Biochemical Pharmacology, Vol. 41, No. 8, pp. 1227–1233, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

EFFECT OF VALPROIC ACID, ITS UNSATURATED METABOLITES AND SOME STRUCTURALLY RELATED FATTY ACIDS ON THE BINDING OF WARFARIN AND DANSYLSARCOSINE TO HUMAN ALBUMIN

MOHAMMAD R. PANJEHSHAHIN, CHRISTOPHER J. BOWMER* and MICHAEL S. YATES
Department of Pharmacology, Worsley Medical & Dental Building, University of Leeds, Leeds LS2 9JT, U.K.

(*Received* 11 *September* 1990; *accepted* 29 *November* 1990)

Abstract—The sites to which valproic acid and its main unsaturated metabolites (2-en-2-propyl pentanoic acid and 4-en-2-propyl pentanoic acid) bind to on human albumin were investigated by (1) measuring their ability to displace the fluorescent probes warfarin and dansylsarcosine and (2) by assessing the extent to which they inhibited the hydrolysis of 4-nitrophenyl acetate. Valproate and its metabolites displaced both warfarin and dansylsarcosine, and they also inhibited the hydrolysis of 4-nitrophenyl acetate. The order of potency for inhibition of both binding and hydrolysis was: 2-en-2-propyl pentanoic acid > 4-en-2-propyl pentanoic acid ≥ valproate. It is concluded that valproic acid and its unsaturated metabolites can displace ligands from the warfarin binding site (site I) and the benzodiazepine/indole binding site (site II), but the primary interaction is with site II. Furthermore, the introduction of a double bond into the carbon backbone of valproate increases affinity for albumin at both sites.

Valproic acid is an eight carbon branched chain fatty acid (2-propyl pentanoic acid) which is widely used to treat various forms of epilepsy [1]. At the plasma concentrations achieved after therapeutic doses it is highly bound to plasma proteins and the bulk of the drug appears to bind in a reversible fashion with albumin [2]. The interaction with human albumin *in vitro* is characterised by two classes of binding sites with an association constant of about $5 \times 10^4 M^{-1}$ for the high affinity sites and $2.5 \times 10^2 M^{-1}$ for the secondary sites, at 37° [3]. There is, however, some confusion in the literature on whether valproic acid's primary binding site corresponds to the warfarin binding site (site I) or to the benzodiazepine/indole site (site II). Sjöholm *et al.* [4] concluded that it interacts principally with site I; but Fleitman *et al.* [5] suggest binding to site II.

In man valproic acid is eliminated mainly by metabolism [6] and several of its metabolites have significant anticonvulsant activity. Most notable in this respect are the unsaturated metabolites 2-en-2-propyl pentanoic acid and 4-en-2-propyl pentanoic acid [1]. Indeed, active metabolites accumulate during chronic therapy with valproic acid and they may be responsible for increased efficacy noted during prolonged treatment and for the persistence of anticonvulsant effect seen when chronic dosage is stopped [7]. Little is known about the disposition of these metabolites, but because their structure is similar to that of the parent drug it can be anticipated that they will also interact with albumin.

The object of the present study was to clarify the conflicting reports on the nature of valproic acid's primary binding site and to determine if its metabolites interact with albumin. This has been done by: (1) investigating the ability of valproic acid and its metabolites to displace fluorescent probes that selectively adsorb to sites I (warfarin) and II (dansylsarcosine), and (2) by studying the effect of these compounds on the hydrolysis of 4-nitrophenyl acetate by human albumin. This latter process is inhibited by ligands which interact selectively with site II and therefore, it provides a test for binding to this site [8]. In addition, we have compared the effects of valproic acid to those of a number of structurally related fatty acids in order to ascertain whether branched chain fatty acids, such as valproate, have different binding characteristics from their straight chain isomers.

MATERIALS AND METHODS

Materials. Defatted human albumin (product no. A-3782), 4-nitrophenyl acetate, the fluorescent probes dansylsarcosine and warfarin, and fatty acids (C5-C8) were purchased from the Sigma Chemical Co. (St Louis, MO). Phenylbutazone and diazepam were gifts from Geigy (Summit, NJ) and Roche Pharmaceuticals (Nutley, NJ), respectively. Sodium valproate and its metabolites were donated by Sanofi U.K. 2-Methyl and 2-ethyl pentanoic acids were synthesized by reaction of either 2-bromopentane or 3-bromohexane, respectively, with Mg to produce the corresponding Grignard reagent. The magnesium salt of the desired carboxylic acid was prepared by slowly pouring the ethereal solution of Grignard reagent onto solid CO_2. This was then converted to the carboxylic acid by acid hydrolysis. The purity of these compounds was about 95% as assessed by high performance liquid chromatography [9]. All other chemicals were obtained from the usual laboratory suppliers.

The sodium salts of valproic and octanoic acid were dissolved directly in the buffers used, but

* Author for correspondence.

metabolites of valproate, other fatty acids together with phenylbutazone and warfarin were dissolved in about 0.5 mL of 0.5 M NaOH. Dissolution of diazepam was achieved with a similar volume of 0.5 M HCl. These solutions were then diluted to the appropriate concentration with buffer.

Fluorescence experiments. All work was done with 0.1 M sodium phosphate buffer, pH 7.4. Fluorescence intensity was measured at 31° with a Perkin–Elmer MPF-3 Spectrofluorimeter. The excitation and emission wavelengths were 350:475 and 320:380 nm for dansylsarcosine and warfarin, respectively and band width for both excitation and emission was 4 nm. Fluorescence measurements were done in triplicate and were corrected where necessary for self-absorption [10]. The methods used to determine the intrinsic molar fluorescences of bound (F_b) and unbound (F_u) probe have been described by us in detail [11]. Values of F_b and F_u for warfarin were 0.63 and 0.058 μM^{-1}, respectively, whereas F_b and F_u were 0.98 μM^{-1} and zero for dansylsarcosine. The equations given by Maes *et al.* [12] were used to calculate the concentrations of bound and unbound probe.

First, the effect of increasing concentrations (0 to 50 μM) of various inhibitors on the fluorescence of bound probes was assessed. Aliquots (20 μL) of a solution containing inhibitor (900 μM), albumin (20 μM) and probe (2 μM) were added to 2 mL of a solution containing 2 μM probe and 20 μM albumin. After each addition fluorescence was measured and the percentage change in intensity was calculated from: $F_i/F_o \times 100$, where F_o is the fluorescence of probe alone and F_i is fluorescence in the presence of inhibitor.

Second, the binding of warfarin and dansylsarcosine was measured by recording the fluorescence that resulted from titration of a fixed concentration of albumin with various concentrations of probes. Aliquots (20 μL) of a solution containing 100 μM probe and 2 μM albumin were added to 2 mL of a 2 μM albumin solution. Nine final concentrations of probe were used ranging from 0.99 to 13 μM. To investigate the effect of potential inhibitors, experiments were repeated using protein solutions containing either 5, 10 or 20 μM inhibitor.

Kinetic experiments. The hydrolysis of 4-nitrophenyl acetate (NPA) by human albumin was studied using the method of Means and Bender [8]. Briefly, NPA was dissolved in acetonitrile to give a 1 mM solution and 10 μL of this were added to 2 mL of 0.05 M Tris buffer, pH 8.0, containing 50 μM albumin. The final concentration of NPA was 5 μM. This solution was rapidly mixed and changes in absorbance were monitored continuously at 400 nm and 24° until the reaction was complete. Inhibitors were added to the Tris–albumin solution to give final concentrations of either 50 or 100 μM. The concentration of albumin was chosen to be tenfold greater than that of NPA to generate a pseudo-first order reaction in which the rate of reaction depends only on the concentration of NPA.

Analysis of results. Scatchard plots [13] for the binding of dansylsarcosine and warfarin showed some non-linearity both in the absence and presence of inhibitors. However, to simplify analysis of these data, binding was assumed to be homogeneous. Thus estimates of the number of binding sites, n, and apparent association constant, K, together with their standard errors, were obtained by non-linear least squares regression analysis [14] using the following equation:

$$r = \frac{nK[D_u]}{(1 + K[D_u])}$$

where r is the molar ratio of bound probe to albumin and $[D_u]$ is the concentration of unbound probe. The above equation can be rearranged to give:

$$(n - r)/r = 1/K[D_u]$$

thus if n is known, a plot of $\log\{(n - r)/r\}$ versus $\log[D_u]$ should be linear and have a slope of -1 if binding is homogeneous [15]. When the data for warfarin and dansylsarcosine were plotted using the logarithmic transformation and the values of n estimated by regression analysis, the graphs were linear with slopes of -0.95 ± 0.05 (N = 9; mean ± SE mean) and -1.01 ± 0.02 (N = 9), respectively. These slopes were not statistically different ($P > 0.05$) from -1 and this indicates that the simple binding model used here is adequate.

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

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

where A_∞ is the absorbance at the end of the reaction; A_o is absorbance at the beginning and A_t the absorbance at time t. Plots of $\ln[(A_\infty - A_o)/(A_\infty - A_t)]$ versus t were constructed and the slope, k, was estimated by linear regression analysis.

Results are given as mean ± SE and Student's non-paired t-test was used to compare means.

RESULTS

Displacement of dansylsarcosine and warfarin by diazepam and phenylbutazone

To check selectivity of binding, the effects of diazepam and phenylbutazone (0–50 μM) were examined on the fluorescence produced by a fixed concentration (2 μM) of probe. The molar ratio of diazepam and phenylbutazone to human albumin varied from 0 to 2.5. Figure 1 shows that as the concentration of phenylbutazone increased the fluorescence intensity of warfarin diminished in a curvi-linear manner. In the presence of 50 μM phenylbutazone fluorescence was reduced to about 40% of the initial intensity. Diazepam, however, caused a linear decrease in fluorescence, but at 50 μM the net decrease was only 17%. With dansylsarcosine as the probe, both the nature and extent of changes in fluorescence were reversed. Increasing concentrations of diazepam elicited a curvi-linear decrease and at 50 μM, fluorescence was reduced to about 35% of its initial value (Fig. 1). By contrast, phenylbutazone had little effect on fluorescence even at a final concentration of 50 μM.

Table 1 lists the binding parameters, n and K, obtained for the two fluorescent probes in the absence and presence of either phenylbutazone or

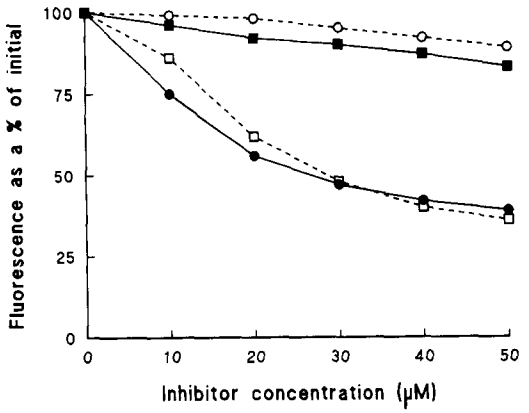

Fig. 1. Effect of increasing concentrations (0–50 μM) of diazepam (□, ■) and phenylbutazone (○, ●) on the fluorescence of dansylsarcosine (2 μM; broken line) and warfarin (2 μM; solid line) bound to defatted human albumin (20 μM). Fluorescence was measured at 31°, pH 7.4. Each point is the mean of three experiments and SE means are too small to be shown.

diazepam (5, 10 and 20 μM). Diazepam had little effect on the parameters for warfarin. However, the benzodiazepine caused a marked, concentration-dependent reduction in K for dansylsarcosine and this change occurred without a concomitant decrease in n. Phenylbutazone substantially reduced K for warfarin and n was also decreased at 5 and 10 μM (Table 1). However, in the presence of 20 μM phenylbutazone n increased from about 1.0, when no phenylbutazone was present, to 2.6. Phenylbutazone also reduced the binding of diazepam, although the extent of inhibition was less than that seen for warfarin (Table 1).

The results from these experiments are similar to those reported in the literature [16] and suggest the presence of two discrete binding sites on human albumin for warfarin and dansylsarcosine.

Effect of fatty acids (C5–C8)

Values of n and K for the binding of the fluorescent probes in the presence of pentanoic, hexanoic, heptanoic and octanoic acids (0–20 μM) are given in Table 2. These carboxylic acids had no major effect on the interaction of warfarin with albumin, but as the carbon chain length increased inhibition of dansylsarcosine's binding became more pronounced. Moreover, these acids mainly reduced K which suggests that inhibition was of a competitive type.

Effect of valproate, some analogues and its metabolites

Figure 2 shows the effect of increasing concentrations (0–50 μM) of valproic acid (2-propyl pentanoic acid); its 2-methyl and 2-ethyl analogues and two of its metabolites 2-en and 4-en-2-propyl pentanoic acid on the fluorescence intensity of the site I and II probes. Valproate diminished the fluorescence of both warfarin and dansylsarcosine such that at a concentration of 50 μM, the reduction of fluorescence for both probes was about 15%. 2-Methyl and 2-ethyl pentanoic acids, however, had no marked effect on the fluorescence of bound warfarin, but these analogues decreased dansylsarcosine's fluorescence to about the same extent as valproate.

Of the five metabolites tested three: 2-propyl glutaric acid, 2-propyl-4-methyl butyrolactone and 5-hydroxy-2-propyl pentanoic acid, had little effect on the fluorescence of either warfarin or dansylsarcosine. Consequently, data for these compounds are not shown in Figure 2. By contrast, the major unsaturated metabolites 2-en and 4-en-2-propyl pentanoic acids reduced the fluorescence intensity of both probes. The effect of 4-en-2-propyl pentanoic acid was similar to that noted with valproate. However, 2-en-2-propyl pentanoic acid caused a more marked and curvi-linear decrease in the fluorescence of the site I and site II probes. With both probes the rank order for potency of inhibition was: 2-en-2-propyl pentanoic acid > 4-en-2-propyl pentanoic acid ≥ valproic acid.

Scatchard plots depicting the interaction of warfarin and dansylsarcosine in the absence and presence of valproate and its unsaturated metabolites are displayed in Fig. 3. From this diagram it is clear that parent drug and its unsaturated metabolites

Table 1. Binding parameters for warfarin and dansylsarcosine obtained at 31° and pH 7.4 in the presence of either diazepam or phenylbutazone

Inhibitor and concentration (μM)	Warfarin		Dansylsarcosine	
	n	$K \times 10^{-5}$ (M^{-1})	n	$K \times 10^{-5}$ (M^{-1})
Diazepam				
0	1.1 ± 0.03*	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
5	1.0 ± 0.03	3.4 ± 0.3	0.80 ± 0.02	1.6 ± 0.1
10	1.0 ± 0.04	3.7 ± 0.4	0.84 ± 0.01	0.89 ± 0.01
20	0.79 ± 0.03	3.4 ± 0.3	0.93 ± 0.02	0.54 ± 0.09
Phenylbutazone				
0	1.1 ± 0.03	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
5	0.89 ± 0.03	0.81 ± 0.03	0.65 ± 0.02	5.1 ± 0.2
10	0.66 ± 0.05	0.47 ± 0.04	0.63 ± 0.01	5.2 ± 0.2
20	2.6 ± 0.1	0.06 ± 0.06	0.59 ± 0.01	3.2 ± 0.2

* Values are given as estimate ± SE estimate (7 degrees of freedom).

Table 2. Binding of parameters for warfarin and dansylsarcosine obtained at 31° and pH 7.4 in the presence of either pentanoic, hexanoic, heptanoic or octanoic acid

Inhibitor and concentration (μM)	Warfarin		Dansylsarcosine	
	n	$K \times 10^{-5}$ (M^{-1})	n	$K \times 10^{-5}$ (M^{-1})
Pentanoic acid				
0	1.1 ± 0.03*	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
10	1.1 ± 0.05	3.3 ± 0.4	0.77 ± 0.01	8.1 ± 0.5
20	0.86 ± 0.03	3.0 ± 0.3	0.80 ± 0.01	5.6 ± 0.2
Hexanoic acid				
0	1.1 ± 0.03	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
10	1.0 ± 0.03	3.3 ± 0.3	0.82 ± 0.01	4.0 ± 0.2
20	1.0 ± 0.03	2.5 ± 0.2	0.77 ± 0.02	3.6 ± 0.2
Heptanoic acid				
0	1.1 ± 0.03	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
10	1.1 ± 0.03	4.1 ± 0.3	0.87 ± 0.03	1.5 ± 0.1
20	0.91 ± 0.02	4.2 ± 0.3	0.85 ± 0.01	1.0 ± 0.02
Octanoic acid				
0	1.1 ± 0.03	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
10	0.91 ± 0.02	3.4 ± 0.3	1.1 ± 0.04	0.48 ± 0.03
20	0.84 ± 0.04	2.9 ± 0.3	1.0 ± 0.04	0.32 ± 0.02

* Values are given as estimate ± SE estimate (7 degrees of freedom).

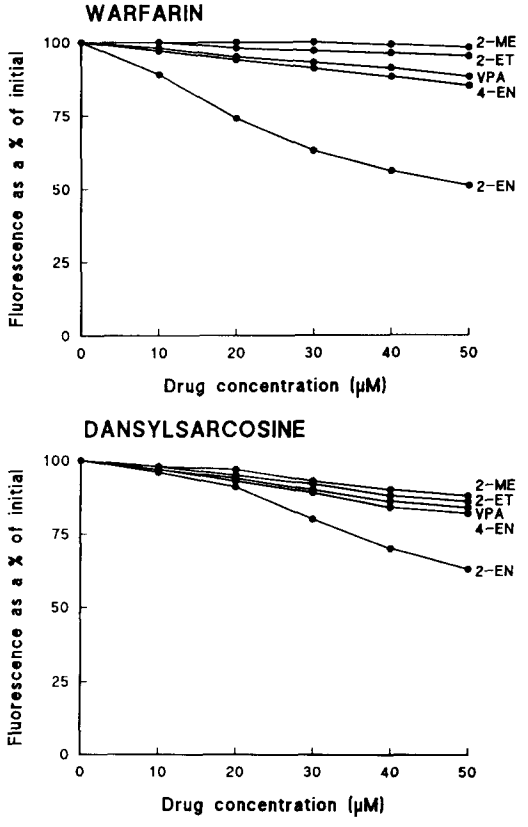

Fig. 2. Effect of increasing concentrations (0–50 μM) of valproic acid (VPA), 2-methyl pentanoic acid (2-ME), 2-ethyl pentanoic acid (2-ET), 2-en-2-propyl pentanoic acid (2-EN) and 4-en-2-propyl pentanoic acid (4-EN) on the fluorescence of warfarin (2 μM; upper) and dansylsarcosine (2 μM; lower) bound to defatted human albumin (20 μM). Fluorescence was measured at 31°, pH 7.4. Each point is the mean of three experiments and SE means are too small to be shown.

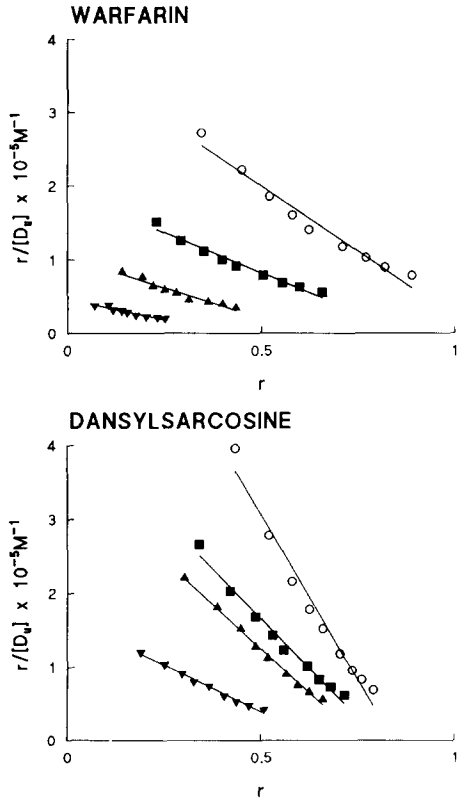

Fig. 3. Scatchard plots of warfarin (0.99–13 μM; upper) and dansylsarcosine (0.99–13 μM; lower) binding to defatted human albumin (2 μM) at 31° and pH 7.4, in the absence (○) and presence of valproic acid (■), 2-en-propyl pentanoic acid (▼) and 4-en-2-propyl pentanoic acid (▲). The concentration of valproic acid and its unsaturated metabolites was 10 μM.

Table 3. Binding parameters of warfarin and dansylsarcosine obtained at 31° and pH 7.4 in the presence of either valproic acid or its unsaturated metabolites

Inhibitor and concentration (μM)	Warfarin		Dansylsarcosine	
	n	$K \times 10^{-5}$ (M^{-1})	n	$K \times 10^{-5}$ (M^{-1})
Valproic acid				
0	1.1 ± 0.03*	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
10	0.91 ± 0.03	2.0 ± 0.2	0.81 ± 0.01	5.4 ± 0.3
20	0.92 ± 0.04	1.8 ± 0.2	0.78 ± 0.02	3.7 ± 0.2
4-En-metabolite†				
0	1.1 ± 0.03	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
10	0.65 ± 0.03	1.5 ± 0.2	0.76 ± 0.01	4.9 ± 0.2
20	0.52 ± 0.03	1.2 ± 0.1	0.76 ± 0.02	3.8 ± 0.2
2-En-metabolite‡				
0	1.1 ± 0.03	3.5 ± 0.3	0.84 ± 0.02	9.5 ± 0.8
10	0.49 ± 0.03	1.0 ± 0.1	0.66 ± 0.02	2.5 ± 0.2
20	0.45 ± 0.02	0.84 ± 0.06	0.66 ± 0.02	1.3 ± 0.1

* Values are given as estimate ± SE estimate (7 degrees of freedom). † 4-en-2-propyl pentanoic acid. ‡ 2-en-2 propyl pentanoic acid.

displaced both probes and in addition, the order of potency observed in the previous studies was preserved. The mechanism of displacement appears non-competitive since both slope and intercept, on the abscissa, of the Scatchard plots were decreased. Table 3 gives the value of n and K for warfarin and dansylsarcosine in the presence of various concentrations (0–20 μM) of valproate and the 2-en and 4-en metabolites. The main effect of valproate was to reduce K for both probes. However, 2-en-2-propyl pentanoic acid, and to a lesser extent its 4-en analogue, elicited a greater decrease in n and K for warfarin and dansylsarcosine than did the parent drug.

Hydrolysis of NPA by albumin

The mean value of the apparent rate constant, k, for the deacetylation of NPA was $4.14 \pm 0.21 \times 10^2$ sec^{-1} (N = 6). Figure 4A shows the effect of site I and site II ligands (50 μM) on the rate of reaction at a molar ratio of ligand to albumin of 1:1. At this ratio and at 2:1 (data not shown), warfarin had no significant ($P > 0.05$) effect on the rate of hydrolysis. Phenylbutazone did not alter k at a molar ratio of 1:1 (Fig. 4A). However, k was decreased by about 40% ($P < 0.05$) at a ratio of 2:1 and this is consistent with the results of the binding studies which showed that phenylbutazone inhibited binding to site II at molar ratios ≥ 2.5 (Table 1). Diazepam and dansylsarcosine decreased k by almost 80% at a molar ratio of 1:1 (Fig. 4A) and at ratio of 2:1, a further reduction in reaction rate of about 10% occurred relative to the control rate.

Pentanoic acid (C5) had no significant effect on the rate of hydrolysis at a molar ratio of 1:1. However, the C6 to C8 acids progressively inhibited the rate of reaction. Rates of reaction as a percentage of control in the presence of each acid were: 54.6 ± 1.7; 35.9 ± 2.5 and 14.0 ± 1.1% (N = 3). Figure 4B shows the effect of 1:1 molar ratios of valproate, 2-methyl and 2-ethyl pentanoic acids, and the 2-en and 4-en-2-propyl pentanoic acids. All these

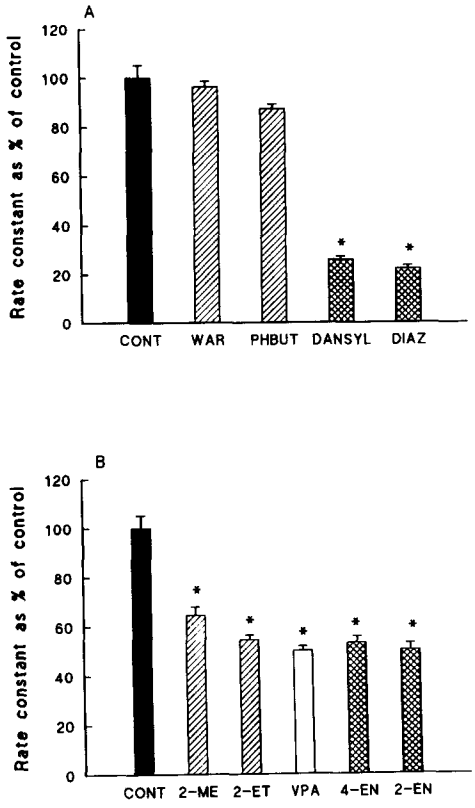

Fig. 4. Rate of hydrolysis of 4-nitrophenyl acetate (5 μM) by defatted human albumin (50 μM) at 24° and pH 8.0 in the absence (CONT) and presence of (A): warfarin (WAR), phenylbutazone (PHBUT), dansylsarcosine (DANSYL) and diazepam (DIAZ) and (B): 2-methyl pentanoic acid (2-ME), 2-ethyl pentanoic acid (2-ET), valproic acid (VPA), 2-en-2-propyl pentanoic acid (2-EN) and 4-en-2-propyl pentanoic acid (4-EN). The concentration of inhibitors was 50 μM and data are given as mean ± SE mean (N = 3–6). Significantly different from control values: * $P < 0.05$.

ligands resulted in a significant ($P < 0.05$) decrease in k of about 50% at a 1:1 ratio. On increasing the ratio to 2:1, these acids further inhibited the reaction and it is noteworthy that the 2-en metabolite had a significantly ($P < 0.05$) greater effect (k reduced to $15.9 \pm 0.8\%$ of control; $N = 3$) than either its 4-en analogue or the parent drug (k reduced to 33.5 ± 0.8 and $30.7 \pm 1.7\%$ of control, respectively; $N = 3$).

DISCUSSION

There are conflicting reports on whether valproate binds to site I or II. Sjöholm *et al.* [4] studied the displacement of diazepam and warfarin by a large number of drugs, and they concluded that valproate interacts with site I. In support of this proposal is the work by Monks *et al.* [17] who reported that valproic acid displaced phenytoin, a site I ligand [4], in what appeared to be a competitive manner. By contrast, Fleitman *et al.* [5] found that warfarin did not displace valproate and furthermore, valproate has been shown to inhibit the interaction of benzodiazepines with human albumin [18, 19]. These data suggest valproate is a site II ligand. However, the present work demonstrates that valproic acid can displace the fluorescent probes warfarin and dansylsarcosine from both site I and II. This agrees with the results obtained by Fehske *et al.* [20] who showed that valproate displaced both warfarin and diazepam to a similar extent.

Valproate inhibited the binding of dansylsarcosine to a greater degree than it displaced warfarin. In the presence of 10 μM valproic acid, the apparent association constant for dansylsarcosine diminished by 44% whereas K for warfarin decreased by 40%. This difference seems small, but the affinity of dansylsarcosine for human albumin is about three times as great as warfarin's (Table 1). Thus it is possible that valproate has a greater affinity for site II than site I. This suggests that the primary interaction of valproic acid with albumin takes place at site II. Data from the hydrolysis experiments also indicates binding to site II as the primary interaction. At a molar ratio of valproate to albumin of 1:1, there was substantial (50%) inhibition of NPA hydrolysis. This was more a feature of known site II ligands, such as diazepam, than of site I ligands. It is also relevant that octanoic acid, of which valproate is a structural isomer, is believed to adsorb selectively to site II [21]. Therefore the balance of evidence suggests that valproate binds to site II, but in addition, it either interacts directly with site I, or on binding to site II it induces a conformational change in the structure of albumin which reduces binding of warfarin to site I. The former is more likely because: (1) octanoic acid did not inhibit the binding of warfarin and (2) the negative charge of valproate's carboxylic acid group is in the centre of the molecule; a structural feature most site I ligands possess [21] and which might permit direct interaction of valproate with site I.

Wanwimolruk and Birkett [21] have suggested that site II is a cleft about 12–16 Å deep and 6–8 Å wide. Using space filling models (Courtauld type) the estimated length and width of valproic acid are about 11.2×6.7 Å, respectively. If these dimensions are correct, then it is possible for valproic acid to interact with site II and in addition, two ways can be postulated for binding to this site. One is that valproic acid penetrates into site II with its entire length embedded and the carboxyl group is actually in the cleft. In this case the acid group probably does not contribute to the binding energy and the total energy of binding comes largely from hydrophobic interactions. The second possibility is that part of the carbon backbone goes into the cleft until the carboxyl group reaches the cleft mouth and then, this acidic group and the carbon chain outside the cleft contribute to binding by interacting with various residues around the mouth of the cleft. If this is the case, it would imply that both the carbon chain outside the cleft and the carboxyl group play a role in the binding process. This suggestion is supported to some extent by the decreasing effectiveness of 2-ethyl and 2-methyl pentanoic acids, and pentanoic acid itself, as inhibitors of both binding to site II and hydrolysis of NPA.

Of the five metabolites tested only two: 2-en and 4-en-2-propyl pentanoic acids displaced warfarin and dansylsarcosine from human albumin. These two metabolites were generally more potent than valproate at inhibiting binding. Hence the introduction of a double bond into the carbon backbone of valproate increases affinity for albumin. This effect is likely to be due to the greater hydrophobicity of the unsaturated metabolites, since hydrophobic interactions make a major contribution to reversible bonding between ligands and albumin [22]. In addition, the position of the double bond is important. The 2-en metabolites was the most potent binding inhibitor and the double bond of this metabolite is near the centre of the molecule. This prevents much of the free rotation of the carbon backbone and makes it more rigid with respect to either the 4-en metabolite or valproate itself. Moreover, space filling models suggest that the 2-en metabolite has a greater width (7.0 Å) than either the 4-en metabolite (6.7 Å) or valproic acid (6.7 Å). These two factors might allow better congruence between the 2-en metabolite and the surface of its binding sites and result in a higher affinity for human albumin.

The saturated straight chain fatty acids C5–C8 did not displace warfarin to any significant extent. By contrast, these acids inhibited both binding to site II and hydrolysis of NPA, and the extent of inhibition increased with carbon chain length. These results are similar to those in the literature [21, 23]. The branched chain acids valproate (2-propyl pentanoic acid) and its 2-methyl and 2-ethyl analogues are structural isomers of C8, C6 and C7. By comparison to their straight chain isomers, the branched chain acids were less effective at displacing dansylsarcosine and inhibiting NPA hydrolysis; but more potent in these respects than pentanoic acid. So substitution of alkyl groups, up to C3, on the 2 position carbon atom of pentanoic acid increases interaction with site II, but not to the same extent as increasing the length of the carbon backbone in a stepwise manner.

The results of the present study provide evidence for the interaction of valproic acid and its two unsaturated metabolites 2-en and 4-en-2-propyl

pentanoic acid with site I and II of human albumin. However, the primary interaction is with site II. The unsaturated metabolites and in particular 2-en-2-propyl pentanoic acid, have a greater affinity than the parent drug and depending on the relative concentrations present *in vivo*, these metabolites might inhibit the binding of valproate to albumin and alter its disposition.

REFERENCES

1. Löscher W and Nau H, Pharmacological evaluation of various metabolites and analogues of valproic acid. *Neuropharmacology* **24**: 427–435, 1985.
2. Patel IH and Levy RH, Valproic acid binding to human serum albumin and determination of free fraction in the presence of anticovulsants and free fatty acids. *Epilepsia* **20**: 85–90, 1979.
3. Barré, Chamouard JM, Houin G and Tillement JP, Equilibrium dialysis, ultrafiltration and ultracentrifugation compared for determining the plasma–protein-binding characteristics of valproic acid. *Clin Chem* **31**: 60–64, 1985.
4. Sjöholm I, Ekman B, Kober A, Pahlman IL, Seiving B and Sjödin T, Binding of drugs to human serum albumin XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. *Mol Pharmacol* **16**: 767–777, 1979.
5. Fleitman JS, Bruni J, Perrin JH and Wilder BJ, Albumin binding interactions of sodium valproate. *J Clin Pharmacol* **20**: 514–517, 1980.
6. Gugler R and von Unruh GE, Clinical pharmacokinetics of valproic acid. *Clin Pharmacokinet* **5**: 67–83, 1980.
7. Löscher W, Anticonvulsant activity of metabolites of valproic acid. *Arch Int Pharmacodyn* **249**: 158–163, 1981.
8. Means GE and Bender ML, Acetylation of human serum albumin by *p*-nitrophenyl acetate. *Biochemistry* **14**: 4989–4994, 1975.
9. Dean HD, Bonser JC, Gent JP, HPLC analysis of brain and plasma for octanoic and decanoic acids. *Clin Chem* **35**: 1945–1948, 1989.
10. Parker TA, *Photoluminescence of Solutions*. Elsevier, Amsterdam, 1968.
11. Panjehshahin MR, Bowmer CJ and Yates MS, A pitfall in the use of double-reciprocal plots to estimate the intrinsic molar fluorescence of ligands bound to albumin. *Biochem Pharmacol* **38**: 155–159, 1989.
12. Maes V, Engelborghs Y, Hoebeke J, Maras Y and Vercruysse A, Fluorimetric analysis of the binding of warfarin to human serum albumin. Equilibrium and kinetic study. *Mol Pharmacol* **21**: 100–107, 1982.
13. Scatchard G, The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–673, 1949.
14. Ralston M, Derivative-free nonlinear regression. In: *BMDP Statistical Software* (Eds. Dixon WJ, Brown MB, Engleman L, Frane JW, Hill MA, Jennrich RI and Toporek JD). University of California Press, Berkeley, 1983.
15. Steinhardt J and Reynolds JA, In: *Multiple Equilibria in Proteins*. Academic Press, New York, 1969.
16. Fehske KJ, Müller WE and Wollert U, The location of drug binding sites in human albumin. *Biochem Pharmacol* **30**: 687–692, 1981.
17. Monks A, Boobis S, Wadsworth J and Richens A, Plasma protein binding interaction between phenytoin and valproic acid *in vitro*. *Br J Clin Pharmacol* **6**: 487–492, 1978.
18. Dhillon S and Richens A, Serum protein binding of diazepam and its displacement by valproic acid *in vitro*. *Br J Clin Pharmacol* **12**: 591–592, 1981.
19. Calvo R, Suárez E, Rodríguez-Sasiaín JM and Aguilera L, Effect of sodium valproate on midazolam distribution. *J Pharm Pharmacol* **40**: 150–152, 1987.
20. Fehske KJ, Schläfer U, Wollert U and Müller WE, Characterization of an important drug binding area on human albumin including the high-affinity binding sites of warfarin and azapropazone. *Mol Pharmacol* **21**: 387–393, 1982.
21. Wanwimolruk S and Birkett DJ, Structure binding relationship for sites I and II on albumin. In: *Protein Binding and Drug Transport* (Eds. Tillement JP and Lindenlaub E), pp. 131–151. FK Schattauer, Stuttgart, 1986.
22. Perrin JH, Bonds between drugs and plasma components. In: *Protein Binding and Drug Transport* (Eds. Tillement JP and Lindenlaub E), pp. 115–127. FK Schattauer, Stuttgart, 1986.
23. Koh S-HM and Means GE, Characterization of a small apolar anion binding site of human albumin. *Arch Biochem Biophys* **192**: 73–79, 1979.