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CHAIN LENGTH DEPENDENCY OF FATTY ACID AND CARBAMATE BINDING TO SERUM ALBUMIN

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Abstract—The binding interactions of bovine serum albumin (BSA) with the unbranched fatty acids (FA) pentanoate (five-carbon chain length: C5) up to nonanoate (C9), and the carbamates *n*-methyl carbamate (equivalent to C3) up to *n*-hexyl carbamate (equivalent to C8) were examined using an ultrafiltration technique. A single, high-affinity site was observed for each of the FA, with an increasing number of secondary sites with increasing chain length. From binding affinity and competition data, there appear to be distinct albumin sites for the short-chain (≤ C7) and the medium-chain (≥ C8) FA. Published data suggest that the medium-chain FA site is one of the major drug-binding sites on human serum albumin (HSA) or BSA, the indole/benzodiazepine site. Competition between the FA and warfarin for BSA or HSA binding was studied by ultrafiltration and fluorescence methods and suggests that the short-chain FA site may lie in the same region as a second major drug-binding site, the large warfarin-binding area. Thermodynamic parameters of the FA–BSA interactions are suggestive of primary binding being a combination of electrostatic and hydrophobic binding and secondary binding being purely hydrophobic in nature. Carbamate interactions with BSA show several primary sites and also suggest a disparity between the binding of ligands of ≤ 7 and ≥ 8 in total length, but there was no evidence of competition between FA and carbamates. A model is proposed to explain these observations, which includes the suggestion that several classes of hydrophobic binding areas exist, each of which is specific for ligands of a restricted range of chain lengths.

Serum albumin binding can be a significant factor in drug distribution and pharmacokinetics and the competition of drugs for albumin-binding sites can be the cause of some adverse drug interactions. In addition, the interaction of small ligands with albumin can display certain properties, including high affinity (e.g. Ref. 1) and stereospecificity (e.g. Refs. 2 and 3) which suggest that such interactions may be models for receptor–agonist/antagonist or enzyme–substrate reactions. Thus, the study of ligand–albumin interactions is of considerable importance in both clinical and basic pharmacology.

The strength and type of binding forces and the location of binding sites on the albumin molecule are the primary characteristics which must be determined in order to understand ligand–albumin interactions. Since many drugs are small, aliphatic anions, the carboxylic fatty acids (FA) have been frequently used in studies of these characteristics, as they provide a convenient homologous series of ligands with similar charge but varying chain length/lipophilicity. In common with other aspects of drug physiological disposition (e.g. absorption [4] and distribution [5]) and receptor interactions, chain length and hydrophobicity have been suggested to be important determinants of ligand–albumin binding [6]. Structure–activity studies have shown linear relationships between lipophilicity (octanol/water partition) and albumin-binding affinity for various ligands, including the FA [7]. It has been suggested, however, that the chain length of FA profoundly affects the location of binding to the albumin molecule, and that binding affinity is not simply related to lipophilicity. Many observations have contributed to the assertion that serum albumin binds long- and medium-chain FA at different sites (see Refs. 8 and 9 for review). A demarkation seems to exist between unbranched FA of 10 carbons [i.e. nine-carbon alkyl chain (decanoate)] or less and those of 12 carbons (dodecanoate) or more [8, 9]. This phenomenon is of additional interest since the primary binding site of the FA with 10 or fewer carbons seems to be identical with one of the major drug-binding sites, the indole/benzodiazepine site ([8], also called the diazepam site [10] or site '2' [9, 11]). It is possible, then, that these medium-chain FA might serve as useful probes of this major binding site.

However, it is not clear if all FA of chain length 10 or less bind to the same site [12]. In addition, it is not known whether chain length dependence is restricted to the FA alone or if other groups may show similar properties. In this investigation, we have examined the characteristics of the primary and secondary albumin binding of short- and medium-chain FA with less than 10 carbons and the corresponding uncharged carbamates. The competition between these classes of ligands for albumin sites has also been studied. In addition, we have examined the binding interaction between these ligands and the drug warfarin, which is a prototype for a second major drug-binding albumin site [8–11].

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MATERIALS AND METHODS

Chemicals. FA (BDH, Poole, U.K.) of at least 98% purity were redistilled prior to use and prepared as sodium salts by dissolving in equimolar NaOH solutions. Radiolabeled FA (1-^{14}C) were purchased as the sodium salts (Radiochemical Center, Amersham, U.K.). *n*-Hexyl and *n*-octyl carbamates and [1-^{14}C]*n*-octyl carbamate were synthesized by the Chemistry Division of the Chemical Defence Establishment (Porton Down, U.K.); other carbamates were from commercial sources. In all cases purity was 99% by GLC. Bovine serum albumin [BSA, Cohn fraction V (Sigma Chemical Co., St. Louis, MO)] was 97% pure and human serum albumin (Albumin Kabi, Kabi, Stockholm, Sweden) was 98% pure. The fatty acid content of both albumins was less than 0.5 moles/mole. Although the exact composition was not known the FA were predominantly stearate and palmitate.

Ultrafiltration. All quantitative binding parameters were obtained from assays of free-ligand concentration in filtrates of ligand–albumin solutions following ultra filtration through regenerated cellulose (Scientific Instrument Center, London, U.K.), i.e. bound ligand was determined from the assay of the free concentration, knowing the total ligand concentration. FA and octyl carbamate concentrations were assayed by liquid scintillation counting; concentrations of the other carbamates were assayed by GLC. Cellulose dialysis tubing (18/32" dia, 22-cm strips) was thoroughly washed prior to use by boiling, followed by several rinses with distilled water. Tubing was formed into a bag, into which 6 ml of albumin–ligand solution were transfered. All solutions were prepared in 66 mM phosphate buffer, pH 7.4. The cellulose bag was tied off and supported by a glass scinter assembly within a 50-ml centrifuge tube for centrifugation at 2000 *g* (ave.) for 1 hr at temperatures of 10–37°. Under these conditions ultrafiltration pressure is low (see Ref. 13 for discussion of the importance of low-pressure ultrafiltration) and less than 10% of the original solution is removed as ultrafiltrate. Free-ligand analyses were performed on replicate 100 μl samples of ultrafiltrate. In all experiments, albumin-free solutions were used to check for recovery and for binding of ligand to the apparatus. In all cases > 95% recovery was obtained.

In all cases, the albumin concentration was 2.9×10^{-4} M (2% w/v) with fatty acid concentrations varying from 1.7×10^{-5} to 5×10^{-2} M and carbamate concentrations from 1×10^{-3} to 1×10^{-2} M. At least four replicate experiments were run at each of 10–15 different total ligand concentrations for each fatty acid. GLC was performed using a Porapac P column (Phase Separations, Flintshire, U.K.) with column temperatures of 160° (methyl and ethyl carbamates) or 180° (all other carbamates) and flame ionization detection. Liquid scintillation counting utilized a dioxane-based scintillant cocktail, external-standard quench correction and a Packard 3320 scintillation counter.

Fluorescence measurements. All fluorescence measurements were made using 1-cm cells in a Perkin–Elmer MPF-3 spectrofluorometer (excitation and emission slit widths of 5 mm), at 20 ± 1°. Data were calculated from uncorrected emission spectra. For protein fluorescence, albumin solutions (2 ml of 1×10^{-5} M BSA in 66 mM phosphate buffer, pH 7.4) were titrated with 1-μl additions of fatty acid solution. Up to 40 μl of 1×10^{-3}–1×10^{-1} M stock solutions were added. The excitation wavelength was 290 nm; the unquenched emission peak was at 341 nm. For albumin-bound warfarin fluorescence, 2 ml of 1.5×10^{-5} M warfarin in 1.0×10^{-5} M BSA solution was titrated with fatty acid, as before. Excitation wavelength was 320 nm and the emission peak wavelength was 378 nm.

Data handling. Binding parameters for FA were calculated from the experimental data using a computerized best-fit to the following Scatchard equation:

$$r = \frac{n_1 K_1 [Df]}{1 + K_1 [Df]} + \frac{n_2 K_2 [Df]}{1 + K_2 [Df]}$$

where r = moles ligand bound/mole of protein, n_1 = number of primary binding sites, K_1 = association constant for primary sites, $[Df]$ = free-ligand concentration, n_2 = number of secondary binding sites, and K_2 = association constant for secondary binding sites. (The authors are grateful to Dr. John Frances, Roche Products, Welwyn Garden City, U.K., for developing and executing the MODFIT 27 program.) Thermodynamic parameters were calculated from affinity constants using standard equations [14]. Enthalpy damage (ΔH) was calculated from binding parameters at 10 and 37°, assuming a linear relationship with temperature. The binding parameters for the carbamates were calculated assuming a single class of binding sites and by linear regression (without weighting) of the Scatchard plots obtained.

RESULTS

Binding of FA to BSA

The binding of the FA to BSA was measured by ultrafiltration and the data analyzed from Scatchard plots. Representative data are shown in Fig. 1 which illustrates the non-linear relationship which was found for each compound, and also an example of the computer-generated best-fit curves. Using a weighted, non-linear regression the data were fitted to a model with variable n_1, n_2, K_1 and K_2 values which yielded estimates of n_1 (number of primary sites) of 1.0–1.67 for the different FA. The model was modified by fixing n_1 as 1, in order to allow more precise comparisons of primary affinities for different FA and for comparisons with published data. The computed primary binding constant ($n_1 K_1$) was not significantly different for the two models (data not shown). Two classes of sites were assumed in all the analyses presented, although a statistically superior fit could be obtained by including a third class of sites. For example, the nonanoate data at 37° were well fitted to a model of $n_1 = 1.3 \pm 0.1$; $n_2 = 8.7 \pm 1.4$; $n_3 = 10.5 \pm 10.2$ and $K_3 = 55.2 \pm 111.9$ M^{-1}. However, little significance could be attached to the coefficients n_3 and K_3 because of consistently large computed errors.

The number of secondary sites (n_2) increase with

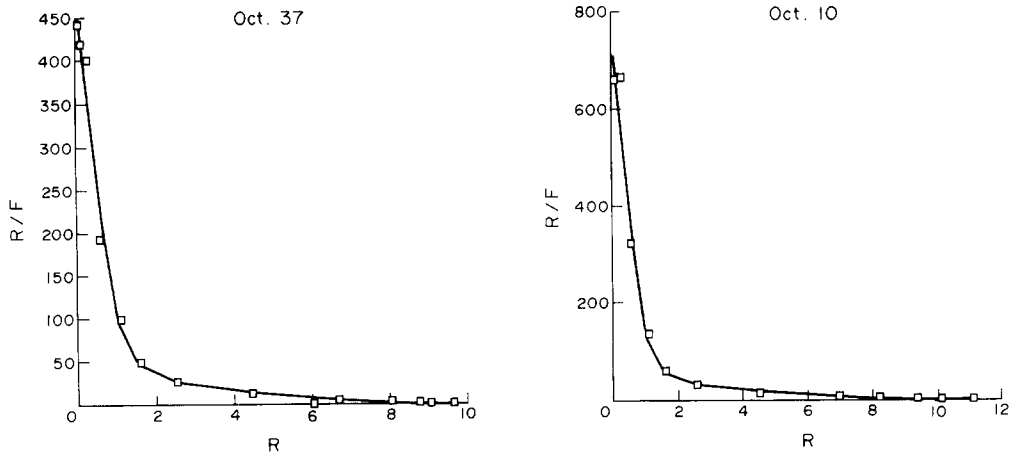

Fig. 1. Scatchard plots of the interaction of BSA with octanoate at 37 and 10°. Each point represents the mean value of r and $r/D\gamma$ determined from four estimates at the same total octanoate concentration. The line is the computer-generated best-fit line assuming two classes of binding site with $n_1 = 1$. Note: R = r, R/F = r/Df.

Table 1. Binding constants for the interaction of FA with BSA*

Temperature (°)	Fatty acid	Binding constants Primary $K_1 \times 10^4$ (M^{-1})	Secondary n_2	Secondary $K_2 \times 10^2$ (M^{-1})
10	Pentanoate	2.30 ± 0.10	1.9 ± 0.7	6.23 ± 3.81
	Hexanoate	4.36 ± 0.17	2.2 ± 0.3	8.73 ± 0.8
	Heptanoate	5.49 ± 0.24	3.8 ± 0.7	25.5 ± 8.67
	Octanoate	73.1 ± 3.95	7.6 ± 0.8	30.4 ± 5.87
	Nonanoate	89.5 ± 4.57	10.3 ± 0.7	43.4 ± 5.49
37	Pentanoate	1.29 ± 0.05	5.3 ± 1.8	3.88 ± 1.33
	Hexanoate	2.39 ± 0.11	2.8 ± 0.4	16.0 ± 3.4
	Heptanoate	4.14 ± 0.42	4.5 ± 0.5	30.8 ± 3.9
	Octanoate	45.5 ± 1.64	7.5 ± 0.7	37.0 ± 4.1
	Nonanoate	49.3 ± 1.52	9.8 ± 0.7	45.6 ± 5.7

* Binding determined by ultrafiltration, as described in Materials and Methods. Primary equilibrium association constants (K_1) were calculated assuming a single primary site ($n_1 = 1$). S.E.s of the estimates are computer generated from the non-linear regression program MODFIT 27.

Table 2. Thermodynamic parameters for fatty acid–BSA interactions*

	ΔG (kcal M^{-1}) 10°	37°	ΔH (kcal M^{-1})	ΔS (kcal M^{-1} degree^{-1})
		Primary		
Pentanoate	5.65	5.83	−3.73	+6.76
Hexanoate	5.90	6.21	−3.88	+7.51
Heptanoate	6.13	6.55	−1.82	+15.2
Octanoate	7.59	8.02	−3.06	+16.0
Nonanoate	7.70	8.07	−3.85	+13.6
		Secondary		
Pentanoate	3.62	3.67	—†	—†
Hexanoate	3.81	4.54	+3.91	+27.3
Heptanoate	4.41	4.95	+1.22	+19.9
Octanoate	4.51	5.08	+1.27	+20.4
Nonanoate	4.71	5.19	+0.32	+17.8

* Parameters calculated from the affinity constants shown in Table 1.
† Not calculated because the number of sites changed with temperature.

chain length from about 2 for pentanoate to 10 for nonanoate (Table 1). With the exception of pentanoate, there is no significant difference between n_2 values determined at 10 and 37°. The n_2 values may be underestimated for the short-chain FA, because there are few data points at high 'r' values due to total fatty acid concentration being kept well below the critical micelle concentration. In addition, these points are given little weight since the fitting program weights data according to the experimental variation in r and r/Df. The greatest experimental variation is at high r values, where there is the lowest percentage binding.

For all the FA, an opposite temperature dependency is observed for primary and secondary binding affinities, with primary affinity decreasing with increasing temperature (Table 1). Because n_2 varies with temperature for pentanoate binding, secondary affinities at different temperatures can not be compared for this compound. From this limited temperature dependency data, the enthalpy (ΔH) and entropy (ΔS) for the reactions were calculated (Table 2). Primary binding is characterized by a relatively constant negative ΔH and an increasingly favorable ΔS with increasing chain length. In contrast, secondary binding seems to be typified by a relatively constant ΔS with a decreasingly unfavourable positive ΔH as chain length increases.

The chain length dependence of primary and secondary binding affinities [or free-energy change (ΔG)] are quite different. For binding at both 10 and 37° the affinity for the primary site increases steadily from pentanoate to heptanoate and from octanoate to nonanoate but there is a disproportionately large increase in affinity between heptanoate and octanoate (Fig. 2A). In contrast, there are no sharp changes in average secondary binding affinities (or ΔG values) with increasing chain length although there is a trend towards lesser incremental increases in K_2 with elongation of chain length at both 10 and 37° (Fig. 2B). It is interesting that the secondary total binding constant (n_2K_2) shows a linear relationship to chain length at either 10 or 37° (Fig. 2C).

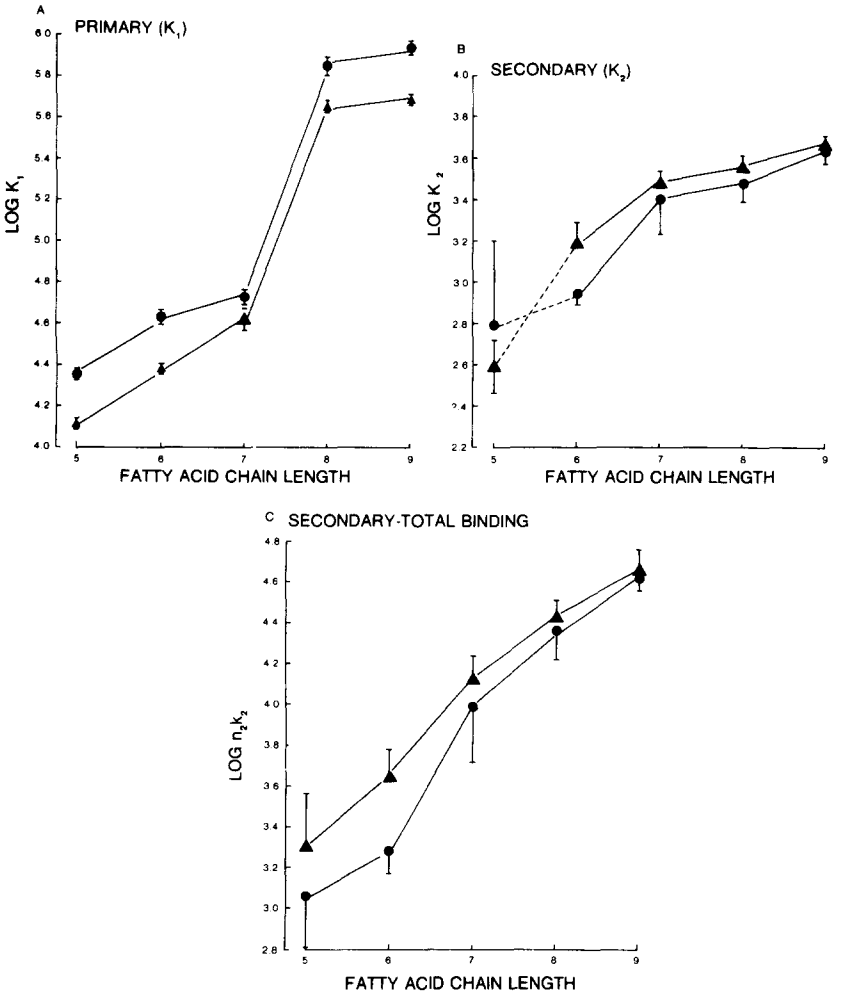

Fig. 2. The influence of fatty acid chain length on primary (K_1) and secondary (K_2) association constants and the secondary total binding constant (n_2K_2) for binding to BSA. Binding curves were determined at 10° (●) and 37° (▲) by ultrafiltration.

Table 3. Competition of octanoate and pentanoate for BSA binding*

A. Pentanoate as ligand					
Octanoate		Percentage of pentanoate bound			
Concentration ($M \times 10^{-4}$)	Competitor/BSA ratio	Concentration ($M \times 10^{-4}$) 0.25	Ligand/BSA ratio 0.1:1	Concentration ($M \times 10^{-4}$) 25.0	Ligand/BSA ratio 10:1
0	—	60.2 ± 0.9		15.8 ± 0.4	
0.25	0.1:1	61.3 ± 0.5		N.D.	
2.5	1:1	58.0 ± 0.4		14.0 ± 0.9	
25.0	10:1	N.D.		5.6 ± 0.9†	
250.0	100:1	5.6 ± 0.8†		N.D.	
B. Octanoate as ligand					
Pentanoate		Percentage of octanoate bound			
Concentration ($M \times 10^{-4}$)	Competitor/BSA ratio	Concentration ($M \times 10^{-4}$) 0.1	Ligand/BSA ratio 0.04:1	Concentration ($M \times 10^{-4}$) 10	Ligand/BSA ratio 4:1
0	—	98.8 ± 0.1		82.1 ± 0.2	
0.5	0.2:1	98.7 ± 0.1		N.D.	
10	4:1	98.6 ± 0.1		70.4 ± 0.1†	
25	10:1	N.D.		68.1 ± 0.1†	
100	40:1	97.5 ± 0.1†		N.D.	

* Percentage bound determined by ultrafiltration at 20°; BSA concentration = 2.5×10^{-4} M. Values are $x \pm$ S.E. ($n = 4$).

† Statistically different from per cent bound without competitor, $P < 0.05$, Student's t-test.

Competition between FA for binding sites

Because the disparate primary binding affinities of heptanoate and octanoate may reflect different binding sites for medium-chain FA of eight or more carbons and short-chain FA of seven or less carbons, competition experiments were performed between short- and medium-chain FA. Also, because of the difficulties in the interpretation of full competition curves where both ligand and competitor bind to primary and secondary sites [15], a simpler experimental design was followed. For each ligand, two concentrations were chosen where binding would be predominantly to primary and predominantly to secondary sites, respectively. A range of competitor concentrations were used under both conditions. The data for the competition between octanoate and pentanoate are presented in Table 3A and B.

At a ligand/BSA ratio where binding of pentanoate is predominantly to primary sites, octanoate has no significant influence on binding at a competitor/BSA ration of 1:1 (Table 3A). Since the K_1 for octanoate is at least 10 × that for pentanoate this would suggest that primary pentanoate and octanoate binding is to different sites. This is confirmed by the finding that pentanoate, at 1000-fold the concentration of octanoate, is unable to markedly displace octanoate from its primary binding site (Table 3B). It seems, however, that the primary pentanoate site may be a secondary site for octanoate: high concentrations of octanoate are able to displace pentanoate from its primary site (Table 3A); in addition, at equimolar concentrations, under conditions where both are bound to both primary and secondary sites (ratio to BSA = 4:1), pentanoate can reduce the binding of octanoate (Table 3B). Consideration of the K_1 and K_2 data shows that the only explanation for this phenomenon is that the primary site for pentanoate ($K_1 = 2.3 \times 10^{-4}\,M^{-1}$) is a secondary site for octanoate ($K_2 = 0.3 \times 10^{4}\,M^{-1}$) The converse seems not to be true, i.e. the primary octanoate site ($K_1 = 73 \times 10^{4}\,M^{-1}$) is not a secondary pentanoate site ($K^2 = 0.06 \times 10^{4}\,M^{-1}$) because a 1000-fold excess of pentanoate, approximately equivalent to the difference in affinities, had no effect on primary octanoate binding. Finally, the marked reduction in the percentage pentanoate bound predominantly to secondary sites (ligand/BSA ratio = 10:1) upon the addition of octanoate at the same concentration suggests that the two acids share some secondary binding sites (Table 3A).

Thus, four conclusions may be drawn from these data: (1) pentanoate and octanoate primary sites are different; (2) the primary pentanoate site is a secondary octanoate site; (3) the primary octanoate site is not a secondary pentanoate site; and (4) octanoate and pentanoate share some secondary sites.

Binding of carbamates to BSA

We wished to establish whether the chain length dependence of fatty acid binding to BSA extended to other short- and medium-chain aliphatic series. Carbamates were chosen because of their lack of charge and because previous work has demonstrated linear relationships between the lipophilicity of carbamates and various biological phenomena (e.g. Ref. 16). Preliminary examination of BSA binding of 12 branched and straight-chain carbamates showed a reasonable correlation of the total binding constant (nK) to the octanol/water partition coefficient (see Ref. 17). However, consideration of straight-chain carbamates alone suggests that this overall trend may mask significant chain length changes.

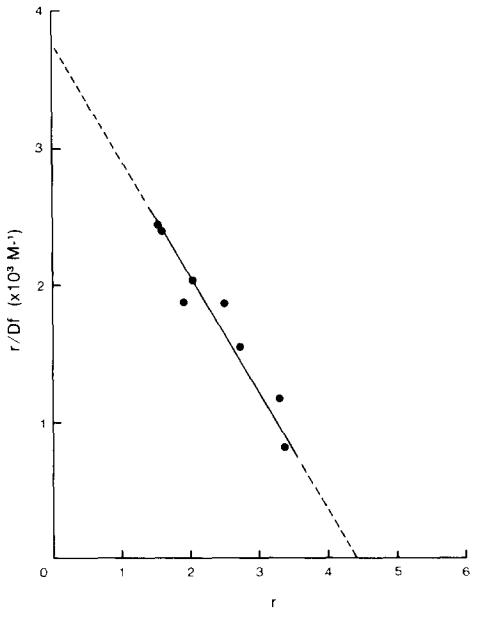

Fig. 3. Scatchard plot of the interaction of BSA with *n*-pentyl carbamate at 37° determined by ultrafiltration. Each point represents the mean of three determinations at the same total carbamate concentration. The lines represent the following linear regression: $Y = 37.4 \pm 3.2 - (8.5 \pm 1.3)X$ [R^2 (correlation coefficient) = 0.97].

The carbamate binding data are less reliable than those of the fatty acids because the gas chromatographic assay system was more variable and less sensitive than the radiometric assay, limiting the lowest total ligand concentrations to 1×10^{-3} M. In addition, the compounds are relatively insoluble, limiting high ligand concentrations to 1×10^{-2} M (low solubility of *n*-heptyl and *n*-octyl carbamates prevented the estimation of full binding curves). Data were typically of the nature illustrated in Fig. 3. This was interpreted as indicating one class of sites, but it is not clear if a wider ligand concentration range would have revealed non-linearity in the Scatchard plots.

From this limited data, it appears that there may be a sharp increase in the binding affinity (K) or the total binding constant (nK) from pentyl carbamate to hexyl carbamate (Table 4, Fig. 4) at either 10 or 37°. It should be noted that the total chain length of hexyl carbamate is 8, i.e. $C_6H_{13}OC(NH_2)O$, which may behave similarly to the fatty acid octanoate $C_7H_{15}C(OH)O$. Furthermore, the temperature dependence of binding for the carbamates yields thermodynamic parameters which are generally consistent with those for primary fatty acid binding to BSA, i.e. negative ΔH and positive ΔS (Table 4). There is some chain length dependence of the number of sites for carbamates binding to BSA (Table 4). Methyl and ethyl carbamates show two sites while longer carbamates show four to six sites, with no pronounced trend in the change with temperature (Table 4).

Competition of carbamates and FA for BSA binding

Since there was a suggestion that carbamates and FA show some similarities in BSA binding, competition between the two groups was examined (Table 5). Two pairs of compounds, *n*-butyl carbamate/pentanoate and nonanoate/*n*-octyl car-

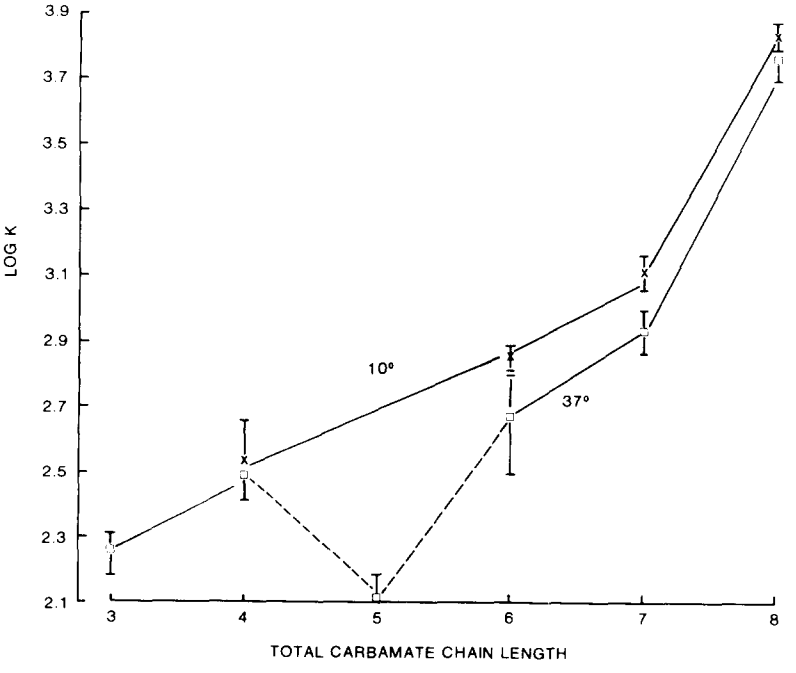

Fig. 4. The influence of carbamate chain length on the association constant for binding too BSA at 10 and 37°. Total chain length is calculated by analogy to the FA, i.e. if $C_4H_9C(OH)O$ (pentanoate) is 5 then $C_3H_7OC(NH_2)O$ (*n*-propyl carbamate) is also 5.

Table 4. Binding parameters of the interaction of carbamates with BSA*

	10°			37°				
	n	K ($\times 10^2\ M^{-1}$)	ΔG (kcal M^{-1})	n	K ($\times 10^2\ M^{-1}$)	ΔG (kcal M^{-1})	ΔH (kcal M^{-1})	ΔS (kcal M^{-1} degree^{-1})
Methyl	N.D.	N.D.	—	1.3 ± 0.1	1.8 ± 0.3	3.19	—	—
Ethyl	2.3 ± 0.6	3.4 ± 1.2	3.27	1.7 ± 0.2	3.1 ± 0.5	3.52	−0.59	+9.5
Propyl	N.D.	N.D.	—	6.7 ± 0.7	1.3 ± 0.2	3.00	—	—
Butyl	6.4 ± 0.4	7.0 ± 0.5	3.67	5.6 ± 1.6	4.7 ± 1.6	3.78	−2.56	+3.9
Pentyl	6.7 ± 0.6	12.8 ± 1.6	4.01	4.4 ± 0.4	8.5 ± 1.3	4.14	−2.63	+4.9
Hexyl	6.7 ± 0.4	66.8 ± 5.2	4.94	3.3 ± 0.6	58.0 ± 8.9	5.32	−0.91	+14.2

* Binding determined by ultrafiltration, as described in Materials and Methods. Data were fitted to a model with one class of binding sites.

bamate were chosen; in each case the carbamate was one greater in total chain length, but within the same putative binding site groups (i.e. short- and medium-chain). This was performed in order to reduce the large difference between affinities of carbamates and FA. Under conditions where pentanoate is bound to its primary site, *n*-butyl carbamate has little effect on percentage binding (Table 5). Similarly, nonanoate has little effect on *n*-octyl carbamate binding (Table 5), even at 20-fold excess and

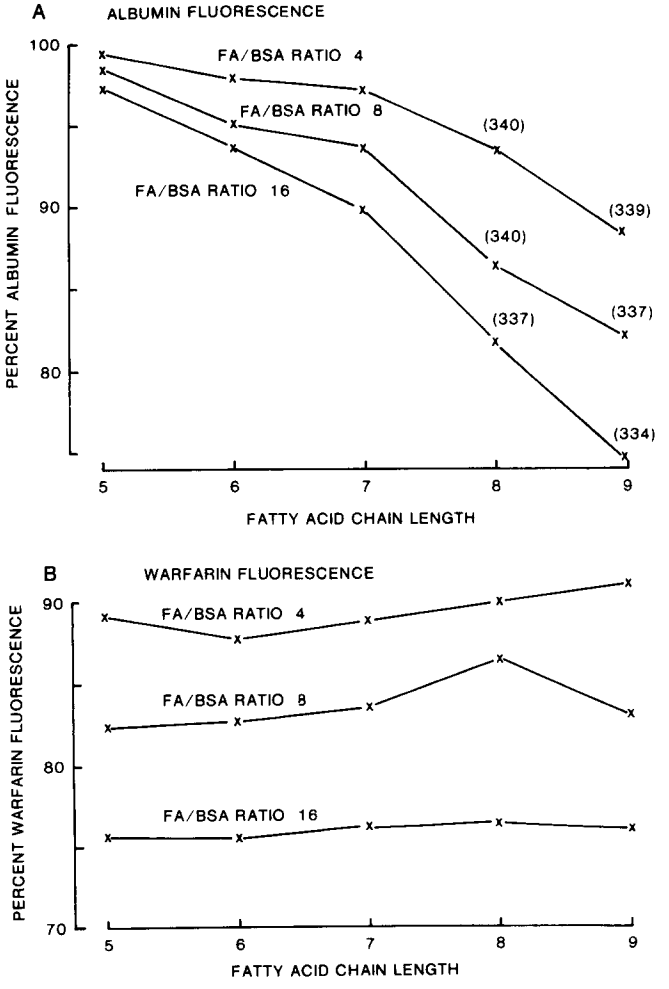

Fig. 5. The influence of FA on intrinsic BSA fluorescence (A) and on BSA-bound warfarin fluorescence (B). In (A), BSA concentration was 1×10^{-5} M and fatty acid was present at 4, 8 or 16×10^{-5} M. Excitation wavelength was 290 nm and emission was at 341 nm, unless otherwise indicated. In (B), BSA was 1×10^{-5} M, warfarin was 1.5×10^{-5} M and fatty acid was present at 4, 8, or 16×10^{-5} M. Excitation wavelength was 320 nm and emission was at 378 nm in all cases. All data are presented as per cent of control fluorescence in the absence of fatty acid. Each point is the mean of four determinations; errors are too small to be presented.

Table 5. Competition for BSA binding between *n*-butyl carbamate/pentanoate and *n*-octyl carbamate/nonanoate*

n-Butyl carbamate/pentanoate				Nonanoate/*n*-octyl carbamate			
n-Butyl carbamate competitor		Pentanoate ligand		Nonanoate competitor		*n*-Octyl carbamate ligand	
Concentration ($M \times 10^{-4}$)	Competitor/BSA ratio	Concentration 0.1×10^{-4} M	Ligand/BSA ratio 0.04:1	Concentration ($M \times 10^{-4}$)	Competitor/BSA ratio	Concentration 0.5×10^{-4} M	Ligand/BSA ratio 0.2:1
		Percentage bound				Percentage bound	
0	—	82.6 ± 0.3		0	—	94.5 ± 0.2	
1.67	0.7:1	82.6 ± 0.1		0.5	0.2:1	95.0 ± 0.2	
8.33	3.3:1	81.6 ± 0.1†		2.5	1:1	93.5 ± 0.4	
16.7	6.7:1	80.5 ± 0.1†		5.0	2:1	93.9 ± 0.3	
33.0	13:1	77.6 ± 0.1†		10.0	4:1	92.7 ± 0.1†	

* Binding was determined by ultrafiltration, at 20°, with a BSA concentration of 2.5×10^{-4} M. Each value is $x \pm$ S.E. ($n = 4$).
† Significantly different from per cent bound without competitor, $P < 0.05$, Students *t*-test.

despite the higher affinity of the fatty acid. Both of these observations suggest that the FA and carbamates do not share common primary binding sites.

Fluorescence studies

BSA fluorescence. The effects of FA on BSA fluorescence at various molar ratios to BSA were examined and the data are summarized in Fig. 5A. Although all the FA quench the fluorescence of BSA, significant effects are obtained only at molar ratios greater than 3. Only octanoate and nonanoate cause a shift in the fluorescence maximum, in these cases a blue-shift to shorter wavelengths. In addition, the quenching of BSA is more pronounced for the binding of the medium-chain FA, octanoate and nonanoate, compared to the short-chain FA (Fig. 5A). Although the disparate effects of the short- and medium-chain FA are consistent with different primary sites for the two groups it should be emphasized that these effects were observed only at high molar ratios to BSA, when binding is to both primary and secondary sites.

Warfarin fluorescence. Warfarin fluorescence was monitored under conditions where warfarin (1.5×10^{-5} M) was bound predominantly to its high-affinity site on BSA (1×10^{-5} PM). Low molar ratios of FA have little effect on warfarin fluorescence but at higher molar ratios there is a significant reduction in BSA-bound warfarin fluorescence (Fig. 5B). There is a tendancy for the short-chain FA to be slightly more effective than the medium-chain FA, particularly at molar ratios of 4 and 8 (Fig. 5B). It is possible that the short-chain fatty acid primary site may be a primary warfarin site but that pronounced competition is not observed at low molar ratios because of the larger primary affinity of warfarin (about 3×10^5 M^{-1} [18]). Since the primary short-chain FA site may be a secondary medium-chain site, this may explain the quenching by the medium-chain FA.

Similar warfarin/FA competition experiments were performed with HSA as the binding protein and with quantification of FA binding by ultrafiltration, using radioactive FA as previously described. Under conditions where the fatty acid is bound predominantly to its primary site (FA/HSA ratio = 0:6:1), increasing warfarin concentrations have little effect upon octanoate and nonanoate binding but significantly reduce the binding of hepanoate (Fig. 6A). It is not possible to tell if this phenomenon is due to short-chain FA and warfarin sharing sites, or if it is a reflection of the warfarin primary affinity being higher than heptanoate but lower than octanoate or nonanoate. Under conditions where FA are bound to both primary and secondary sites (FA/HSA ratio = 6:1), warfarin significantly reduces the binding of heptanoate, octanoate and nonanoate by similar amounts (Fig. 6B). This is consistent with the primary short-chain FA site being both the warfarin site and a secondary medium-chain FA site. It is also likely that the FA and warfarin share some secondary sites.

DISCUSSION

The ultrafiltration analysis of short- and

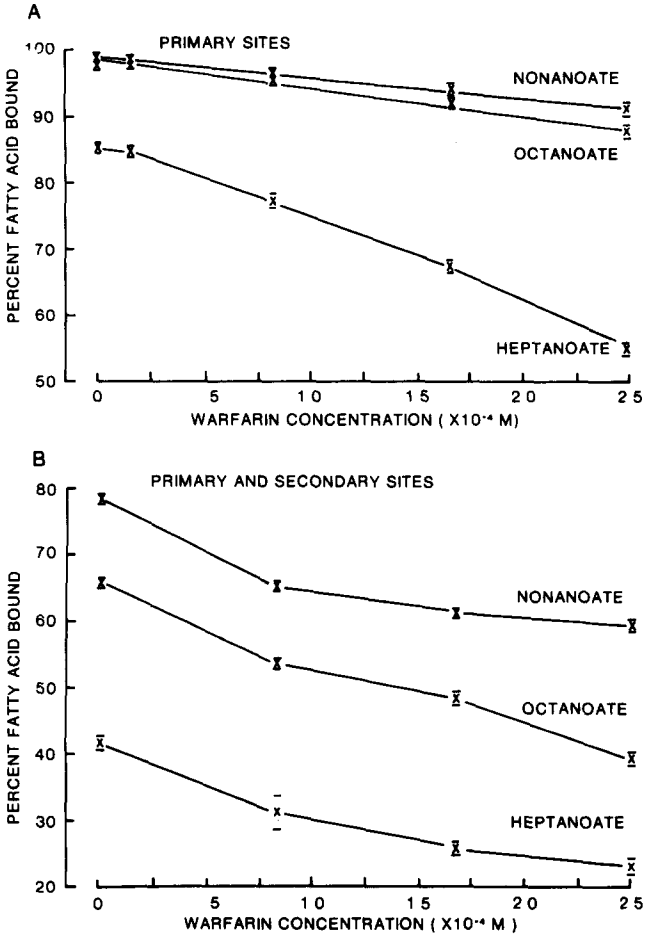

Fig. 6. The influence of warfarin on the per cent of fatty acid bound to HSA determined by ultrafiltration. (A) Primary fatty acid sites: fatty acid 1.67×10^{-4} M, HSA 2.5×10^{-4} M. (B) Primary and secondary sites: fatty acid 16.7×10^{-4} M, HSA 2.5×10^{-4} M. Each data point is $\bar{x} \pm$ S.E. ($n = 4$).

medium-chain fatty acid interactions with BSA shows that the FA have a single primary binding site on the protein. This is in contrast to early studies of FA–BSA binding in which four to seven primary sites were reported by other investigators [19] but consistent with a recent investigation, which also reports a single primary site [12]. It is likely that the primary sites reported in the former studies actually represent secondary sites, with primary binding being overlooked, due to the absence of data at low ligand–albumin ratios. This view is supported by binding affinities in those studies (10^2–10^3 M^{-1}) [19] being of the same order of magnitude as those reported here for secondary binding.

The number of secondary binding sites increased with increasing chain length and was not dependant upon temperature (Table 1). Similar increases in the number of sites with chain length has been previously observed for FA [12] and other ligands [20]. As will be discussed, these secondary interactions are hydrophobic in nature and it is possible that there are more sites available to longer chain length FA because the longer chains permit greater (hydrophobic) binding to areas of weak hydrophobicity, stabilizing an interaction which is too weak with the shorter molecules. Alternatively, the longer FA may have a greater 'flexibility' [21] to conform to weak hydrophobic areas on the protein.

The major observation of this study is the non-linear increase in primary binding affinity with increasing FA chain length, with a surprisingly large increase in affinity between C7 (heptanoate) and C8 (octanoate). This may be explained by either: (i) different primary sites for short-chain FA with less than eight carbons and for medium-chain FA with eight or more carbons or (ii) a single site for both groups of FA but one which has a much greater affinity for FA with eight or more carbons. Although we have insufficient data to conclusively prove either hypothesis, we favor the explanation of different sites, based upon the competition data for pentanoate and octanoate. These data show that there is no mutual competition of pentanoate and octanoate for primary binding sites on BSA (Table 3). These data also suggest that the primary pentanoate site is one secondary octanoate site but that the primary octanoate site is not a secondary pentanoate site. We suggest the following model to explain these observations. The short-chain FA site is a relatively small binding area which can accommodate FA up

to C7, providing increasing hydrophobic interactions with increasing chain length. Longer-chain FA do not fit this site ideally, although binding can take place, with reduced affinity. The medium-chain FA site does not bind short-chain FA either because the conformational adaptability [21] of longer chain length is required for efficient interaction with the binding site or because the site is composed of a cationic residue and a strongly hydrophobic area separated by a distance such that the short-chain FA could not bridge the gap.

This chain length influence on binding sites or binding affinity is contrary to previous characterization of FA–BSA interactions as linearly correlated to lipophilicity [7]. However, the results reported here are similar to a recent study of FA–BSA interactions [12]. Rodrigues de Miranda *et al.* [12] reported that the free energy of the FA–BSA interaction does not increase linearly with increasing chain length, but that there is a plateau between C5 and C6 and a further increase at C7. From competition data they concluded that BSA posesses two types of hydrophobic site, which can accommodate alkyl chains of up to pentanoate and octanoate respectively. These data vary from those reported here only in the 'break' in affinity or free-energy change being at C6–C7 rather than C7–C8, as we have observed.

In this study we did not examine FA of chain length greater than C9 because of their tendency to associate in aqueous solution and because they do not pass freely through dialysis membranes [22]. Therefore, we can not be certain of the upper limit for the length of FA which bind to the proposed medium-chain site. However, from published observations described later, it seems likely that FA < C10 and those < C10 bind to different sites. These same data suggest that both HSA and BSA have similar medium-chain FA binding properties. The affinity of the first binding site from a multiple equilibrium analysis of FA–HSA interactions shows a sigmoidal relationship to chain length, with a disproportionately large increase between C10 and C12 [23]. FA < C10 competitively displace a bilirubin spin label from HSA while those > C10 enhance the binding [24]. Similarly, only FA < 10 carbons inhibit the reaction of paranitrophenyl acetate with HSA [25] and the same FA, only, compete with a range of drugs for one HSA binding site [26]. This binding site is the indole–benzodiazepine or diazepam site [8] which has been most thoroughly studied using HSA but also seems to exist on the BSA molecule. Octanoate competes for BSA binding with chlorophenoxybutyric acid [27] and also with tryptophan [28] both of which are diazepam site ligands. Other data which indicate that medium- and long-chain FA have different sites and that medium-chain FA, particularly octanoate, are ligands for the diazepam site have been recently reviewed [8, 9, 29]. In summary, we conclude that the primary HSA or BSA binding site for FA of 8–10 carbons is the indole–benzodiazepine site.

Since our data suggest that FA of less than eight carbons bind to a different site, it was of interest to see if these short-chain FA might bind to another major drug-binding site. The azapropazone/warfarin binding site ([8, 11], also called site '3' [10] or site '6' [9]) is the second major drug-binding site on serum albumin. We observed that the quenching of BSA-bound warfarin fluorescence appeared to be more efficient for the shorter FA, and warfarin displaced heptanoate from its primary HSA site much more efficiently than it displaced either octanoate or nonanoate. This suggests that the short-chain FA may be bound to the same site as warfarin, although the data are far from conclusive. The fact that there was not a direct 1:1 competition in either of the earlier experiments may be explained by the fact that the azapropazone/warfarin site is thought to be a large binding 'area' of overlapping sites [8].

The thermodynamic parameters for the interaction of the FA with BSA seem to reflect electrostatic/hydrophobic interactions for primary binding and purely hydrophobic binding for secondary binding. For primary binding, the relatively constant enthalpy change with different chain length FA is probably due to binding of the carboxylate group to the protein. The increasingly favorable free-energy change with increasing chain length is due to increasing positive entropy. Positive entropy changes in ligand binding are normally assumed to reflect hydrophobic interactions [30] although Klotz [31] has shown that electrostatic interactions can also be entropically driven. It may be significant that heptanoate showed a lower ΔH than the other FA, perhaps reflecting impaired interaction with the binding site for this fatty acid, the longest which binds to the putative short-chain site. The thermodynamic parameters for secondary fatty acid binding must be considered less reliable since K_2 values are more variable and are also average values for several sites. Secondary binding was accompanied by positive enthalpy and entropy changes, which is characteristic of hydrophobic binding. The binding of carbamates to BSA was characterized by negative ΔH and positive ΔS changes, as for primary fatty acid binding, but showing no discernable trend with chain length. In this case it is possible that combined hydrogen binding and hydrophobic interactions account for the thermodynamic changes.

The apparent increase in affinity for hexyl carbamate in comparison to pentyl carbamate (Fig. 4) occurs at the same overall chain length as the 'break' for FA binding and suggests that the phenomenon of short- and medium-chain sites is not restricted to the FA. However, we did not observe any competition between the FA and carbamates suggesting that they do not share binding sites. The similar 'break' may reflect the fact that hydrophobic binding areas, in general, are limited to certain sizes. We suggest that different classes of hydrophobic binding areas may exist including one class which requires a chain length of 8–10 carbons and one which binds, ideally, chains of 5–7 carbons. This concept has also been proposed by Rodrigues de Miranda *et al.* [12] based upon their observations described earlier. They conclude that hydrophobic binding areas on proteins cannot accommodate groups with a carbon chain of more than four atoms [12]. Thus, a third class of sites, for chains of four carbons or less may also represent a general feature of protein hydrophobic binding areas.

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