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A COMPARISON OF DRUG BINDING SITES ON MAMMALIAN ALBUMINS

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Abstract—The fluorescent probes warfarin and dansylsarcosine are known to selectively interact with binding sites I and II, respectively, on human albumin. This paper investigates whether similar binding sites exist on bovine, dog, horse, sheep and rat albumins. Binding sites on albumins were studied by: (1) displacement of warfarin and dansylsarcosine by site I (phenylbutazone) and site II (diazepam) selective ligands; (2) the effects of non-esterified fatty acids (carbon chain lengths: C_5–C_{20}) and changes in pH (6–9) on the fluorescence of warfarin and dansylsarcosine; and (3) the ability of site selective ligands to inhibit hydrolysis of 4-nitrophenyl acetate. For bovine, dog, horse, human and sheep albumins the fluorescence of bound warfarin and dansylsarcosine was selectively decreased by phenylbutazone and diazepam, respectively. For these albumins medium chain fatty acids (C_7–C_{12}) reduced the fluorescence of dansylsarcosine (maximum inhibition with C_9) whereas long chain acids (C_{12}–C_{20}) enhanced the fluorescence of warfarin (maximum increases with C_{12}). In addition, changes in pH from 6 to 9 increased the fluorescence of warfarin and although site I ligands (warfarin/phenylbutazone) had no pronounced effects on 4-nitrophenyl acetate hydrolysis, site II ligands (dansylsarcosine/diazepam) significantly inhibited this reaction. Rat albumin behaved differently from the other albumins studied in that the C_{12}–C_{20} fatty acids and changes in pH did not enhance the fluorescence of warfarin. Moreover, the differential effects of site I and site II ligands on the fluorescence of warfarin/dansylsarcosine and hydrolysis of 4-nitrophenyl acetate were less apparent with rat albumin. The results suggest bovine, dog, horse and sheep albumins have binding sites for warfarin and dansylsarcosine with similar properties to sites I and II on human albumin. By contrast, the warfarin binding site and to a lesser degree the dansylsarcosine site, of rat albumin have different characteristics from these sites on the other albumins studied.

It is well established that there are two major and structurally selective drug binding sites on human albumin [1–4] and Sudlow *et al.* [2] have termed these sites I and II. Both sites were distinguished on the basis of experiments which revealed differences in the pattern of ligand binding and displacement [1–4]. In addition, changes in pH over the range 6 to 9 enhanced binding of fluorescent probes to site I but not to site II [5] and medium chain fatty acids (C_8–C_{11}) reduced binding to site II without altering adsorption to site I [6]. The esteratic properties of human albumin have also been used to discriminate between sites I and II. Substrates such as 4-nitrophenyl acetate (NPA†) are hydrolysed at a locus which is either identical to or is in close proximity to site II [7, 8]. Hence ligands which bind to site II inhibit hydrolysis of NPA [7, 8].

The locations of sites I and II in the human albumin molecule are not known for certain, but site I is believed to exist on loop 4 of domain II in close proximity to tryptophan 214 [9]. The ligands that adsorb here are mainly weak acids with bulky structures that have a negative charge in the centre of the molecule [2]. Phenylbutazone and warfarin are typical of the ligands which interact with site I and indeed, this site is often referred to as the warfarin binding site. It seems likely though that site I is not a discrete locus, but more probably a binding area since the warfarin and phenylbutazone binding sites only partially overlap [4]. By contrast to site I, site II is thought to be on loop 7 of domain III and tyrosine 411 is one of the important amino acids at this locus [9, 10]. The OH group of tyrosine 411 is preferentially acetylated during hydrolysis of NPA [9]. Ligands with an elongated shape with or without a negative charge bind to this site [2, 6] and diazepam, together with dansylsarcosine, are examples of substances that interact selectively with site II. This binding locus is often referred to as the benzodiazepine/indole binding site [9].

In view of the likely structural similarities between albumins [9], and observations that human albumin has two major drug binding sites, it might be anticipated that other mammalian albumins have binding sites analogous to those on human albumin. Therefore the aim of this work was to answer the question: do sites equivalent to I and II of human albumin exist on other albumins? To address this question the effects of selective site I (phenylbutazone) and site II (diazepam) ligands; non-esterified fatty acids (NEFAs) (C_5–C_{20}) and pH on the binding of warfarin (site I probe) and dansylsarcosine (site II probe) to six different mammalian albumins have been investigated by fluorescence spectroscopy. The ability of these

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† Abbreviations: k, apparent first-order rate constant for hydrolysis of 4-nitrophenyl acetate; N-B, neutral-to-base; NEFAs, non-esterified fatty acids; NPA, 4-nitrophenyl acetate.

albumins to hydrolyse NPA in the absence and presence of site-selective ligands and NEFAs (C_5–C_{20}) has also been examined. Albumins studied were bovine, dog, horse, sheep and rat, and the results obtained for these proteins have been compared to those for human albumin.

MATERIALS AND METHODS

Materials. The following albumins were purchased from the Sigma Chemical Co. (Poole, U.K.): human (lot No. 86F-9310), bovine (126F-9350), dog (37F-327), horse (66F-9381), rat (114F-9379) and sheep (127F-9306). All albumins except dog and rat were supplied as fatty acid free. Dog and rat albumins were defatted by the method of Chen [11] and the fatty acid content of all albumins used was estimated, using the colorimetric method of Duncombe [12], to be <0.1 mole fatty acid per mole of protein. Dansylsarcosine, NPA, NEFAs (C_5–C_{20}) and warfarin were also obtained from Sigma. Phenylbutazone and diazepam were gifts from Geigy Pharmaceuticals (Macclesfield, U.K.) and Roche Products Ltd (Welwyn Garden City, U.K.), respectively. All other chemicals were of analytical grade and were obtained from the usual suppliers.

Fluorescence experiments. The effect of 20 μM phenylbutazone, diazepam or NEFAs (C_5–C_{20}) on the fluorescence intensity of a fixed concentration of either dansylsarcosine or warfarin (2 μM) bound to various defatted albumins (20 μM) was measured at pH 7.4 and 31°, according to procedures described by us in detail previously [13]. The excitation/emission wavelengths for dansylsarcosine and warfarin were: 350/475 and 320/380 nm, respectively. Some NEFAs (C_{16}–C_{20}) had to be added in ethanol. The percentage of ethanol in the final solutions did not exceed 1% (v/v). This concentration of ethanol reduced the fluorescence of dansylsarcosine by about 3%, but it had a much greater effect on the fluorescence of warfarin (30% reduction). However, for each probe the effect of ethanol was taken into account by titrating warfarin– or dansylsarcosine–albumin solutions with appropriate volumes of solvent. Changes in fluorescence in the presence of C_{16}–C_{20} NEFAs were then expressed as a percentage of the fluorescence intensity of the probe–albumin solution of equivalent ethanol concentration. To investigate the influence of pH on the fluorescence of dansylsarcosine and warfarin, defatted albumin solutions were prepared in 0.1 M sodium phosphate buffers whose pH varied from 6.0 to 9.0. Final concentrations of probe and albumin in these experiments were 2 and 20 μM, respectively.

Kinetic experiments. Hydrolysis of NPA by defatted albumin was studied using the method of Means and Bender [14]. Briefly, 10 μL of NPA (1 mM in acetonitrile) were added to 2 mL of 0.05 M Tris buffer, pH 8.0, containing 50 μM albumin. This solution was mixed and resultant changes of absorbance were measured continuously at 400 nm and 24° until the reaction was complete. Inhibitors were added to the Tris–albumin solution to give a final concentration of 50 μM. The concentration of albumin was set 10-fold greater than that of NPA (5 μM) in order to generate a pseudo-first-order reaction, the rate of which depends only on the concentration of NPA.

Analysis of results. The apparent rate constant, k, for hydrolysis of NPA by albumin was calculated using the expression:

$$\ln[(A_\infty - A_0)/(A_\infty - A_t)] = kt$$

where A_∞ is the absorbance at the end of the reaction; A_0 is the absorbance at the start and A_t the absorbance at time t. Linear regression analysis was used to estimate k from graphs of $\ln[(A_\infty - A_0)/(A_\infty - A_t)]$ versus t.

All results are reported as mean ± SEM and statistical comparisons were made with Student's non-paired t-test.

RESULTS

Effects of phenylbutazone, diazepam and NEFAs on the fluorescence of warfarin and dansylsarcosine

Table 1 shows the effect of either phenylbutazone or diazepam (20 μM) on the fluorescence intensity of warfarin and dansylsarcosine (2 μM) bound to bovine, dog, horse, human, sheep and rat albumins (20 μM). Phenylbutazone significantly ($P < 0.001$) decreased the fluorescence of warfarin bound to human albumin; but it had no statistically significant ($P > 0.05$) effect on the fluorescence of dansylsarcosine. By contrast, diazepam had no significant ($P > 0.05$) effect on the fluorescence intensity of warfarin. However, the benzodiazepine substantially decreased the fluorescence of dansylsarcosine bound to human albumin (Table 1). These data agree with those reported in the literature [1–4] and they illustrate: (1) binding of warfarin and dansylsarcosine to separate locations on human albumin and (2) selective displacement of these fluorescent probes by phenylbutazone and diazepam, respectively.

A similar pattern of results was obtained for bovine, dog, horse and sheep albumins in that phenylbutazone selectively diminished the fluorescence of warfarin and diazepam decreased the fluorescence intensity of only dansylsarcosine (Table 1). Rat albumin, however, was atypical because phenylbutazone significantly ($P < 0.05$) decreased the fluorescence of both probes and diazepam had no statistically significant ($P > 0.05$) effect on the binding of each fluorescent probe. The rank orders for net reductions in fluorescence were: (a) for warfarin–phenylbutazone, human (44%) > bovine (23%) > horse (20%) > sheep (15%) ≥ dog (13%) > rat (9%) and (b) for dansylsarcosine–diazepam, human (37%) > horse (18%) ≥ dog (15%) ≥ bovine (14%) > sheep (9%) = rat (9%) (Table 1).

The effect of NEFAs (C_5–C_{20}, 20 μM) on the fluorescence of the site I and II probes (2 μM) bound to various albumins (20 μM) is illustrated in Fig. 1. It is clear from this figure that NEFAs have similar qualitative effects on the fluorescence of dansylsarcosine (site II probe) for all albumins studied. Short chain acids (C_5–C_6) had no marked effect on fluorescence, but from C_7 onwards the decrease in fluorescence induced by NEFAs progressively increased with maximum reductions seen in the presence of C_9. Thereafter the reductions

Table 1. Effect of phenylbutazone and diazepam on the fluorescence of either warfarin or dansylsarcosine bound to various albumins at pH 7.4 and 31°

Albumin (20 μM)	Warfarin (2 μM)		Dansylsarcosine (2 μM)	
	Phenylbutazone (20 μM)	Diazepam (20 μM)	Phenylbutazone (20 μM)	Diazepam (20 μM)
Human	56 ± 2‡	94 ± 2	97 ± 1	63 ± 3‡
Bovine	77 ± 3†	98 ± 1	100 ± 1	86 ± 4*
Dog	87 ± 3*	98 ± 1	98 ± 1	85 ± 4*
Horse	80 ± 3†	98 ± 1	95 ± 2	82 ± 3†
Rat	91 ± 1*	96 ± 2	91 ± 1*	91 ± 2
Sheep	85 ± 2‡	98 ± 2	100 ± 1	91 ± 2*

Values are fluorescence as a percentage of control; mean ± SEM (N = 3–6). The molar ratio of phenylbutazone and diazepam to albumin was 1:1. *P < 0.05; †P < 0.01; ‡P < 0.001 relative to control fluorescence (*t*-test).

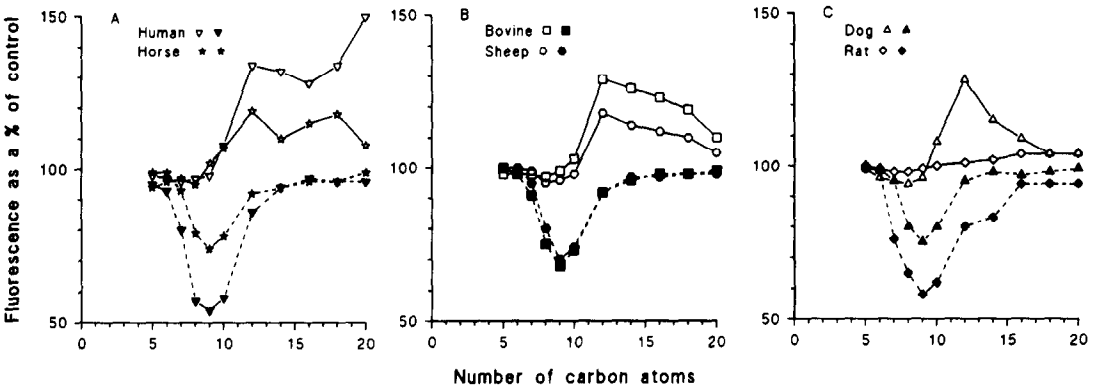

Fig. 1. Effect of NEFAs (C_5–C_{20}) (20 μM) on the fluorescence intensity of either warfarin (2 μM; solid line) or dansylsarcosine (2 μM; dashed line) in the presence of various albumins (20 μM). Fluorescence was measured at 31° and is expressed as a percentage of fluorescence intensity in the absence of fatty acid. Data are the mean of three observations and SEM values are too small to be shown.

in fluorescence diminished, such that the C_{16}–C_{20} acids had no pronounced effects on fluorescence intensities. There were, however, some quantitative differences among albumins with respect to the size of the inhibitory effect induced by C_9. For example, the largest net decrease in fluorescence occurred with human albumin (46%; Fig. 1A) whereas the smallest decrement was observed with dog albumin (25%; Fig. 1C).

By contrast to dansylsarcosine, NEFAs with carbon chain lengths greater than C_{10} tended to increase the fluorescence of warfarin (Fig. 1). For human and horse albumin, C_5–C_9 fatty acids had no striking effects on fluorescence intensity; but the C_{10}–C_{20} fatty acids enhanced fluorescence with peaks occurring at C_{12} and C_{20} for human albumin, and C_{12} and C_{18} for horse albumin (Fig. 1A). With bovine, dog and sheep albumins, NEFAs up to C_9 had little effect on warfarin's fluorescence (Fig. 1B and C). However, longer chain acids (C_{10}–C_{20}) increased fluorescence intensities with maximum increments occurring at C_{12}. Thereafter, fluorescences decreased such that with C_{20} present fluorescence intensities had almost returned to control values. By contrast to the other albumins, the entire range of NEFAs had no distinct effect on the fluorescence of warfarin bound to rat albumin (Fig. 1C). The rank order for the net enhancement of fluorescence induced by C_{12} was: human (33%) > bovine (28%) = dog (28%) > horse (19%) ≥ sheep (18%) > rat (0%).

Effect of pH on the fluorescence of dansylsarcosine and warfarin

Unbound dansylsarcosine (2 μM) had no detectable fluorescence in phosphate buffer over the pH range 6.0 to 9.0 and the fluorescence of unbound warfarin (2 μM) remained constant in this range. Figure 2 shows the influence of pH on the fluorescence intensity of the two probes (2 μM) in the presence of the various albumins (20 μM). The fluorescence intensity of bound dansylsarcosine remained constant for all six albumins (Fig. 2A and B). However, for most albumins the fluorescence of warfarin increased with pH. With bovine, sheep and horse albumins the enhancement of warfarin's fluorescence appeared

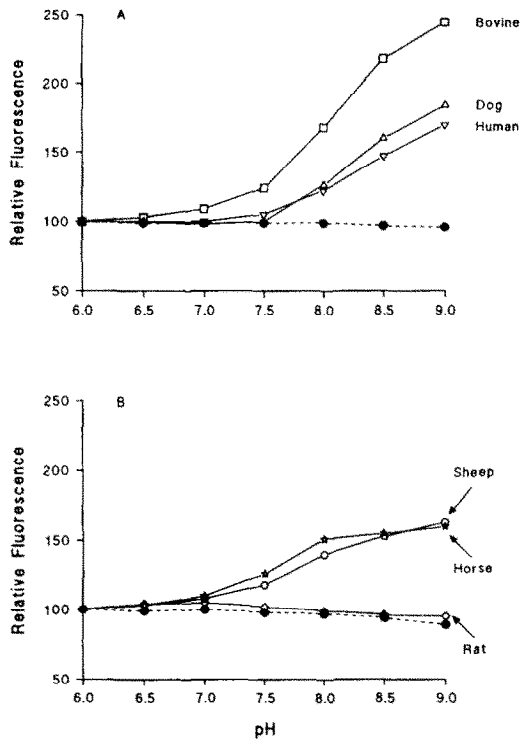

Fig. 2. Effect of pH on the fluorescence intensity of either warfarin (2 μM; solid line) or dansylsarcosine (2 μM; dashed line) in the presence of various albumins (20 μM). Fluorescence was measured at 31° and is expressed as a percentage of fluorescence at pH 6.0. Data are the mean of three observations and SEM values are too small to be shown. Data for dansylsarcosine represent combined results for the three albumins shown in each caption.

to start as soon as the pH changed from 6.0 to 6.5 and the rate of increase tended to slow as the pH approached 9.0 (Fig. 2A and B). The fluorescence intensity of warfarin bound to human or dog albumins, remained constant up to pH 7.5 and then it started to increase at what seemed to be a constant rate until pH 9.0 was reached (Fig. 2A). With bovine albumin the net increment in fluorescence was pronounced; 145% at pH 9.0 relative to the value at pH 6.0 (Fig. 1A). In the case of the other albumins net increases of between 60 and 80% were observed over the same pH range. By contrast to other albumins, the fluorescence of warfarin bound to rat albumin did not alter over the range pH 6.0 to 9.0 (Fig. 2B). The order of change in fluorescence at pH 9.0 relative to pH 6.0 was: bovine > dog > human > sheep ≥ horse > rat.

Effects of site I (warfarin and phenylbutazone) and site II (diazepam and dansylsarcosine) ligands on the hydrolysis of NPA

The first-order rate constant, k, for hydrolysis of NPA (5 μM) by different albumins (50 μM) showed marked inter-species differences (control values; Table 2). There was a 24-fold difference between the fastest (human) and slowest (horse) rates of hydrolysis and the rank order for the magnitudes of k was: human > bovine > dog > rat > sheep > horse. Table 2 also shows the effects of site I (warfarin and phenylbutazone) and site II (dansylsarcosine and diazepam) selective ligands on the rate of NPA hydrolysis at a molar ratio of 1:1 with albumin. Warfarin had no significant ($P > 0.05$) effect on the hydrolysis of NPA by human albumin. Phenylbutazone, however, inhibited hydrolysis ($P < 0.05$), but the extent of this effect (k reduced to about 88% of control value) was small compared to the pronounced inhibition of the reaction caused by both diazepam and dansylsarcosine (Table 2). Both these site II ligands reduced k to about 25% of its control value ($P < 0.001$). A similar pattern of results was obtained for bovine, dog, horse and sheep albumins, in that the site I ligands had little or no effect on NPA hydrolysis; but site II ligands all diminished k to at least 70% of the respective control value. However, in the case of rat albumin there was little difference between the effects of site I and II ligands on k (Table 2).

Effect of NEFAs on the hydrolysis of NPA by various albumins

Figure 3 shows the influence of different NEFAs (C_5–C_{20}), at a 1:1 molar ratio to albumin, on the rate of NPA hydrolysis. It is clear from the graph that the general pattern for the effects of NEFAs up to C_{10} was similar for all albumins. These fatty acids (C_5–C_{10}) inhibited hydrolysis and the extent of inhibition increased with carbon chain length. Maximum inhibition was found with C_8 for rat albumin and with C_{10} for dog albumin (Fig. 3A). However, for bovine, horse, human, and sheep albumins maximum inhibition occurred with C_9 present (Fig. 3). The rank order for maximum inhibition produced by the C_8–C_{10} acids was human (91%) > bovine (85%) > dog (75%) > sheep (65%) > rat (45%) > horse (28%). As the NEFA carbon chain length exceeded C_{10}, inhibition of hydrolysis became less marked, although k was still reduced to between 60 and 70% of its control value in the presence of C_{20} with all albumins except horse (Fig. 3). It is notable that horse albumin had the smallest value of k (Table 2) and NEFAs were much less effective as inhibitors of NPA hydrolysis for this albumin when compared to the other proteins studied.

DISCUSSION

A binding locus comparable to site I of human albumin seems to exist on albumins from cow, dog, horse and sheep. This conclusion is based on the following evidence. First, phenylbutazone reduced the fluorescence of warfarin bound to these albumins; but diazepam, which is known to interact selectively with site II on human albumin [1–4], had no significant effect on the fluorescence of warfarin. Second, some NEFAs with carbon chain lengths >C_{10} enhanced the fluorescence of warfarin bound to bovine, dog, horse, human and sheep albumins. For these albumins, a peak increase in fluorescence intensity occurred in the presence of C_{12}. Third for all albumins except rat, increases in pH from 6.0 to 9.0 augmented warfarin's fluorescence. Over this pH

Table 2. Effect of site I (warfarin and phenylbutazone) and site II (diazepam and dansylsarcosine) ligands on the hydrolysis of NPA by various albumins at pH 8.0 and 24°

Albumin (50 μM)	Control $k(sec^{-1} \times 10^2)$	Site I ligands: Warfarin (50 μM) k ($sec^{-1} \times 10^2$)	Site I ligands: Phenylbutazone (50 μM) $k(sec^{-1} \times 10^2)$	Site II ligands: Diazepam (50 μM) $k(sec^{-1} \times 10^2)$	Site II ligands: Dansylsarcosine (50 μM) $k(sec^{-1} \times 10^2)$
Human	4.1 ± 0.01	4.0 ± 0.10	3.6 ± 0.10*	0.91 ± 0.03†	1.06 ± 0.03†
Bovine	1.9 ± 0.10	1.8 ± 0.10	1.9 ± 0.20	0.97 ± 0.02†	0.58 ± 0.01†
Dog	1.07 ± 0.01	0.96 ± 0.02*	0.85 ± 0.02*	0.21 ± 0.01†	0.25 ± 0.01†
Horse	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.12 ± 0.01*	0.12 ± 0.01*
Rat	0.64 ± 0.02	0.58 ± 0.01	0.53 ± 0.01*	0.53 ± 0.02*	0.52 ± 0.01*
Sheep	0.55 ± 0.02	0.44 ± 0.01*	0.45 ± 0.01*	0.30 ± 0.01†	0.23 ± 0.01†

The molar ratio of ligand to albumin was 1:1 and the initial concentration of NPA was 5 μM.
Values are mean ± SEM (N = 3).
* $P < 0.05$; † $P < 0.001$ relative to control rate of reaction (t-test).

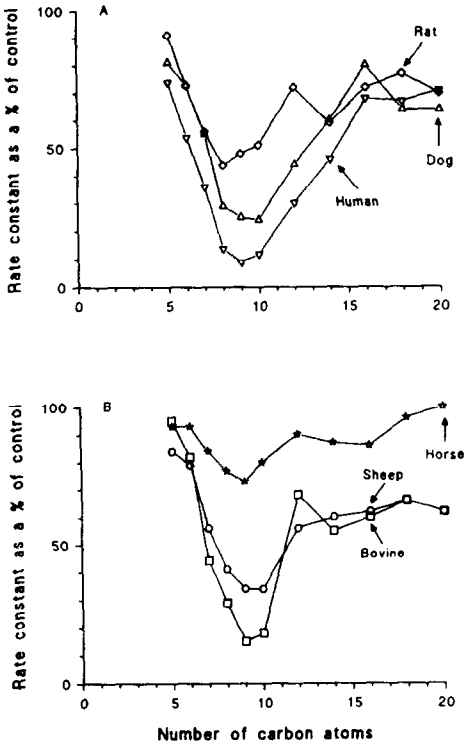

Fig. 3. Effect of NEFAs (C_5–C_{20}) (50 μM) on the apparent first-order rate constant for hydrolysis of NPA (5 μM). The rate of reaction was measured at 24° and pH 8.0. Data are the mean of three observations and SEM values are too small to be shown.

range albumin undergoes a conformational change referred to as the neutral-to-base (N–B) transition [9, 15]. Both the N–B transition, and long chain fatty acids, increase the affinity of warfarin for its binding site on bovine and human albumin [6, 16]. As the fluorescence of warfarin bound to dog, horse and sheep albumins increased with pH, it seems likely that these albumins also undergo an N–B transition which leads to enhanced fluorescence. Although all three of the above manoeuvres produced similar qualitative changes in the fluorescence of warfarin, there were some quantitative differences between albumins. For example, phenylbutazone reduced the fluorescence of warfarin bound to human albumin by 44%; but with dog albumin fluorescence intensity decreased by only 13%. Moreover, the NEFA C_{20} enhanced warfarin's fluorescence when bound to human albumin by 150%, yet this NEFA had little effect in the presence of bovine, dog, horse and sheep albumins. Such quantitative differences probably reflect variations between these albumins with respect to their affinities for warfarin, phenylbutazone and NEFAs.

The results of this study suggest the warfarin binding site of rat albumin is different from its counterpart on the other albumins investigated. The extent to which phenylbutazone reduced the fluorescence of warfarin bound to rat albumin was lowest when compared to the reductions noted with bovine, dog, horse, human and sheep albumins. Moreover, there were no detectable changes in fluorescence in the presence of C_{10}–C_{20} NEFAs or in response to changes in pH. Fluorescence intensity of a bound fluor is governed by both the extent of binding and fluorescence quantum yield. Differences in these factors between rat albumin and the other albumins studied might account for the anomalous behaviour of the warfarin binding site on rat albumin. There is evidence that the affinity of warfarin for rat albumin is higher than for human albumin. The affinity of warfarin for its primary binding site on human albumin ranges from 3.2×10^5 at 6° to $2.0 \times 10^5\ M^{-1}$ at 37° [17] but for rat albumin, Chignell [18] reported, at an unspecified temperature, an affinity of $8.2 \times 10^5\ M^{-1}$. Furthermore, the fluorescence quantum yield of warfarin bound to rat albumin has been reported to be greater than the quantum yields for the other albumins used in this study (rat = 0.153, horse = 0.088, dog = 0.083, sheep = 0.076, human = 0.070 and bovine = 0.069 [18]).

The various methods used to establish the presence of site II on human albumin [1–8], suggest that a corresponding site is present on bovine, dog, horse and sheep albumins. Diazepam significantly diminished the fluorescence of dansylsarcosine bound to bovine, dog, horse, human and sheep albumins whereas the site I selective ligand, phenylbutazone, had no statistically significant effect. In addition, the medium chain NEFAs (C_7–C_{12}) decreased the fluorescence intensity of dansylsarcosine when bound to these albumins and the maximum reduction occurred in the presence of nonanoic acid (C_9). Similar observations by Wanwimolruk *et al.* [6], and consideration of the molecular size of NEFAs and other ligands which interact with site II, lead them to suggest that site II on human albumin is a hydrophobic cleft of depth 12–16 Å and width about 6–8 Å. The limited data obtained here with C_7–C_{12} NEFAs are consistent with a site of comparable dimensions existing on bovine, dog, horse and sheep albumins.

Studies of the effect of selective site I and site II ligands on the esterase activity of bovine, dog, horse, human and sheep albumin showed good agreement between these proteins. Hydrolysis of NPA was inhibited by the selective site II ligands diazepam and dansylsarcosine; but warfarin and phenylbutazone had little or no effect on this process. In addition, the medium chain NEFAs (C_7–C_{12}) inhibited deacetylation of NPA and the maximum effect was observed with either C_9 (bovine, horse, sheep) or C_{10} present (dog). These results also support the presence on bovine, dog, horse and sheep albumins of a binding site analogous to site II of human albumin. Despite the similarities between these albumins, there were some quantitative differences with respect to the size of the k, the rate constant for hydrolysis of NPA. Horse albumin in particular reacted slowly with NPA and compared to other albumins, the medium chain NEFAs were the least effective inhibitors of NPA hydrolysis. The OH group on tyrosine 411 of human albumin is thought to be preferentially acetylated during hydrolysis of NPA [9]. In horse albumin tyrosine 411 is replaced by valine which has no OH group [19]. It is possible that this change in amino acids accounts for the slow rate of NPA hydrolysis observed with horse albumin.

The least discrimination between the effects of selective site I and II ligands on the fluorescence of bound dansylsarcosine and NPA hydrolysis was obtained with rat albumin. It is not clear from the present work why this occurred. However, rat albumin behaved like the other albumins studied in regard to the influence of medium chain NEFAs (C_7–C_{12}) on the fluorescence of dansylsarcosine and hydrolysis of NPA. This suggests better congruity, than was seen for warfarin, between the dansylsarcosine binding site on rat albumin and its binding locus on bovine, dog, horse, human and sheep albumins.

Based on studies with the dye 2-(4′-hydroxybenzeneazo)benzoic acid, Witiak and Whitehouse [20] concluded that "the rat may be a singularly unrepresentative species as far as drug-binding to its albumin is concerned". The results from the present investigation support this conclusion. The warfarin binding site, and to a lesser extent the diazepam/dansylsarcosine binding site, of rat albumin appear to have different attributes from these sites on the other albumins studied. Further work is necessary to clarify the reasons for this anomaly. However, it is possible such differences might contribute to inter-species variations in pharmacological and toxicological responses to albumin-bound drugs.

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