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DRUG BINDING IN HUMAN SERUM ALBUMIN AS ASSAYED BY DIAFILTRATION AND FLUORIMETRY

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Abstract—The binding of salicylate has been investigated by equilibrium ultrafiltration at various concentrations of human serum albumin up to 50 mg/ml. Very pronounced differences in the binding of the ligand at the different protein concentrations were observed, notably the amount bound per protein molecule decreased at constant levels of free (not total) salicylate, as the protein concentration was increased. A detailed investigation of the effect of the binding of the first molecules of salicylate to the protein by fluorescence indicated that the initial binding of salicylate was very tight indeed and induced a minor conformational change in the albumin allowing further molecules to be bound. This initial binding was also dependent on the protein concentration.

It is normal procedure when analysing protein–ligand binding to utilise low concentrations of protein thereby minimising non-ideality effects in the protein solution [1]. However the results of such studies are often extrapolated, at least by imputation, to the so-called "physiological" situation of high protein concentrations. Nevertheless a number of studies have shown that binding of ligand can vary with protein concentration. For example the binding of progesterone and cortisol to various proteins varied with protein concentration [2]. Further Lester *et al.* [3] showed that the binding of salicylic acid to human plasma varied markedly with the protein concentration of the plasma while Hvidberg *et al.* [4] noted a "discrepancy" between the association constants for the binding for indomethacin to human serum albumin (HSA) at physiological concentrations (50 mg/ml) and at 10 mg/ml.

It was therefore relevant to examine the binding of ligand to protein over a range of protein concentrations. Serum albumin was selected because it is easily available in a highly purified state and most previous studies have used dilute solutions of the protein. It was decided to utilise salicylic acid as the ligand because its binding to serum albumin has been intensively investigated and this drug is the most used clinically, at least in New Zealand, of the common anti-inflammatory drugs.

A "normal" level for the albumin content of blood was taken as 40–50 mg/ml [5]. Approximate levels of total salicylate in plasma with clinical significance are 0.05–0.1 mg/ml for analgesia, 0.2–0.4 mg/ml for treatment of rheumatic fever and above 0.5 mg/ml symptoms of poisoning may be obvious [6]. Levels of free (i.e. ultrafiltrable) salicylate in human plasma may vary by up to approximately 0.3 mg/ml [7].

MATERIALS AND METHODS

HSA crystallised and lyophilised (batch Nos. 34C-8121 and 65C-8320) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. and was used as supplied. Salicylic acid, analar grade (B.D.H. Ltd., Poole, U.K.) was dried at 110° and stored desiccated, until use. [^{14}C]Salicylic acid (61 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, U.K., and dissolved in glass distilled water to an approximate activity of 25 μCi/ml before use. Solutions were buffered with 0.01 M phosphate adjusted to an ionic strength of 0.05 with sodium chloride [8].

Albumin solutions were prepared by weight and the concentrations were checked by absorbance assuming $E^{1\%}_{1\,cm} = 5.8$ [9].

Ligand–protein binding was measured directly by equilibrium diafiltration using the Amicon Micron-Ultrafiltration System Model 8MC and Amicon Diaflo ultrafiltration membranes type PM10. (Amicon Corp., Lexington, MA U.S.A.). Typically 5 ml of buffered HSA (1–5 g %) was subjected to a gradual "wash-in" of 50 of salicylate (5–100 mg %) with 50–100 μl of added radioactive salicylate. The fractions (approx. 30) were collected by fraction collector in pre-weighed tubes and their exact volume determined by weighing. Radioactivity was measured by the technique described by Graham and Neuberger [10].

Fluorescence measurements were made with an Amino–Bowman Spectrophotofluorometer (Model J-4-8911AE) fitted with the Ellipsoidal Condensing System and operated in the Ratio mode. Arc wander was minimised by positioning a small electric motor close to the lamp-housing. Fluorescence measurements were made up to a concentration of 10 mg/ml for HSA since above this concentration significant concentration quenching occurred.

Typically 2.00 ml of HSA solution were placed in a 1 cm square quartz cell in the fluorimeter and salicylate solution added in 0.01 ml aliquots. Fluorescence was measured after mixing. Duplicate determinations of all binding profiles were made and the machine setting was adjusted throughout each experiment against an HSA standard. Corrections of the observed relative fluorescence were made for attenuation of the excitation light by absorption by free salicylate, and for the slight contribution of salicylate fluorescence at the emission wavelengths. It should be noted that both the maximum excitation (282–289 nm) and emission (348–354 nm) wavelengths increased as the protein concentration was increased (1–10 mg/ml).

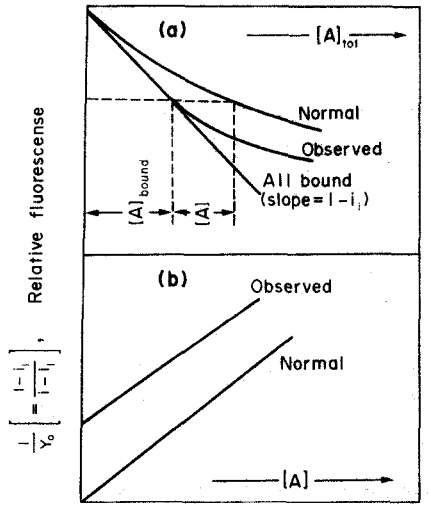

Fig. 1. Interpretation of fluorescence results (See Reference 11). In diagrams (a) the initial slope equals $1-i_1$ (see text) which is required for the construction of diagram (b). "Normal" binding refers to that reported previously for a different ligand-binding system [11]. The interpretation of the observed binding where $1/Y_0 \neq 0$ at $[A]=0$ is described in Results and Discussion.

Results of the fluorescence studies were calculated by the method of Steiner *et al.* [11]—see Fig. 1. The quantity i_1 is obtained as the limiting or initial slope of the relative fluoresence decrease versus amount of ligand added. This slope indicates the decrease in fluorescence to be expected if *all* the ligand molecules added were bound to the protein (Fig. 1a). By careful analysis of experiments results plotted as in Fig. 1a it is possible to calculate both $[A]$, the concentration of free ligand and the quantity

$$^1/Y_0 \left(= \frac{1-i_1}{i-i_1}\right)$$

(where i is the experimentally observed relative fluorescence at any particular point in Fig. 1a). The apparant binding constant (K) is obtained as the initial slope of the plot of $^1/Y_0$ versus $[A]$ (Fig. 1b) [11].

All albumin solutions were in phosphate buffer of pH 7.5 and ionic strength of 0.05.

RESULTS AND DISCUSSION

The effect of protein concentration upon binding of salicylate may be seen in Fig. 2. It is clear that there is no major variation in binding in the range 10–30 mg/ml HSA. (However a fine analysis of the initial binding of salicylate does indicate significant changes even below this range—Fig. 4). Above 30 mg/ml (i.e. in the "normal" plasma range) the binding is appreciably altered—particularily that involving the lower affinity sites. Overall, the total binding of salicylate falls off markedly with increasing protein concentration. For example, at a concentration of free salicylate of 0.5 mg/ml (3.62 molar) there is a 30 per cent reduction in the amount per molecule. Thus for any particular free

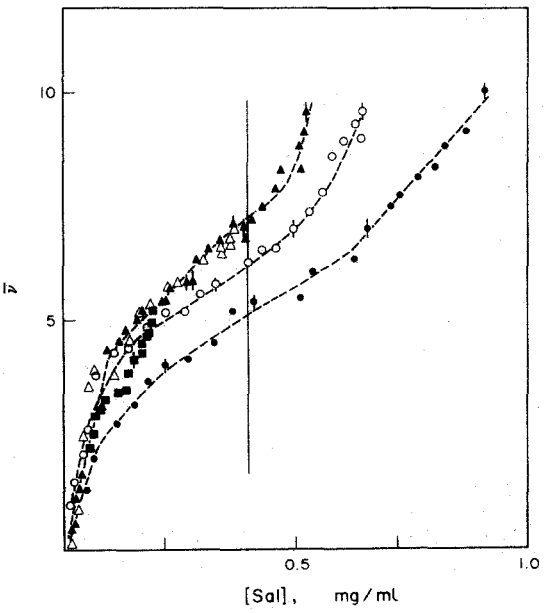

Fig. 2. The binding of salicylate at different concentrations of HSA as assayed by diafiltration. Protein concentrations were 10 (■), 20 (△), 30 (▲), 40 (○) or 50 (●) mg/ml at pH 7.5 and ionic strength 0.05. $\bar{\nu}$ is the measured average number of salicylate molecules bound per HSA molecule. Typical error bars are indicated for selected points. The solid vertical line represents an approximate upper level for the range in which clinical effects are observable [7].

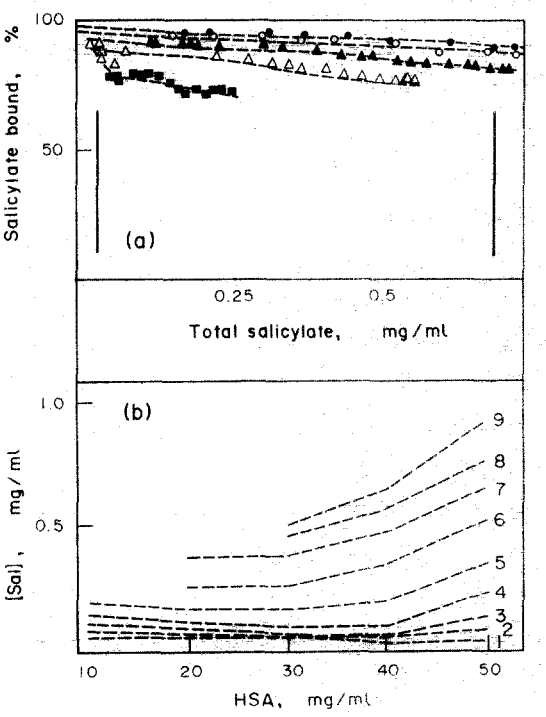

Fig. 3. The binding of salicylate as affected by HSA concentration (symbols as in Fig. 2). The vertical lines in (a) represent range of normal clinical interest [6]. The effect of HSA concentration at fixed binding levels on the concentration of free salicylate ([SAL]) is shown in (b). Numbers in the diagram are values of $\bar{\nu}$ – the average number of salicylate molecules bound per HSA molecule. (Data calculated from Fig. 2 by interpolation.)

salicylate concentration, appreciably less is bound to the HSA than would be predicted from experiments utilising low protein concentrations. However, when the same data are plotted in terms of total salicylate bound versus total salicylate (Fig. 3a) then it is clear that with respect to the *total* salicylate available, more is bound by a high protein concentration than by a low one. This merely reflects the rapid saturation of the available tightly-binding sites at the lower concentrations of albumin. Figure 3b complements Fig. 2 in that it is obvious from the figure that generally a greater concentration of free salicylate is required at higher albumin levels to cause the same level of ligand–protein binding. The explanation for this phenomenon clearly lies in the non-ideality of solution behaviour at these higher concentrations. Recent reports of the amount of water bound by biological macromolecules suggest that lg H_2O/g macromolecule is a reasonable level [12–14]. If so, 50 mg/ml of albumin would bind 5 per cent of its total water. Binding of this level would be relected in changed interactions of the protein molecules with one another. In particular w, the electrostatic interaction factor, is very sensitive to alterations in the "atmosphere" surrounding molecules and thus there would be significant changes in the apparent association contants, and therefore in the amount of ligand bound [15]. (It is worth noting that blood, having a much higher ionic content than the buffer system used in these experiments, would be expected to produce an even more extreme effect on the binding of salicylate to albumin than has been indicated by these experiments).

It was tempting to plot our data in the form of the traditional Scatchard plots [16]. However the difficulties of interpreting results obtained for such a complex system with multiple binding sites (and also at such high protein concentrations) are well illustrated by the varied results already in the literature.

For example, a simple comparison of figures depicting the same binding of serum albumin and salicylate, but measured by different techniques, illustrates well the dangers of interpretating curved Scatchard plots at low concentrations. Mais *et al.* [17] obtained their data by gel filtration [18] while Otagiri and Perrin [19] utilised induced changes in circular dichroism. Both sets of data, however (Figure 1 in reference 17 and Figure 3 in reference 19), show a paucity of data in the very low concentration ranges. Nevertheless although the initial slopes of the Scatchard plots are similar, and thereby their appearent initial binding constants, there is a vast discrepancy in their intercepts on both the abscissa and ordinate and hence a vast difference in the actual number of sites measured. It is noted that these authors used impure HSA (Fraction V) [19] or did not provide details on the purity of the sample [17]. Since we used crystallised protein and since no undue curvature was noted in the data analysis for obtained the diffusion coefficient from laser dynamic light-scattering [12] we concluded that our sample was both pure and low in content of dimers etc. We therefore conclude that the data reports in this paper refer to the pure HSA molecule and any discrepancies with the results of previous authors arise because of their use of impure samples of human serum albumin.

In conclusion it is clear from Figs. 2 and 3 that regardless of the albumin concentration there is initial rapid tight binding of salicylate. This followed by a

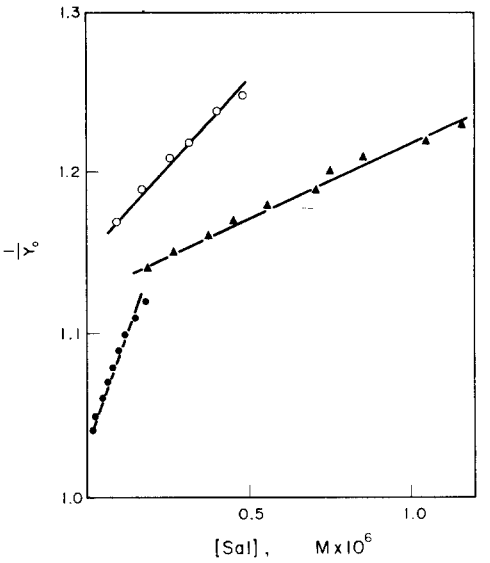

Fig. 4. Analysis of the results of salicylate–HSA binding measured by fluorescence. Protein concentrations were 1 (●), 5 (○) and 10 (▲) mg/ml at pH 7.5 and ionic strength 0.05. [SAL] is the concentration of free salicylate in solution. For explanation see text and Fig. 1.

region of weaker but nonetheless extensive binding up to approximately $\bar{\nu} = 8$. Above this level the plots of bound versus free salicylate take an upper curvature indicative of further tighter binding presumably caused by a conformational change induced by the binding of large amounts of salicylate. Additionally, at all levels including those of clinical interest, it is clear that the more albumin present the weaker the binding is. The region of particular clinical interest (using 0.2 mg/ml as being an approximate upper limit [6]) is also the region of tight-binding and is limited to the binding of the first 3–5 molecules of salicylate depending upon the concentration of the albumin. Since this region is of particular interest it has been studied in a more detailed manner by fluorescence. This is a particularly suitable method for this region of tight ligand binding and correspondingly low concentrations of free salicylate (see Fig. 4) since the measured quantity is basically a property of the protein and can therefore be measured with great sensitivity.

The results (Fig. 4) show several very interesting features. Firstly, it is clear that the concentration of albumin grossly affects its binding of salicylate. Secondly, none of the lines pass through the origin. This observation clearly indicates that, as observed by fluorescence, the salicylate molecules added initially to the albumin solutions are all totally bound (See Fig. 1). It is only after a certain amount has been bound that the "normal" multiple equilibrium-type binding pattern is adopted and this critical amount of very tight binding is related to the total albumin concentration in a complex manner as indicated by the varied intercepts on the $1/Y_0$ axis. This could be interpreted as indicating the presence of a small number of sites with extremely high binding constants. This seems unlikely because of the apparent initial increase in the number of these sites with increasing protein concentration. A more likely

explanation is that albumin undergoes a minor conformational change upon binding of salicylate after a critical amount of salicylate has been tightly bound. This critical amount is also dependent upon the protein concentration. The dependence upon protein concentration of binding of small molecules to albumin has been observed before (see for example, the binding of thyroxine to both bovine and human serum albumin at concentrations between approximately 0.1 and 3.5 g/l [11]). However the fine conformational change has not previously been reported and owes it observation to the sensitivity of the fluorescence technique. It is observed during the binding level of the first one or two molecules of salicylate where Scatchard and other plots which rely on the measurement of free ligand are obviously insensitive (cf. Fig. 2). A fine conformational change between $\bar{\nu} = 0$ and $\bar{\nu} = 2.5$ has been observed for the binding of salicylate to the closely related bovine serum albumin by laser dynamic light-scattering measurements of the diffusion coefficient (R. Geddes, J. D. Harvey and P. R. Wills, unpublished observations). These latter results indicate that the diffusion coefficient increased with the binding of the initial molecules of salicylate indicating that the albumin molecule adopted a more compact conformation as more ligand was bound.

The explanation for this phenomenon and the fluorescence observation described above is clearly related to the fact that the albumin monomer does not exist in a single rigid conformation [20]. Indeed, recent evidence suggests that the HSA molecule is composed of up to nine parallel α-helical rods, which have a high degree of structural integrity and are able to exist independently of the whole molecule [21, 22]. Further, the fluorescent probe 8-anilino-naphthalane-sulphonate appears to be bound in clefts [21] between the various domains [23] of the albumin molecule.

Thus it is reasonable to conclude that the first molecules of salicylate bound by the albumin are enfolded within the outer layers of the molecule, causing a conformational change in the protein, which in turn affects the binding of subsequent salicylate molecules.

A measure of the strength of this secondary binding may be gained by measuring the slopes of the lines in Fig. 4. The results are shown in Table 1. It is clear that the strength of this binding decreases with increasing protein concentration in agreement with the conclusions from the diafiltration experiments.

In summary, it may be concluded that the binding of salicylate to HSA is a complex process even in the region of clinical relevance. The initial binding of salicylate causes a distinct conformational change in the protein molecule which affects the binding of further molecules. Further, both the initial and subsequent binding is grossly affected by the actual protein concentration in a complex manner.

Table 1. Apparant association contants (K) for salicylate–HSA binding calculated from Fig. 4

Concentration of HSA (mg/ml)	K ($M^{-1} \times 10^{-3}$)
1	560
5	230
10	92

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