available which are specific for aminopeptidase M and for leucine-aminopeptidase [3], it appears that the tools are becoming available with which to investigate the possible roles of these enzymes in various pathological conditions. The (phenylthio)-phenylacetic and propionic acid compounds might also prove useful in studying the catabolism of certain biologically active peptides, such as the polypeptide hormones, and the more recently discovered hormone releasing factors.

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Binding of nonsteroidal anti-inflammatory agents to proteins—I. Ibuprofen–serum albumin interaction

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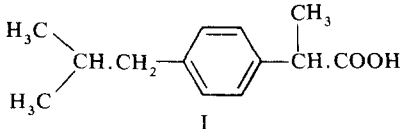
A generally accepted explanation of the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAID) is the inhibition of prostaglandin synthesis [1–5]. Other explanations which have been proposed include displacement of corticosteroids from serum proteins [6], displacement of endogenous anti-inflammatory polypeptides [7] and stabilization of protein structure [8].

Whether nonsteroidal anti-inflammatory drugs act by a process of plasma protein stabilization, or of displacement, or by inhibition of prostaglandin synthetase, an evaluation of their protein binding characteristics is directly relevant to all mechanisms. Binding to the active site of prostaglandin synthetase might be expected to parallel albumin binding although the latter may require a much smaller degree of ligand structural specificity than the enzyme. Furthermore, binding of drugs to serum and tissue proteins is known to be an important determinant of their disposition kinetics and pharmacodynamics [9–13].

Ibuprofen (I), reported in 1967 [14], is widely used in the treatment of rheumatoid arthritis and osteoarthroses. Mills *et al.* [15] have reported that ibuprofen at a concentration of 20 μg ml^{-1} was 99 per cent bound in whole human plasma. It was reported that interaction occurred with a single primary albumin binding site with an association constant of 10^5 molar^{-1}; however, it is not clear how this was established using whole plasma.



I

Ibuprofen, 2-(4-isobutylphenyl)-[3-^{14}C] propionic acid with a specific activity of 13.11 ± 0.19 μCi mg^{-1}, was a gift from The Boots Pure Drug Co. Ltd., Nottingham, U.K. The pK_a was determined, by the solubility technique of Albert and Serjeant [16], to be 4.13 ± 0.05. Binding of ibuprofen to bovine serum albumin (BSA) (fraction V, Sigma Chemical Co.) and human serum albumin (HSA) (fraction V, Behringewerke A.G.) was determined by equilibrium dialysis using 10-ml sterilized glass cells. The "Visking" cellophane membrane was boiled in several changes of distilled water before use.

Each cell was equilibrated for 36 hr at the required temperature (5–45° ± 0.1). Binding of ibuprofen was characterized alone and in the presence of bilirubin, uric acid, cholesterol and palmitate. The molecular weight of albumin was taken to be 69,000. Apparent contributions to binding by osmotic pressure and Donnan membrane effects were negligible under the experimental conditions. Where necessary albumin was defatted by the charcoal treatment described by Chen [17]. Palmitate was added by the technique of Avigan [18] and assayed [19]. Solutions were analyzed by liquid scintillation counting of [^{14}C]ibuprofen in Brays solution, to <3.5 per cent standard deviation in a Hewlett Packard scintillation spectrometer (model 3375). Correction for quenching was made using an automatic external standard technique.

Data were fitted to the generalized Scatchard equation [20] using FUNFIT, an interactive program for nonlinear regression [21]. Binding parameters were compared by a two-tailed t-test ($P \leq 0.05$). Each test parameter was assumed to be normally distributed.

Ibuprofen binding to HSA (Fig. 1) and BSA was determined in 0.033 M phosphate buffer at 37°. Correction for possible electrostatic interactions between binding sites, resulting from ligand binding and subsequent protein valency changes, were performed according to the Debye–Huckel–Born model [20]. However, the Scatchard plots remained curved, suggesting multiple class binding.

The quality of fit of the generalized Scatchard model with and without electrostatic correction was distinguished by comparison of residual variances. No improvement in fit was achieved by electrostatic correction or using numbers of classes of sites greater than 2($P \leq 0.05$). The data were thus best described by binding to two classes of sites. Binding parameters obtained using this model are summarized in Table 1 together with the coefficient of variation as an indication of the precision of each estimate. Ibuprofen binds strongly to a single primary site on HSA with an association constant of 2.73 × 10^6 molar^{-1}, and six to seven secondary sites with an association constant of 1.95 × 10^4 molar^{-1}.

Comparison of the binding parameters estimated at 1% and 0.4% HSA reveals an apparent protein concentration dependence.* Lowering the albumin concentration results in an apparent increase in the binding capacity (n_1 and n_2) and a decrease in the strength of binding (k_1 and k_2). These

* One of the reviewers directed our attention to a previous report [J. Cassel, J. Gallagher, J. A. Reynolds and J. Steinhardt, *Biochemistry* **8**, 1706 (1969)] which established that apparent protein concentration-dependent binding of dodecylsulfate and other compounds to BSA was due instead to the very slow approach to equilibrium of this and other ligands particularly when the ionic ligand was dialyzed into concentrated protein solutions. In all our experiments, the drug was dialyzed from the protein compartment and a preliminary study using 1% HSA established that thermodynamic equilibrium was established well within the 36-hr equilibration period.

Table 1. Binding parameter estimates characterizing the interaction of ibuprofen with serum albumin at 37°, pH 7.4

Buffer	Protein	n_1	k_1 (molar^{-1} × 10^{-6})	n_2	k_2 (molar^{-1} × 10^{-4})	Sum of squares of residuals
Phosphate, 0.033 M	1.0% HSA	0.80 (7.7)*	2.73 (17.1)	6.27 (2.1)	1.95 (7.1)	0.065 (28)†
	0.4% HSA	1.00 (8.3)	2.47 (19.7)	7.06 (1.3)	1.47 (6.6)	0.355 (43)†
Phosphate, 0.067 M	1.0% HSA	0.72 (10.0)	2.41 (22.4)	6.17 (2.6)	2.25 (8.4)	0.012 (10)†
	0.4% HSA	0.91 (10.7)	1.89 (26.5)	6.86 (1.4)	1.68 (7.1)	0.021 (12)†
Phosphate, 0.033 M	1.0% BSA	0.92 (10.8)	1.37 (21.1)	6.66 (5.1)	1.94 (14.3)	0.007 (11)†
	0.4% BSA	1.01 (11.5)	1.31 (29.3)	7.32 (1.6)	1.76 (8.0)	0.021 (12)†
Tromethamine, ionic strength = 0.1	1.0% HSA	0.80 (4.7)	0.83 (10.0)	6.99 (1.1)	1.43 (3.8)	0.002 (14)†

* Per cent coefficient of variation.
† Number of data points.

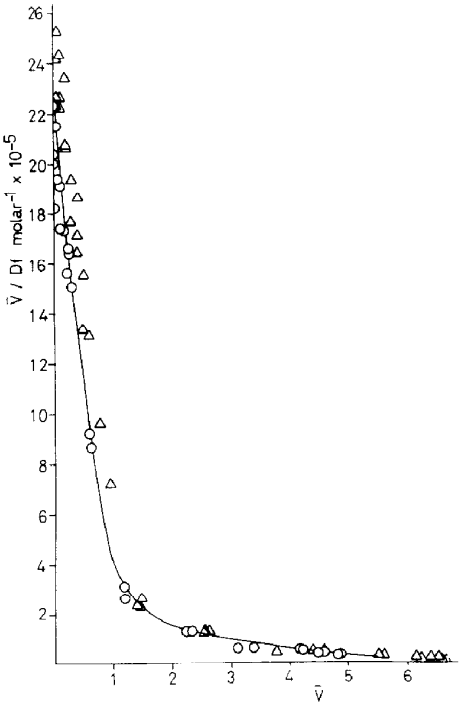


Fig. 1. Scatchard plots for the binding of ibuprofen to 1% HSA (○) and 0.4% HSA (△) in 0.033 M phosphate buffer at pH 7.4 and 37°. Points are experimental values. The solid curve was generated from binding parameters estimated for 1% HSA.

differences are significant ($P \leq 0.05$) for all four parameters in 0.033 M phosphate buffer. It is difficult to explain the observed differences in the number of binding sites. However, the HSA concentration-dependent differences in k_1 and k_2 can be explained in two ways. There is evidence that phosphate buffer may compete with ligand molecules for binding sites [22–24] although this competition is considered to be only very slight compared with other buffer systems. However, the molar concentration ratio of phosphate to ibuprofen ranges from 10^2 to 10^3; thus, it is likely that there would be a small competitive effect which would vary with protein concentration. In an attempt to substantiate this explanation the binding was estimated in the presence of double strength (0.067 M) phosphate buffer (Table 1). The primary association constants were further decreased at both concentrations of protein although there appears to have been a slight increase in values for k_2. These findings are consistent with buffer competition for binding sites. Alternatively, there is the possibility that purified protein contains small amounts of contaminants [25, 26]. The competitive displacing effect of such contaminants would also increase with decreasing albumin concentration.

Tromethamine buffer reduces the primary association constant 3-fold, while the secondary association is diminished only slightly. This is consistent with previous findings [27]. It is of interest that binding to primary sites on BSA is significantly weaker than to HSA ($P \leq 0.05$). This suggests that BSA is unsuitable as a model protein for ibuprofen binding to HSA.

Primary site binding is highly temperature sensitive (Table 2); a decrease in temperature from 45 to 5° causes a 7-fold increase in association constant. The effect of temperature variation on the secondary sites is much smaller, and difficult to rationalize. However, it is unlikely that the secondary sites are homogeneous and, therefore, it would not be expected that all binding sites would respond uniformly to changes in temperature.

Table 2. Influence of variation in temperature on binding parameters characterizing the interaction of ibuprofen with 1% HSA in 0.033 M phosphate buffer, pH 7.4

Temperature (°)	n_1	k_1 (molar^{-1} × 10^{-6})	n_2	k_2 (molar^{-1} × 10^{-6})	Sum of squares of residuals
5	0.84 (7.2)*	7.08 (19.5)	6.78 (2.3)	1.82 (6.0)	0.016 (12)†
12	0.79 (9.1)	4.56 (20.9)	6.38 (2.8)	2.19 (8.9)	0.017 (12)†
20	0.81 (10.3)	3.69 (21.9)	6.03 (2.3)	2.38 (8.5)	0.033 (13)†
37	0.80 (7.7)	2.73 (17.1)	6.27 (2.1)	1.95 (7.1)	0.065 (28)†
45	1.00 (19.6)	1.04 (35.4)	6.51 (5.5)	1.41 (20.9)	0.043 (14)†

* Per cent coefficient of variation.
† Number of data points.

Table 3. Thermodynamic constants governing the interaction of ibuprofen with primary and secondary sites of HSA

Temperature (°)	Primary site $\Delta G°$ (kcal·mole^{-1})	$\Delta H°$ (kcal·mole^{-1})	$\Delta S°$ (e.u.)	Secondary sites $\Delta G°$ (kcal·mole^{-1})	$\Delta H°$ (kcal·mole^{-1})	$\Delta S°$ (e.u.)
5	−8.72	−7.06	5.96	−5.42	−1.15	15.36
12	−8.69	−7.06	5.72	−5.67	−1.15	15.84
20	−8.81	−7.06	5.97	−5.87	−1.15	16.11
37	−9.14	−7.06	6.69	−6.09	−1.15	15.93
45	−8.76	−7.06	5.35	−6.04	−1.15	15.37

Thermodynamic constants governing the interaction were calculated using the standard relationships [20]. The free energy of association with the primary site is composed of an 80 per cent contribution from the negative enthalpy change and 20 per cent positive entropy (Table 3). The negative enthalpic component is generally believed to result from van der Waals' dispersion or dipolar forces, while the entropic component results from electrostatic and/or hydrophobic bonding [28]. Thermodynamic parameters alone do not permit a more detailed interpretation. It is significant that tromethamine buffer, of ionic strength equivalent to phosphate, produced more than a 3-fold reduction in k_1. It was shown recently that, with tromethamine containing 0.033 M chloride ion, seven molecules of chloride are bound per albumin molecule [20]. Chloride ion may interfere with ibuprofen anion binding by direct competition and/or by increasing the net negative charge on the albumin molecule, thereby increasing the electrostatic potential which must be overcome before further binding can take place [29]. Thus, the displacing effect of tromethamine buffer perhaps suggests that the 20 per cent entropic contribution to ΔG^0 is due largely to ion pair formation between ibuprofen anions and cationic loci on the albumin molecule rather than hydrophobic bonding.

Binding to the second class of sites is characterized by a much greater (80 per cent) entropic contribution to ΔG^0 (Table 3). Thus, hydrogen and/or van der Waals' bonds are of minor importance in this interaction. In addition, binding is only slightly affected by chloride ion in tromethamine buffer (Table 1), suggesting that ion pair formation is also of little significance at these sites. Therefore, it is likely that hydrophobic bonding is responsible for the seconary binding.

Vallner [13] has stressed the importance of investigating the displacing effects of endogenous constituents of serum. Binding data, determined using only purified serum albumin, may not reflect accurately *in vivo* albumin binding. To this end the interaction of ibuprofen has been investigated in the presence of bilirubin, cholesterol and urate. Palmitic acid was chosen, in addition, to represent the effects of endogenous nonesterified fatty acids (NEFA).

The concentration of 15 μg ml^{-1} bilirubin used in this study is representative of unconjugated bilirubin in jaundice [30]. An elevated concentration of 100 μg ml^{-1} of uric acid was chosen to represent hyperuricaemia associated with gouty arthritis. Serum cholesterol concentrations may be extremely high, up to 5 mg ml^{-1}. However, much of this is bound to the lipo-protein fraction of serum. Use of the Celite 545 adsorption–desorption technique of Avigan enables preparation of albumin solutions containing up to 36 μg ml^{-1} of cholesterol in 1% HSA [18].

Data for ibuprofen binding to HSA in the presence of the additives are illustrated in Fig. 2. Palmitate at a concentration of 118 μg ml^{-1}, corresponding to 3.21 equivalents mole^{-1} of albumin, produced the greatest reduction in ibuprofen binding in both classes of sites. These data indicate the

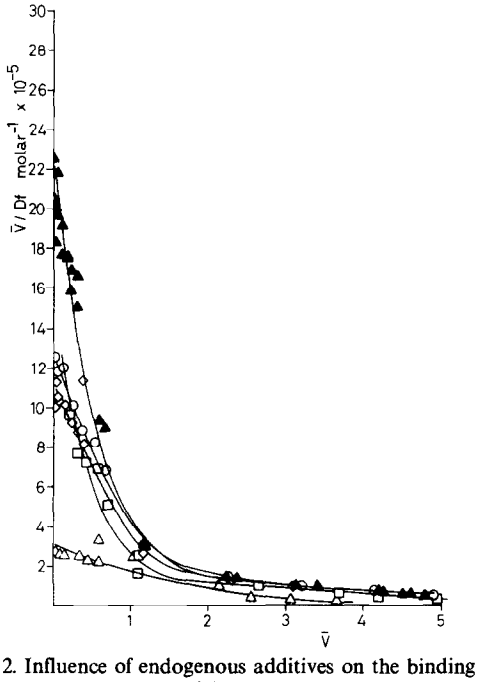


Fig. 2. Influence of endogenous additives on the binding of ibuprofen to 1% HSA in 0.033 M phosphate at pH 7.4 and 37°. Key: (▲) ibuprofen alone; ibuprofen in the presence of bilirubin, 15 μg ml^{-1} (◇); cholesterol, 36 μg ml^{-1} (approx.) (□); urate, 100 μg ml^{-1} (○); and palmitate, 118 μg ml^{-1} (△).

Table 4. Binding parameters for the interaction of ibuprofen at 37° with 1% HSA in 0.033 M phosphate buffer at pH 7.4 in the presence of bilirubin (15 μg·ml^{-1}), cholesterol [36 μg·ml^{-1} (approx.)], urate (100 μg·ml^{-1}) and palmitate (118 μg·ml^{-1})

Endogenous compound	n_1	k_1 (molar^{-1} × 10^{-6})	n_2	k_2 (molar^{-1} × 10^{-4})	Σnk (molar^{-1} × 10^{-6})	Sums of squares of residuals
Nil	0.80 (7.7)*	2.73 (17.1)	6.27 (2.1)	1.95 (7.1)	2.30	0.065 (28)†
Urate	0.87 (8.2)	1.81 (16.3)	6.26 (2.4)	1.86 (8.0)	1.69	0.014 (15)†
Bilirubin	0.84 (12.4)	1.54 (22.8)	6.08 (3.3)	1.68 (11.5)	1.40	0.026 (14)†
Cholesterol	0.60 (25.4)	2.45 (62.6)	5.45 (4.2)	2.24 (17.7)	1.60	0.111 (13)†
Palmitate	2.37 (13.3)	0.14 (21.7)	2.95 (36.5)	0.39 (62.7)	0.33	0.105 (13)†

* Per cent coefficient of variation.
† Number of data points.

importance of serum NEFA in modulating binding of drugs to albumin. Urate, bilirubin and cholesterol all produce similar degrees of displacement although very much less than that of palmitate.

Binding constants for ibuprofen with the various additives are shown in Table 4. Also included are values for the parameter Σnk which is useful as an index of binding overall, combining values for both the capacity and strength of the association.

Palmitate and, to a lesser extent, cholesterol alter the number of binding sites available in each class. Direct competition for common binding sites is expected to diminish the association constant of both drug and competitor; however, the number of sites of association is not expected to change. Certainly, small variations in values of n_1 and n_2 are likely to result from iteration on data having varying amounts of experimental error. These are indicated by the coefficients of variation. However, with palmitate the changes in n_1 (0.8 to 2.37) and n_2 (6.27 to 2.95) are much too large to be explained in this way. It seems more likely that the changes in the numbers of sites in addition to the 20-fold and 5-fold reduction in k_1 and k_2, respectively, indicate a conformational change in the albumin molecule. Other workers have also proposed conformational changes in albumin caused by binding of NEFA [31, 32].

The purpose of studying the influence of endogenous materials is to establish the possible significance that fluctuations in their serum concentrations may have on drug disposition. When the data are adjusted for a physiological serum albumin concentration of 4%, the percentage of free ibuprofen is 0.1% for a total serum concentration of 20 $\mu g\ ml^{-1}$. Based on the data in Table 4, the displacing effects of urate, bilirubin and cholesterol are negligible at this drug concentration. At the high albumin concentration present in serum, less than 17 per cent of available primary sites are occupied; thus, the likelihood that both drug and displacer will compete for the same sites is remote. In the unlikely event that competition occurs, the displaced drug molecule will immediately associate with one of the excess free sites. In contrast, palmitate (118 $\mu g\ ml^{-1}$) would increase the percentage of free ibuprofen 5-fold (from 0.1 to 0.5%).

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Effects of adrenergic agonists on gastric secretion in the rat

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Many organ systems, the gastrointestinal tract among them, are controlled in opposite directions by the sympathetic and parasympathetic nervous systems. However, little investigation has been done on gastric secretion under the control of the sympathetic nervous system, because it is usually thought that gastric secretion is controlled mainly by hormones such as gastrin and the parasympathetic nervous system.

Some contradictory results about gastric secretion by adrenergic agonists have been reported [1, 2]. Curwain and Holton [3] mentioned that isoproterenol and norepinephrine inhibited gastric acid secretion stimulated by pentagastrin in the dog, and Daly and Stables [4] obtained the same results; however, Hsu and Cooper [5] reported that epinephrine and isoproterenol raised the serum gastrin level in the rat.