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BINDING OF PHENYTOIN, L-TRYPTOPHAN AND *O*-METHYL RED TO ALBUMIN. UNEXPECTED EFFECT OF ALBUMIN CONCENTRATION ON THE BINDING OF PHENYTOIN AND L-TRYPTOPHAN

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Abstract—The binding of phenytoin and *o*-methyl red to HSA and the binding of L-tryptophan to BSA has been studied by equilibrium dialysis at 37°, pH 7.4. The data for the binding of *o*-methyl red to HSA studied by either variation of the *o*-methyl red concentration or variation of the albumin concentration gave identical Scatchard plots. Scatchard plots of the data obtained for phenytoin and L-tryptophan, at constant ligand concentration, but with a range of albumin concentrations, were unusual and had a positive slope. Values for the apparent association constant (k) and number of binding sites (n) could not be obtained from these plots, but it was apparent that n and/or k decrease as albumin concentration increases.

Drug–albumin interactions are usually studied under experimental conditions where the albumin concentration is constant and the ligand concentration is varied. This approach assumes that the apparent association constant (k) and the number of binding sites (n) are independent of albumin concentration. The possibility that for some ligands n and k may be dependent upon protein concentration has not received much attention although there is evidence that albumin concentration may be important in this respect for acidic and basic drugs [1–8], dyes [9–14], surfactants [15], and neutral compounds [15, 16–18].

A preliminary account of the findings for L-tryptophan has appeared and this paper describes in detail the binding of phenytoin and *o*-methyl red to human serum albumin (HSA) and the binding of L-tryptophan to bovine serum albumin (BSA) [19].

MATERIALS AND METHODS

Chemicals. Crystalline human serum albumin fraction 5 (Sigma Chemical Co. HSA batch nos. 24C-1632-8, 62C-1301-8 and 84C-0152-1) and crystalline bovine serum albumin (Sigma BSA batch no. 44C-8000) were used. The purity of the *o*-methyl red (Hopkin and Williams Ltd.) was examined by ascending paper chromatography using the method of Burkhard and others (1961). R_f of *o*-methyl red = 0.43. Unlabelled sodium phenytoin was kindly donated by Parke Davis and Co; unlabelled L-tryptophan and all other reagents were of 'Analar' grade from B.D.H. Ltd.

The radiochemical purity of 4-[^{14}C]phenytoin (New England Nuclear Corporation, specific radioactivity = 5.21 μ Ci/m-mole, radiochemical purity > 99%) and L-[^{3}H]tryptophan (Radiochemical Centre, Amersham, specific radioactivity = 1.0 Ci/m-mole, radiochemical purity > 96 per cent) was checked by radiochromatogram scanning (Tracerlab 4 π scanner, argon–isobutane 98:2). [^{14}C]Phenytoin was chromatographed on thin-layer plates (silica gel 60/Kieselguhr F_{254}, 0.2 mm, Merck) with CH_3 OH-CH_3 COOH(98:2, v/v), R_f of phenytoin = 0.81. L-[^{3}H]Tryptophan was chromatographed by descending paper chromatography (Whatman no. 1) in C_2H_5 $OHNH_3$-H_2O (80:4:16, v/v), R_f of L-tryptophan = 0.54.

Solutions for dialysis. Experiments with L-tryptophan and *o*-methyl red were done with 0.05 M sodium phosphate buffer pH 7.4. In the phenytoin experiments the 0.05 M phosphate buffer was diluted with 5×10^{-4} M NaOH (containing phenytoin) to give 0.025 M sodium phosphate buffer pH 7.4, containing 2.5×10^{-4} M NaOH. Equal volumes of buffer containing the required concentration of HSA and unlabelled sodium phenytoin solution were mixed together with [^{14}C]phenytoin in ethanol. Ethanol concentrations were constant at 0.3% v/v at the start of equilibrium dialysis. [^{3}H]Tryptophan in aqueous solution was mixed with unlabelled L-tryptophan in phosphate buffer to give the required total tryptophan concentration. HSA and BSA solutions were made up in phosphate buffer and a partial specific volume correction was made, allowing 0.74 ml per g of albumin [20].

Pre-dialysis. Aliquots (25 ml) of HSA and BSA solutions were each pre-dialysed through Visking tubing (Medicell International) against 2×2 l of phosphate buffer for 40 hr at 2–4°, with 20 hr mixing and 20 hr standing. Albumin solutions (4.8 and 4% w/v) were routinely pre-dialysed in this manner and then diluted with phosphate buffer to the required protein concentration prior to the equilibrium dialysis for determination of binding. Previous experience had shown that pre-dialysis produced a measurable increase in binding and for example, the per cent binding (± S.D.) of phenytoin (21.7 μM) to 2% w/v HSA increased from 48.3 ± 0.6 (N = 13)

to 54.8 ± 0.8 after pre-dialysis [14]. Possible dilution of the albumin solution by the pre-dialysis procedure was investigated by estimation of protein concentration by the Biuret reaction and also by measurement of the binding of *o*-methyl red to a diluted (1% w/v) 3 ml aliquot of the HSA solution using equilibrium dialysis.

Equilibrium dialysis. The binding of phenytoin, L-tryptophan and *o*-methyl red to albumin was measured at 37° by equilibrium dialysis with a "Dianorm" apparatus (MSE Fisons Ltd). This apparatus is based upon the design of Weder and Bickel [21]. Visking tubing, flat width 44 mm was used as the dialysis membrane. Segments of membrane were soaked for 24 hr in water, immersed for 30 min in a 30% v/v ethanol–water mixture, thoroughly rinsed with water and then transferred to phosphate buffer before use. Dialysis times were 2 hr for *o*-methyl red and 4 hr for phenytoin and L-tryptophan. The concentration of *o*-methyl red was measured spectrophotometrically at 525 nm [22]. There was no measurable binding of the ligands to the dialysis membrane and the mean (± S.D.) recovery of *o*-methyl red was 94.4 ± 2.5 per cent (N = 7), and recoveries of radioactivity were 91.4 ± 1.0 per cent (N = 5) and 92.8 ± 6.9 per cent (N = 231) for [^{3}H]tryptophan and [^{14}C]phenytoin respectively.

Measurement of radioactivity. Radioactivity in samples (0.25 ml) from the albumin and non-albumin compartments was measured by liquid scintillation counting in a Nuclear Chicago Unilux II scintillation counter. Samples were counted in plastic insert vials (Sterilin Ltd.) using one of the following scintillator systems (1) 0.25 ml sample + 0.75 ml H_2O + 2.5 ml xylene-Triton X-114 (Sigma) (2:1, v/v) containing 0.3% w/v PPO (2) 0.25 ml sample + 0.75 ml H_2O + 2.5 ml PCS (Amersham, Searle) liquid scintillator (3) 0.25 ml sample + 2.5 ml NE260 liquid scintillator (Nuclear Enterprises Ltd). Internal standards of [^{3}H]water (New England Nuclear), [^{3}H]*n*-hexadecane (Radiochemical Centre) were used for assessment of quenching and appropriate correction made for tritium decay.

Calculation of results. The results were analysed by means of the Scatchard [23] equation for the law of mass action $r/D_u = nk - rk$, where r is the molar ratio of bound ligand to albumin, D_u is the concentration of unbound ligand at equilibrium, k is the apparent association constant and n the number of binding sites. Results are expressed as the mean ± the S.D. of three or more experiments. The mol. wt of HSA and BSA were assumed to be 69,000.

RESULTS

Effect of pre-dialysis. Pre-dialysis of the albumin preparations was routinely used to remove diffusible contaminants, such as *N*-acetyl-L-tryptophan, and the possibility that this may have diluted the albumin solutions was investigated [24]. The mean HSA concentration of a 4.0% w/v solution measured by the Biuret method was 3.95 ± 0.23% w/v (N = 6) and 3.83 ± 0.11% w/v (N = 14) before and after pre-dialysis respectively. The difference was not statistically significant although the results imply a small dilution of the HSA solution during pre-dialysis.

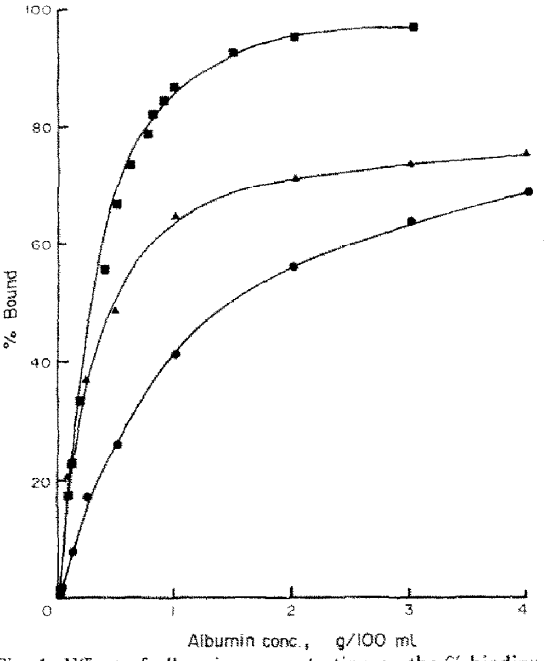

Fig. 1. Effect of albumin concentration on the % binding of phenytoin, L-tryptophan and *o*-methyl red at pH 7.4 and 37°. ● phenytoin (21.7 μM) binding to HSA (batch no. 24C-1632-8); ▲ L-tryptophan (9.8 μM) binding to BSA; ■ *o*-methyl red (133 μM) binding to HSA. Each point is the mean of at least three experiments.

Equilibrium dialysis experiments with *o*-methyl red showed that prior to pre-dialysis 84.7 ± 0.2% (N = 5) was bound and that after pre-dialysis 84.8–0.2% (N = 5) of the dye was bound. This suggests that the apparent dilution of HSA by pre-dialysis is due to loss of diffusible substances capable of giving a positive Biuret reaction.

Binding of phenytoin to HSA. Figure 1 shows that the per cent binding of phenytoin (21.7 μM; 5.9 μg/ml) increased from 0.5 ± 0.5 per cent with 0.025% w/v HSA to 69.1 ± 0.2 per cent with 4% w/v HSA. Odar-Cederlöf [25] reported phenytoin to be 88.1 per cent bound to human plasma at 37° over the concentration range 10–15 μg/ml phenytoin (36–50 μM). The greater per cent binding to whole plasma is probably accounted for by binding of the drug to other plasma proteins in addition to albumin [26].

Figure 2 is a Scatchard plot of the data for the binding of a range of concentrations of phenytoin (0.37–739 μM) to 1% w/v and 2% w/v HSA at 37°. The low aqueous solubility of phenytoin makes it difficult to determine n and k separately but values for nk were 4.74×10^3 l/mole and 3.92×10^3 l/mole for 1 and 2% w/v HSA respectively. These results are similar to those previously published but a satisfactory comparison is difficult because of discrepancies in several earlier reports [27–29].

Figure 3 is a Scatchard plot of the data, obtained from a separate series of experiments, for the binding of a single concentration of phenytoin (21.7 μM) to HSA (batch no. 24C-1632-8) over a range of concentrations from 0.125 to 4.0% w/v. This is an atypical plot with a positive slope. As can be seen in Fig. 3 the data (from Fig. 2) obtained for the

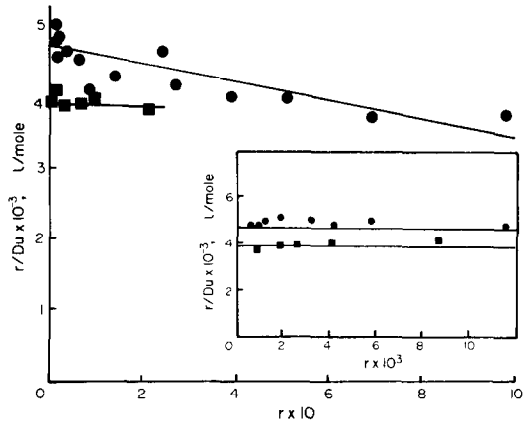

Fig. 2. Scatchard plots for the binding of various concentrations of phenytoin to 1 and 2% w/v HSA at pH 7.4 and 37°. ● phenytoin (0.37–739 μM) binding to 1% w/v HSA (lot no. 24C-1632-8). ■ phenytoin (0.69–185 μM) binding to 2% w/v HSA (batch no. 84C-0152-1). The inset is a plot of the remaining data, expanded for r, up to $r = 1.2 \times 10^{-2}$. Each point is the mean of 4–16 experiments.

binding of a range of phenytoin concentrations to 1% w/v (batch no. 24C-1632-8) intersects with the corresponding data point when the HSA concentration was varied. The data obtained with 2% w/v HSA (batch no. 84C-0152-1) and varying concentrations of phenytoin does not agree so closely with the corresponding data point for 2% w/v HSA, obtained when the HSA concentration was varied (Fig. 3). This was because the per cent binding of phenytoin differed to the extent of about 3 per cent between the two batches of albumin used, for example, at 2% w/v HSA phenytoin (21.7 μM) was 56 per cent bound to HSA batch no. 24C-1632-8 and 53 per cent to HSA batch no. 84C-0152-1. Nevertheless it is clear that an increase in albumin concentration from 1 to 2% w/v results in a decrease in nk. Artifacts due to osmosis are unlikely because protein estimations carried out before and after equilibrium dialysis on 4, 2 and 1% w/v HSA solutions showed no significant change.

Binding of L-*tryptophan to BSA.* The binding of

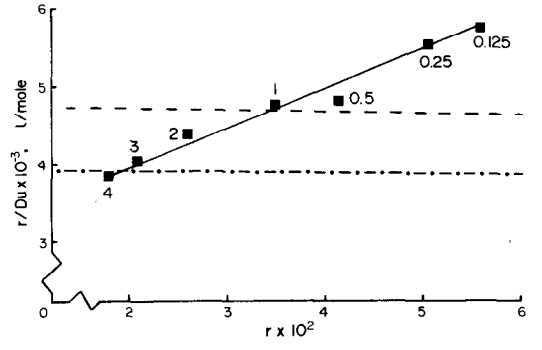

Fig. 3. Scatchard plot for the binding of a single concentration of phenytoin (21.7 μM) to a range of concentrations of HSA (batch no. 24C-1632-8) at pH 7.4 and 37°. Numerals are the HSA concentration (% w/v) at the point indicated. Regression lines of the data from Fig. 2 are included for comparison: – – – 1% w/v HSA (lot no. 24C-1632-8); – · – · – 2% w/v HSA (lot no. 84C-0152-1). Each point is the mean of 5–9 experiments.

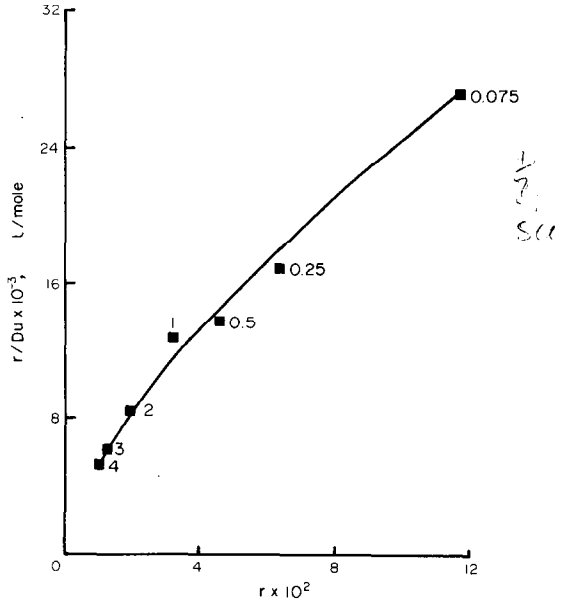

Fig. 4. Scatchard plot for the binding of a single concentration of L-tryptophan (9.8 μM) to a range of concentrations of BSA at pH 7.4 and 37°. Numerals are the BSA concentration (% w/v) at the point indicated. Each point is the mean of five experiments.

L-tryptophan (9.8 μM) to BSA increased from 20.7 ± 3.9 per cent with 0.075% w/v BSA to 75.4 ± 0.5 per cent with 4% w/v BSA (Fig. 1). This shows a similar relationship to that for the binding of phenytoin to HSA. Figure 4 is a Scatchard plot of the data for the binding of a single concentration of L-tryptophan (9.8 μM) to a range of concentrations (0.075–4% w/v) of BSA. This Scatchard plot also shows an atypical positive slope similar to that obtained for phenytoin when the albumin concentration was varied (Fig. 3).

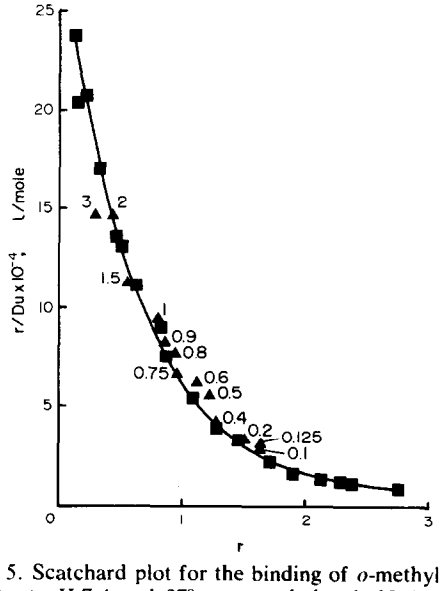

Fig. 5. Scatchard plot for the binding of *o*-methyl red to HSA at pH 7.4 and 37°. ■ *o*-methyl red (20–1,000 μM) binding to 1% w/v HSA; ▲ *o*-methyl red (133 μM) binding to a range of concentrations of HSA, the numerals are the HSA concentration (% w/v) at the point indicated. Each point is the mean of 3–5 experiments.

Binding of o-methyl red to HSA. The per cent binding of *o*-methyl red (133 μM) to various concentrations of HSA is shown in Fig. 1. The per cent bound increased from 17.7 ± 0.8 per cent with 0.1% w/v HSA to 97.0 ± 0.1 per cent with 3% w/v HSA. Figure 5 is a Scatchard plot of data obtained in two separate series of experiments where either the dye or the HSA concentration was varied. It can be seen from Fig. 5 that the data obtained for the binding of *o*-methyl red (20–1000 μM) to 1% w/v HSA and that for the binding of *o*-methyl red (133 μM) to 0.2–3% w/v HSA gave identical Scatchard plots. The curvilinear nature of the Scatchard plot for *o*-methyl red binding to HSA makes interpretation and analysis difficult but linear regression analysis of the first steep portion of the curve, up to $r = 1.0$ (Fig. 5) gave $n_1 = 1.21$ and $k_1 = 1.99 \times 10^5$ l/mole. The binding of *o*-methyl red to HSA at pH 7.4 has not been studied previously but Burkhard and others [30] studied *o*-methyl red binding to BSA at pH 6.8 and 0° and found $n_1 = 0.84$ and $k_1 = 2.25 \times 10^5$ l/mole.

DISCUSSION

The results for phenytoin and L-tryptophan show that the binding of these two ligands is dependent upon protein concentration. The positive slopes of the Scatchard plots indicate a decrease in n or k values, or both, as the albumin concentration increases. In contrast no such effect was found for *o*-methyl red when studied under identical experimental conditions. The dependence of nk upon protein concentrations has been observed with HSA, BSA and whole plasma or serum, and the ligands involved have considerable structural diversity. Several explanations for this phenomenon are possible.

Gibbs–Donnan effect. The calculated theoretical Donnan ratios ranged from 1.0035 (0.125% HSA) to 1.11 (4% w/v HSA), in the experiments with phenytoin, assuming HSA to have a net negative charge of 18 at pH 7.4 [31]. If a pK_a of 8.3 is assumed for phenytoin [32], only 12.6 per cent of the drug is ionized at pH 7.4. Rudman and others [27] using rabbit serum and 0.05 M sodium phosphate buffer at a similar ionic strength and the same pH as this study, found no more than 2 per cent difference in sodium concentration across the dialysis membrane. Similarly, Odar-Cederlöf [25] estimated the Gibbs–Donnan effect to give a theoretical maximum error of 5 per cent in unbound phenytoin concentration for human plasma and suggested that this would not significantly affect the results.

Several ligands, including oxazepam, lorazepam [7], 2-(4′-hydroxybenzeneazo) benzoic acid [13], and methyl orange [10] were studied by methods where no membrane was employed and so the Gibbs–Donnan effect could not operate. In view of this it seems unlikely that the Gibbs–Donnan effect had any major influence on the binding of phenytoin and L-tryptophan.

Inhibition of binding by endogenous ligands. Commercial albumin preparations may contain quantities of highly bound ligands as contaminants e.g. *N*-acetyl-L-tryptophan and other indoles [24] and fatty acids [33, 34]. At constant ligand concentration and increasing albumin concentration the contaminant–ligand ratio will increase and may result in decreased binding if the contaminant is an inhibitor of binding. This would cause an apparent decrease in n and/or k as the protein concentration was increased. The HSA used in our own studies was found by gas–liquid chromatography (Bowmer and Lindup, unpublished results) to contain principally oleic acid (66.4 ± 2.8% [4]) palmitic acid (17.3 ± 4% [4]) and stearic acid (8.0 ± 2.2% [4]) and the molar ratio of total fatty acid (C_{12}–C_{18}) to HSA was 0.51 ± 0.09 [4]. The routine pre-dialysis used in these experiments should remove diffusible contaminants e.g. *N*-acetyl-L-tryptophan but the fatty acids would not be so easily removed. The binding of *o*-methyl red to HSA is strongly inhibited by fatty acids such as lauric, myristic and palmitic [22] but since the Scatchard plots obtained by variation of either *o*-methyl red or HSA concentration were identical, it is unlikely that fatty acids present in the albumin preparations are responsible for the unusual binding behaviour of phenytoin and L-tryptophan. Contaminants have been offered as an explanation for unusual Scatchard plots with cortisol [16] and decanol [15] but there was no direct experimental proof. Furthermore, replacement of the buffer surrounding the dialysis bag, thereby decreasing the concentration of possible contaminants, did not alter the binding of dodecylsulphate to 0.1 and 1% w/v BSA [35]. Scholtan [1] found that drug binding in whole plasma with pathologically lowered albumin concentration did not decrease as much as anticipated and this suggests that dilution of endogenous inhibitory ligands cannot fully explain the increase in n and k with more dilute albumin solutions.

Cooperative effects. Positive cooperativity has been implicated in various unusual ligand–protein interactions [36–39]. These cooperative effects have all been observed at one protein concentration or at most, with only a small range of protein concentrations. If cooperativity is responsible for the protein concentration dependent binding observed with phenytoin, L-tryptophan and the other ligands it would also be expected to be manifest when the ligand concentration, as opposed to the albumin concentration, was varied. However no such cooperative effects were apparent in the studies where the ligand concentration was also varied.

Protein–protein interaction. Protein–protein interaction may serve to decrease the availability of binding sites and/or the affinity as the albumin concentration increases. It may take the form of some non-specific molecular aggregation or it may involve the formation of polymers. Commercial preparations of albumin used *in vitro* have been found to contain polymers of albumin and removal of these may prove necessary before consistent results can be obtained [39]. Brock [40] found that digitoxin binding to HSA dimers was less than to HSA monomers. Excessive concentrations of digitoxin did not influence the HSA monomer–dimer ratio [40].

Albumin polymers may be an artifact of commercial albumin preparations but there is evidence which suggests that albumin polymers also occur *in*

vivo. Paper electrophoresis of plasma has shown that albumin can exist in a variety of polymeric forms, particular as a dimer [41]. Radioactive cobalt (^{60}Co) was bound mainly to albumin monomer and to a smaller extent to albumin dimer after intravenous administration of ^{60}Co to the rabbit [42, 43].

Scholtan [1] observed that the decreased binding of 2-(*p*-aminobenzenesulphonamido)-5methoxy pyrimidine in pathological human sera was not as great as would have been predicted from the lowered albumin concentrations of the sera. The *n* and *k* values for the interaction of two long-acting sulphonamides with human and ox albumin were also found to be dependent upon protein concentration [2], with *n* and *k* decreasing with increasing albumin concentration. Several other workers [3, 7, 8, 11] view albumin polymerisation as an explanation for their findings.

An increase in the aggregation or polymerisation of albumin molecules as the protein concentration increases does offer an explanation for the binding behaviour of phenytoin, L-tryptophan and the other ligands. Ligand binding sites near or on the site of polymerisation may be partially or completely obscured as polymerisation occurs. Teller [44] has predicted that each monomer unit loses 28.6 per cent of accessible surface when globular proteins interact to form a square planar tetramer of identical monomers. The theoretical kinetic implications of the way in which binding and polymerisation may interact have been considered by Nichol and others [45]. The plasma protein apolipoprotein A1 has to be in monomeric form to bind lipids readily *in vitro*, and the state of association of this protein in solution may play a major role in determining its binding capacity [46].

Phenytoin inhibits the binding of L-tryptophan to HSA [47] to a small extent and so these two compounds may share a common binding site, which may explain their similar binding behaviour. If the binding site(s) for *o*-methyl red is unaffected by polymerisation this would account for the different results obtained with this ligand. An alternative explanation is that phenytoin and L-tryptophan do not inhibit albumin polymerisation while *o*-methyl red does because of its higher affinity for HSA. There is evidence that bendazac and phenylbutazone are able to inhibit the polymerisation of BSA *in vitro* [48].

General aspects. The dependence of *n* and/or *k* upon protein concentration has several implications. It may no longer be sufficient to use a single albumin (or other protein) concentration to experimentally determine a single set of binding constants to characterise the interaction. The dye binding methods used routinely by clinical biochemists for determining plasma albumin concentration give erroneously high values at low plasma albumin concentration and this is at least partially explicable in terms of the results discussed above [49]. When albumin concentration falls *in vivo*, as a result of disease or trauma, an increase in *nk* may serve as a homeostatic mechanisms to dampen fluctuations in the concentrations of unbound biologically active ligands.

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