot}1 \times 10^{-5}$ M and another of 'low concentration', $8{\cdot}1 \times 10^{-6}$ M. Both solutions were prepared directly by weighing and dissolution assuming a molecular mass for HSA of 69 000 g mol^{-1}. A solution of $5{\cdot}0 \times 10^{-4}$ M salicylate was also prepared. Three fluorescence cuvettes were employed: the first contained 3 mL of phosphate buffer; the second 1·5 mL of buffer and 1·5 mL of the 'high concentration' HSA solution, and the third contained 1·5 mL of buffer plus 1·5 mL of the 'low concentration' HSA solution. To

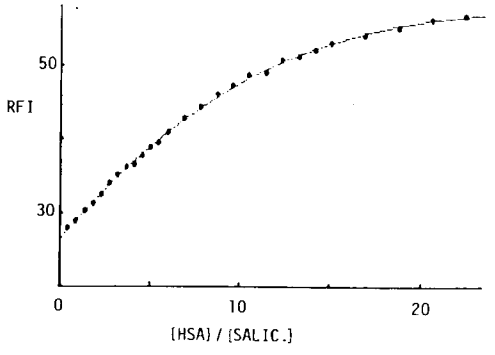

FIG. 1. Variation in the relative fluorescence intensity (RFI) with molar ratio HSA/Salicylate. Excitation wavelength, 320 nm; emission wavelength, 410 nm. The concentration of salicylate is $5{\cdot}0 \times 10^{-6}$ M.

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Table 1. Thermodynamic binding parameters of salicylate to HSA.

T/K	$K_1/10^6$	$\Delta G_1^\circ/10^4$ Jmol^{-1}	$K_2/10^4$	$\Delta G_2^\circ/10^4$ Jmol^{-1}
293·2±0·1	3·30±0·2	−3·65±0·02	3·1±0·3	−2·57±0·05
208·2±0·1	1·90±0·08	−3·59±0·02	2·7±0·2	−2·52±0·04
303·2±0·1	0·79±0·04	−3·43±0·02	2·6±0·2	−2·54±0·04
308·2±0·1	0·42±0·02	−3·33±0·02	2·8±0·2	−2·61±0·04
310·2±0·1	0·37±0·02	−3·30±0·02	1·5±0·1	−2·45±0·03
313·2±0·1	0·34±0·02	−3·31±0·02	1·2±0·1	−2·44±0·03
318·2±0·1	0·13±0·01	−3·10±0·02	0·94±0·04	−2·42±0·03

each of these successive amounts of 10 μL of the salicylate solution were added, in each case measuring the fluorescence intensity at 15 min after each addition. Previously, it was ascertained that this time was sufficient for the fluorescence intensity to stabilize; i.e. for binding equilibrium to be established. The fluorescence intensities of the three cuvettes, measured at the above-indicated wavelengths and slit widths were designated as I1, I2 and I3, respectively. The concentration of the salicylate was between $1·7 \times 10^{-6}$ M and $3·0 \times 10^{-5}$ M. In each case, the absorbance of the salicylate was so small that the 'inner filter' effect was negligible. The concentration of the 'high concentration' HSA solution was so high that the salicylate, under the conditions in which measurements were made, was completely bound to the protein; this was apparent on observing good linearity between the fluorescence intensity and the concentration of salicylate. Measurements were taken at seven temperatures between 20·0±0·1 and 45·0±1°C (see Table 1) and in all cases at least three replicate determinations were made.

Calculation of the fraction of ligand bound to the protein, X, was performed according to the following equation (Laurence 1952).

$$X = \frac{I2 - I3}{I2 - I1}$$

From the value of X, concentration of free ligand in each case, c, and the degree of occupancy, r, (moles of ligand bound mol^{-1} of protein) could be calculated.

Results and discussion

The non-linearity of the Scatchard plots (r/c versus r) shows that there is not a single type of binding site. The results were therefore fitted by a non-linear regression calculation program based on the 'Simplex' algorithm using an IBM-XT personal computer to the mathematical equation corresponding to a model that considers two independent types of binding site:

$$r = \frac{n1\ K1\ c}{1 + K1\ c} + \frac{n2\ K2\ c}{1 + K2\ c}$$

where n1 and n2 are the numbers of sites in each type, K1 and K2 are the corresponding binding constants and c is the concentration of free salicylate. The values leading to the best fit of the experimental results proved to be n1 = 1 and n2 = 2. Fig. 2 shows the results of r against c obtained together with the theoretical curves calculated with the binding constants K1 and K2 obtained in the fittings. These values are shown in Table 1 together with their standard deviations. The results have been plotted for five temperatures ranging between 20 and 45°C. The values of ΔG° were calculated from the equation:

$$\Delta G^\circ = -RT \ln K$$

Fig. 3 shows the Van't Hoff plots corresponding to the two types of binding sites:

$$\ln K = -\frac{\Delta H^\circ}{RT} + C$$

It is possible to observe good linearity in both cases, with correlation coefficients of 0·990 for the first site and 0·920 for the second. From the slopes of the straight lines we obtained the binding enthalpies; for the two kinds of sites these proved to be: $\Delta H_1^\circ = -90·3 \pm 0·2$ kJ mol^{-1} and $\Delta H_2^\circ = -38·5 \pm 0·1$ kJ mol^{-1}. Binding entropies are $\Delta S_1^\circ = -184 \pm 1$ J/K mol and $\Delta S_2^\circ = -44 \pm 2$ J/K mol, which are calculated from these enthalpies and the values of ΔG°.

The linearity of the Van't Hoff plots seems to show that the binding sites of salicylate to HSA are not changed conformationally within this temperature range. The values of the binding constants, which are relatively high, and the negative signs of the enthalpies and entropies lead one to assume that the interaction between both substances is mainly of the ionic kind (Nemthy & Scheraga 1962). However, a significant difference can be seen between the thermodynamic constants of the two kinds of sites.

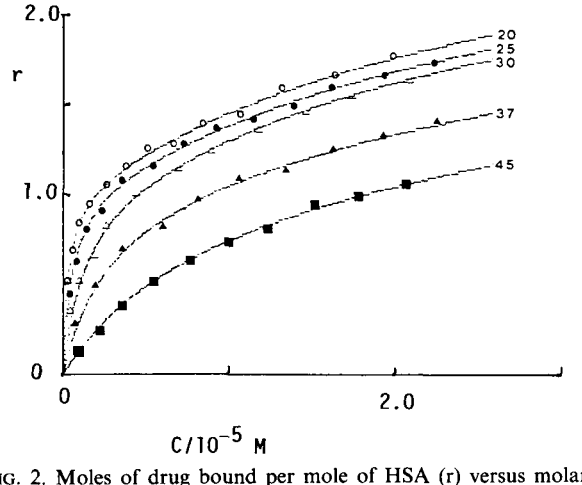

FIG. 2. Moles of drug bound per mole of HSA (r) versus molar concentration of free drug at different temperatures (°C).

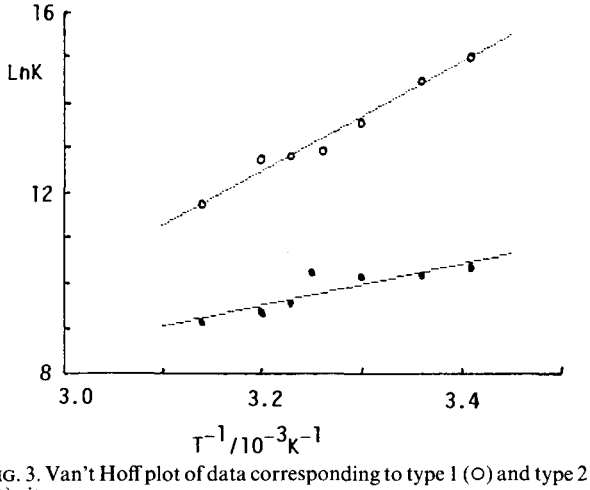

FIG. 3. Van't Hoff plot of data corresponding to type 1 (○) and type 2 (●) sites.

In the second, the binding constant is two orders of magnitude smaller; the process is much less exothermic and the entropy is much less negative. This suggests that, although an ionic interaction is maintained, in the second type of binding site there is also an important hydrophobic contribution which is much greater than in the first type of site.

In view of the variation in the quantum yield of fluorescence of salicylate upon binding to HSA, the presence of another substance that might compete with the salicylate for the same binding sites on the protein should give rise to a decrease in the intensity of emission. Measurements of the fluorescence of salicylate ($5{\cdot}0 \times 10^{-6}$ M) in the presence of HSA ($5{\cdot}0 \times 10^{-5}$ M) were performed with different concentrations of imidazole corresponding to molar imidazole/salicylate ratios of 5, 2, 1 and 0·5. In no case were any differences detectable in the intensity of fluorescence on comparing solutions with the same concentration of salicylate in the absence and presence of imidazole. It should be noted that there is no interference due to absorbance of imidazole at an excitation wavelength of 310 nm. This finding is in agreement with the observation, found using ultrafiltration measurements, that imidazole does not compete with salicylate for the same binding sites on HSA (Rodrigo et al 1988).

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Enhancement of phenytoin binding to tissues in rats by heat treatment

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Abstract—Phenytoin binding to heat-treated tissue homogenates has been examined to characterize the phenytoin binding to tissues. The binding to the heat-treated tissue homogenates was enhanced in all tissues studied compared with controls. The heating might produce the changes in conformation of proteins in tissues and then enhance phenytoin binding to tissue homogenates.

Phenytoin is a widely used anticonvulsant agent in clinical drug therapies and we succeeded in estimating in-vivo tissue-to-blood partition coefficients of phenytoin in variously aged rats from in-vitro binding data using serum and tissue homogenates (Kato et al 1987). However, even in brain which is a target organ for phenytoin, the factors governing the tissue distribution of phenytoin are not clarified and conflicting data have been reported regarding the binding characteristics of phenytoin to brain membranes. Burnham et al (1981) reported that phenytoin binds in a saturable and reversible manner to at least two sites in brain membrane fractions and after heat pretreatment, binding was greatly enhanced, but the saturable binding could no longer be seen. These data agree well with the results of Shah et al (1981) who pointed out, however, that heat treatment enhanced the specific binding of phenytoin to brain membranes. On the other hand, Geary et al (1987) and Goldberg & Todoroff (1976) reported that there are no specific binding sites for phenytoin in brain. Furthermore, Geary et al (1987) pointed out that the treatment of brain section with both formalin and heat enhanced the nonspecific binding compared with the control.

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It is well recognized that drug binding to blood or tissue constituents greatly affects the disposition of the drug. Recently, drug binding to the tissue constituents has stressed as an important pharmacokinetic determinant (Terasaki et al 1984; Harashima et al 1984). Whether this phenomenon found in heat-treated brain is specific to brain, led us to make a preliminary experiment to characterize the phenytoin binding to tissues, so phenytoin binding to heat-treated tissue homogenates was examined.

Materials and methods

Adult male Wistar rats (Shizuoka Laboratory Animal Center, Japan), 270–300 g, were cannulated in the portal vein under ether anesthesia. After washing blood from the lumbar vein by pumping ice-cold saline (0·9%) into the cannulae to exsanguinate tissues, the tissues (brain, lung, liver, kidneys and muscle) were excised, blotted dry and frozen at −20°C until study. 10% w/v tissue homogenates were prepared in 0·01 M phosphate buffer containing 0·15 M KCl (pH 7·0) on ice. Part of the tissue homogenates were bubbled mildly with nitrogen gas to avoid denaturation (oxidation) in phospholipids during heating and then the vessels were sealed with Saran Wrap (Asahi Kasei Kogyo Co., Ltd, Japan). Heat-treated tissue homogenates were prepared by placing them in boiling water for 10 min. [^{14}C]phenytoin (New England Nuclear, USA) was diluted with non-radioactive compound (Aldrich Chemical Company, USA) and spiked into the homogenates at the initial concentration of 10 μg mL^{-1} (20·4 μCi L^{-1}). Phenytoin binding to tissue homogenates was determined by equilibrium dialysis as described previously