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Valproate and Palmitate Binding to Human Serum Albumin: An Hypothesis on Obesity

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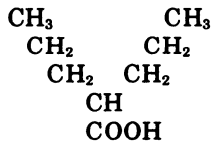
SUMMARY

Binding equilibria of valproate (2-*n*-propyl-pentanoic acid anion) with defatted human serum albumin were studied by equilibrium dialysis in a 66 mM sodium phosphate buffer, pH 7.4, 37°. Three hundred and fifty-six observed points for bound versus free valproate concentration were obtained and analyzed in terms of stepwise binding. It was found that the best fit resulted from a model in which 67% of the albumin was capable of binding valproate, whereas 33% did not bind. Thirty acceptable variants of the curve fitting were generated in order to assess the variation of the binding constants. The binding albumin component combines with three molecules of valproate with high affinity and with at least seven additional molecules that are loosely bound. Saturation of the protein cannot be reached. At very high concentrations of free valproate (above 10 mM) irreversible changes in the albumin take place, resulting in poor reproducibility in the amount of bound valproate. In the presence of palmitate, 0.5, 1, and 1.5 mol/mol of albumin, binding of valproate is decreased by a competitive mechanism. It is hypothesized that obesity, developing as a complication of valproate treatment of epilepsy, results from increased availability of long-chain fatty acids due to competitive valproate binding.

Valproate binding. Valproic acid is a branched octanoic acid,



In previous work, we have described albumin binding of *n*-hexanoate, *n*-octanoate, and *n*-decanoate in terms of multiple stepwise equilibria (1). Four models were considered. The simplest model was expressed by the general binding equation,

$$r = \frac{cK_1 + 2c^2K_1K_2 + 3c^3K_1K_2K_3 + \ldots + Nc^NK_1K_2K_3 \ldots K_N}{1 + cK_1 + c^2K_1K_2 + c^3K_1K_2K_3 + \ldots + c^NK_1K_2K_3 \ldots K_N} \quad (1)$$

assuming that albumin is homogeneous with respect to binding of the fatty acid. In this equation, r is the molar concentration of bound fatty acid (or anion) divided by that of albumin, c is the unbound fatty acid (or anion) concentration, K_i are stoichiometric binding constants (step constants), and N is the maximal number of fatty acid molecules bound per molecule of albumin.

Alternatively, the protein might consist of a mixture of two components, one component binding the fatty acid, in one high affinity step, more than the other,

$$r = f\frac{cK_1 + 2c^2K_1K_2 + 3c^3K_1K_2K_3 + \ldots + Nc^NK_1K_2K_3 \ldots K_N}{1 + cK_1 + c^2K_1K_2 + c^3K_1K_2K_3 + \ldots + c^NK_1K_2K_3 \ldots K_N} + (1-f)\frac{cK_2 + 2c^2K_2K_3 + 3c^3K_2K_3K_4 + \ldots + (N-1)\,c^{(N-1)}K_2K_3K_4 \ldots K_N}{1 + cK_2 + c^2K_2K_3 + c^3K_2K_3K_4 + \ldots + c^{(N-1)}K_2K_3K_4 \ldots K_N} \quad (2)$$

where f is the fraction of high affinity albumin.

A third model had one albumin component binding, in two high affinity steps, more than the other component.

$$r = f\frac{cK_1 + 2c^2K_1K_2 + 3c^3K_1K_2K_3 + \ldots + Nc^NK_1K_2K_3 \ldots K_N}{1 + cK_1 + c^2K_1K_2 + c^3K_1K_2K_3 + \ldots + c^NK_1K_2K_3 \ldots K_N} + (1-f)\frac{cK_3 + 2c^2K_3K_4 + 3c^3K_3K_4K_5 + \ldots + (N-2)\,c^{(N-2)}K_3K_4K_5 \ldots K_N}{1 + cK_3 + c^2K_3K_4 + c^3K_3K_4K_5 + \ldots + c^{(N-2)}K_3K_4K_5 \ldots K_N} \quad (3)$$

In a final model the protein was considered to consist of two components, one binding the fatty acid anion, and another not binding this ligand at all,

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ABBREVIATION: NEFA, nonesterified fatty acids.

$$r = f\,\frac{cK_1 + 2c^2K_1K_2 + 3c^3K_1K_2K_3 + \ldots + Nc^NK_1K_2K_3 \ldots K_N}{1 + cK_1 + c^2K_1K_2 + c^3K_1K_2K_3 + \ldots + c^NK_1K_2K_3 \ldots K_N} \quad (4)$$

It was found that binding of octanoate and decanoate could be described equally well in terms of models 2 and 4 and that hexanoate binding observations were fitted best by models 3 and 4. The best values of f, the fraction of the high affinity binding albumin component, ranged from 0.64 to 0.74, irrespective of which model was used. The experimental observations could in no case be fitted well by the simple model 1, assuming homogeneous albumin.

In the present study we want to examine binding of valproate in terms of the same models.

Obesity and availability of fatty acid. In studies of protein binding of soluble ligands, the tightness of binding is usually expressed by the ratio of bound to free ligand concentrations. This is not possible for fatty acids with a chain-length of 16 carbon atoms or longer, due to their immeasurably low solubility at physiological pH (2). Recent development of a dialysis exchange rate technique has opened the possibility of measuring how tightly palmitate and stearate are bound to albumin (3). We avoid using the freey fatty acid anion concentration and express the availability of a fatty acid in a plasma sample by C/p, i.e., the concentration, C, of albumin-bound fatty acids divided by p, the concentration of reserve albumin for binding of the fatty acid. A detailed deduction of the availability has been given in a former paper (3). The concentration of reserve albumin for binding of a ligand has previously been defined as the concentration of a pure standard albumin in buffered solution that binds a trace amount of the ligand as tightly as it is bound in the sample.

The concentration of albumin, P, of NEFA, and of reserve albumin for binding of palmitate, p, has been measured in 66 healthy adults (4). The availability of fatty acid, $[NEFA]/p$, increases, as expected, with increasing ratio of NEFA to albumin and, in addition, varies considerably from one individual to another, as illustrated in Fig. 1

We have speculated whether this individual variation of fatty acid availability could influence the tendency to obesity, our simplistic hypothesis being that an increased availability of

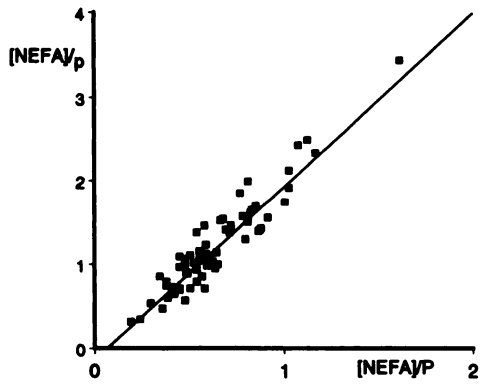

Fig. 1. Fatty acid availability in healthy adults as a function of NEFA/albumin ratio. Availability of fatty acids in serum samples from 66 healthy adults was calculated as $[NEFA]/p$, where p is the concentration of reserve albumin for binding of palmitate. A regression line is shown. The availability of fatty acids increases linearly with the NEFA/albumin ratio and, in addition, shows an individual variation.

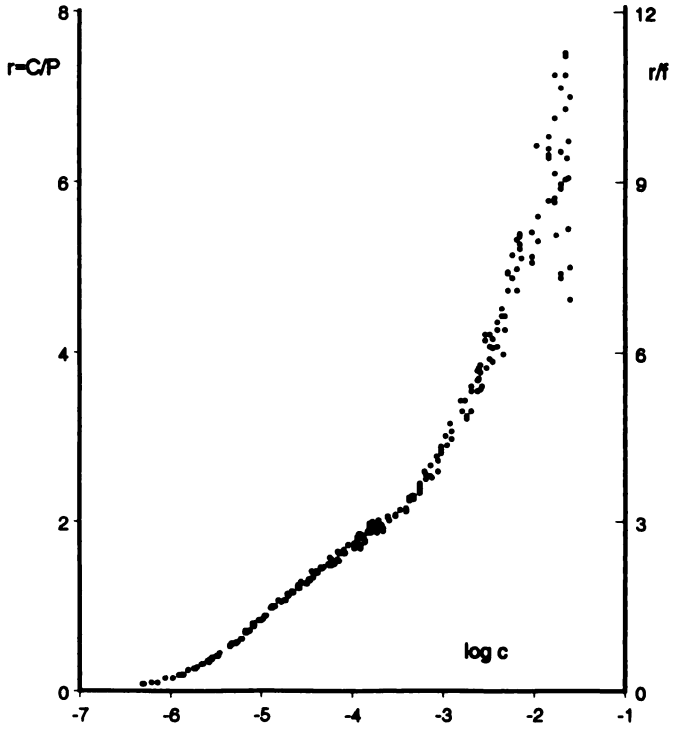

Fig. 2. Binding isotherm for valproate to defatted human serum albumin, Bjerrum plot. Three hundred and fifty-four observed points from equilibrium dialysis experiments were plotted as r, the number of valproate molecules bound per molecule of albumin (*left ordinate*), versus the logarithm ($\log_{10}$) of c, the free valproate concentration (molar). *Right ordinate*, r/f, the average number of valproate molecules bound per molecule of binding albumin in model 4. Buffer was 66 mM sodium phosphate, pH 7.4, 37°.

fatty acids to the tissues might cause obesity. Work is in progress in our laboratory with the aim of elucidating this question. One obvious way of doing so would be to find a substance that could compete with long-chain fatty acids for binding to albumin and, thus, increase the availability of fatty acids in plasma. Our hypothesized relation would be supported if administration of such a substance should prove to cause obesity. Valproate appears to fulfil these criteria. A previous study has shown that reserve albumin for binding of palmitate decreases when valproate is added *in vitro* to buffered solutions of albumin or serum (5). Epileptic patients treated with valproate have high plasma concentrations of the drug, around 0.7 mM (6), similar to the molar concentration of albumin, and should, thus, have markedly increased availabilities of fatty acid. It is already known that such patients often develop obesity (7, 8).

In the present study we have investigated the binding of valproate to albumin and the influence of addition of palmitate, to see whether valproate competes with binding of palmitate. A mechanism for the development of obesity in valproate-treated patients is hypothesized.

Experimental Procedures

Materials

Serum albumin. Human serum albumin was obtained from AB Kabi, (Stockholm, Sweden) (lot RFM 57) and was defatted with charcoal in acid solution (9). Sulfuric acid was used for the acidification. The albumin was desalted by dialysis against water, lyophilized, and

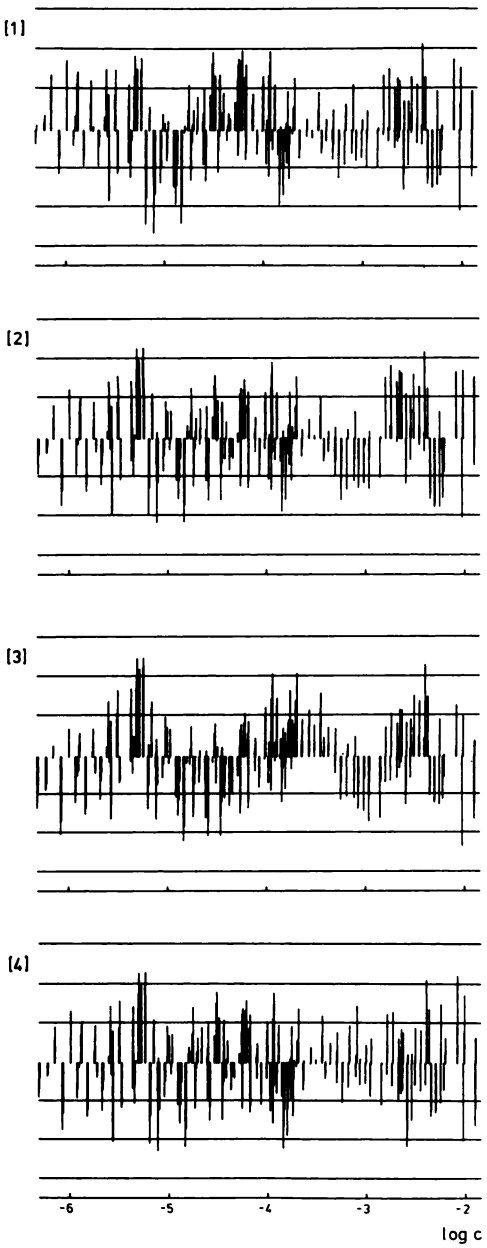

Fig. 3. Weighted residuals for best fits, obtained with each of models 1–4.

stored over silica gel at 4°. Fatty acid content was below 0.1 mol/mol of albumin.

Fatty acids. Unlabeled sodium valproate was obtained as a gift from Ciba Geigy A/S. Palmitic acid was from Fluka (Buchs, Switzerland) (purissimum, >99% by gas-liquid chromatography).

To prepare the palmitic acid-albumin solutions, we dissolved albumin in water to a concentration of 300 μM and adjusted the pH to 8 with sodium hydroxide. A 180 mM ethanolic solution of palmitic acid was prepared. One volume of this solution was added to 100 volumes of albumin solution, with stirring. The molar ratio of palmitic acid to albumin was 6. The solution was lyophilized and the dried material was stored over silica gel at 4°. Before use, the dry palmitic acid-albumin was dissolved in 66 mM sodium phosphate, pH 7.4, forming a clear solution that was mixed with solutions of albumin to obtain various ratios of fatty acid to protein.

Radiolabeled valproate. Sodium [1-^{14}C]valproate (specific activity, 0.74 MBq/mg) was obtained as a gift from Ciba Geigy A/S. Radiochemical purity was 97% according to the manufacturer. After purification by thin layer chromatography on silica gel (Merck 60, F254) in toluene/dioxane/acetic acid (10:6:1 by volume), the content of dialyzable, loosely bound, radioactive impurities was measured as described previously (10) and accounted for 0.32% of the radioactivity. An aqueous solution of the valproic acid was added, with stirring, to the buffered albumin solution.

TABLE 1

Binding constants for valproate to human serum albumin

Values were obtained in 66 mM sodium phosphate buffer, pH 7.4, 37°, best-fit values and ranges of 30 acceptable sets. The probability limit was 0.75. Model 4, $f = 0.67$, was used.

	Binding constants	
	Best fit	30 acceptable sets, ranges
	M^{-1}	
K_1	2.75×10^5	2.59×10^5–2.90×10^5
K_2	7.74×10^4	7.01×10^4–8.85×10^4
K_3	1.30×10^4	9.86×10^3–1.56×10^4
K_4	8.01×10^2	2.69×10^2–2.25×10^3
K_5	1.07×10^3	2.46×10^{-1}–5.68×10^3
K_6	7.19×10^2	1.89×10^2–2.14×10^6
K_7	6.40×10^{-1}	1.43×10^{-2}–6.75×10^2
K_8	4.39×10^2	1.10×10^{-1}–4.90×10^5
K_9	2.33×10^4	4.07×10^1–3.98×10^6
K_{10}	8.83×10^0	5.09×10^{-3}–2.64×10^5

Methods

Equilibrium dialysis. Binding of valproic acid was studied by equilibrium dialysis in 1-ml dialysis chambers, as previously described (11). Cellulose membranes (type 10.17) from Dianorm Diachema AG (Zürich, Switzerland) were used. A 66 mM sodium phosphate buffer, pH 7.4, was placed on both sides of the membrane. Albumin (100, 300, or 600 μM) with varying concentrations of valproate (ranging from 20 μM to 50 mM), was present on the left side, together with [^{14}C]valproic acid at low concentrations (3 μM). The temperature was 37°. The time necessary for obtaining equilibrium was tested. Constant concentrations were present for time intervals from 1 to more than 3 hr; 2 hr was chosen. A volume of 0.8 ml was withdrawn from each compartment and mixed with scintillation fluid (LSC Cocktail Maxi Fluor; J.T. Baker Chemicals B.V. Deventer, Holland) and the radioactivity was determined in an LKB Wallac 1218 Rackbeta liquid scintillation counter. The concentration of unbound valproic acid, c, and the molar ratio of bound valproic acid to albumin, r, was calculated.

Numeric analysis of binding data. Experimental data (free versus bound valproate) were analyzed by iteration of K values in models 1–4, generating a best fit set of stoichiometric binding constants for each model. Iteration was continued to minimize the sum of squared and weighted residuals, as previously described (12). The residuals were weighted by

$$w = c^q$$

An optimal distribution of weighted residuals was obtained when q was given the value of 0.4. Weighted residuals,

$$(r_{obs} - r_{calc})/\sqrt{w}$$

are pictured in Fig. 3. In order to obtain an idea of the variation of the binding constants, we generated 30 sets of binding constants that were acceptable within chosen probability limits, according to an F test.

Results

Binding of Valproate

Binding isotherm, Bjerrum's plot. Three hundred and fifty-six observations of valproate-albumin binding equilibria are presented in Fig. 2 as r, the concentration of bound valproate divided by that of albumin, on the left ordinate versus

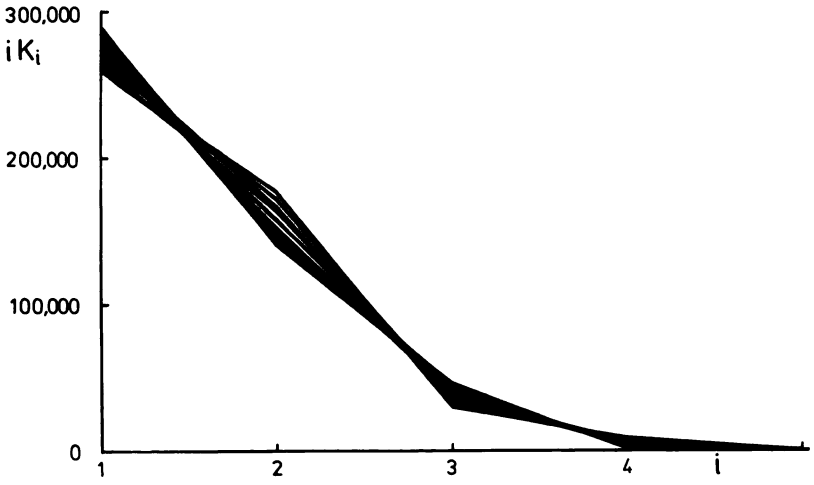

Fig. 4. Klotz' affinity profiles from 30 acceptable sets of stoichiometric binding constants for valproate to human serum albumin, according to model 4.

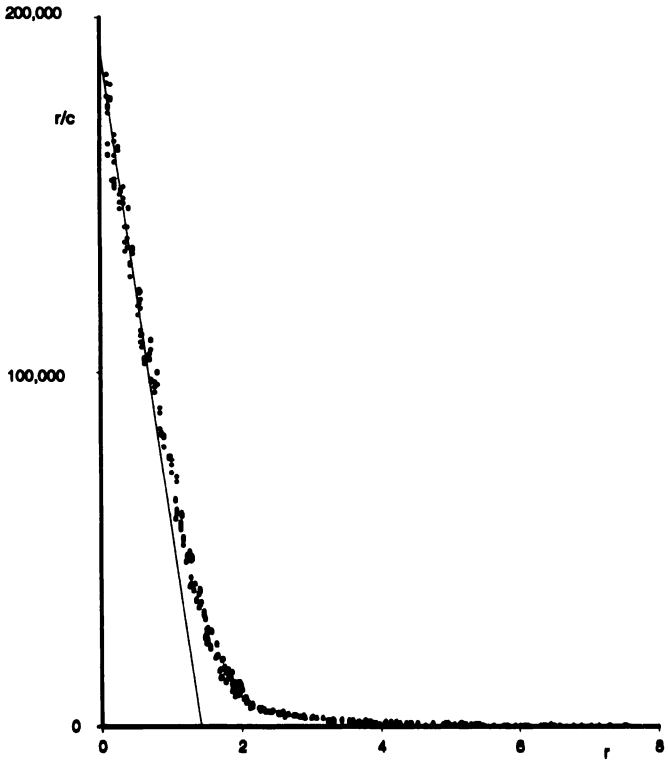

Fig. 5. Scatchard plot. The same data as in Fig. 2, are depicted as *r/c* versus *r*.

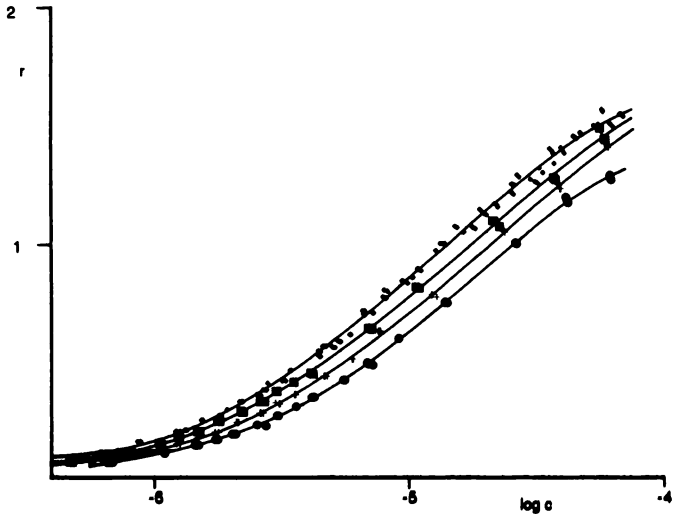

Fig. 6. Cobinding of palmitate and valproate. Binding isotherms for valproate to defatted human serum albumin, with conditions as in Fig. 1, in the presence of varying amounts of palmitate, are shown. ♦, Without palmitate; ■, with 0.5 mol of palmitate/mol of albumin; +, 1 mol of palmitate/mol of albumin; ●, 1.5 mol of palmitate/mol of albumin.

the free valproate concentration, *c*, on a logarithmic scale. Experiments were conducted with three different albumin concentrations, 100, 300, and 600 μM. All observed points are scattered equally around one continuous curve, demonstrating the absence of reversible albumin-albumin interaction.

It is noted that saturation of the albumin could not be obtained. Free valproate concentrations above 10 mM cause irreproducible results, indicating slow irreversible changes taking place in the albumin molecule. The following analysis of the binding data has, accordingly, been carried out using the 330 points observed at free valproate concentrations below 10 mM.

Least squares fitting of *K* values in the equations presented in the Introduction showed that model 4 gave the best fit to the observed points. In this model, it is presumed that a fraction of the albumin can bind multiple valproate molecules, whereas

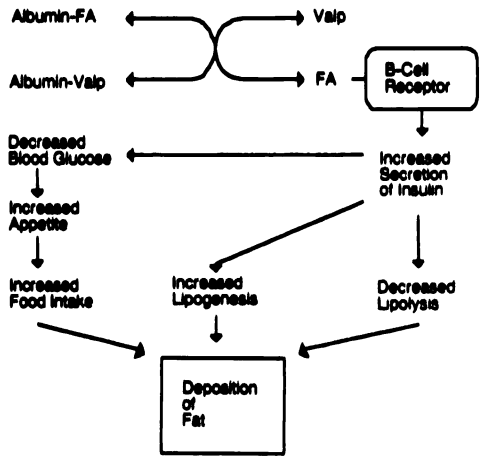

Fig. 7. Obesity pathogenesis. Proposed mechanism for development of obesity in valproate-treated patients. *FA*, Fatty acid; *Valp*, valproate.

the rest of the albumin is nonbinding. An optimal fit was obtained on the presumption that 67% of the albumin was capable of binding valproate whereas 33% was nonbinding ($f = 0.67$). The weighted residuals are pictured in Fig. 3, model 4. An ideal fit is signified by an even distribution of weighted residuals, positive and negative, throughout the range of free ligand concentrations, as found in this case.

Model 1, with homogeneous albumin, gave a fairly good fit to be the observed data, with, however, an uneven distribution of residuals, as seen in Fig. 3, *model 1*. At the lowest concentrations of free valproate, around $\log c = -6$, residuals are predominantly positive; at higher concentrations, about $\log c = -5$, residuals are mostly negative and again positive around $\log c = -4.3$, etc. The finding of such a wave pattern of residuals in our experience indicates that a poor model was chosen. The result was improved when it was presumed that less than 100% of the albumin was capable of binding valproate, as in model 4.

Models 2 and 3, with two unequally binding albumin components, gave inferior fits: model 3 produced an obvious wave pattern of the residuals, as shown in Fig. 3, *model 3*, a clear indication for rejection of this model.

The commercial albumin used in our work consists, as previously found (1), of two proteins, in approximately the proportion 2:1, one component binding *n*-octanoate and *n*-decanoate with high affinity and the other binding less or not at all. It was, accordingly, expected that valproate, an *iso*-octanoate, would be bound with different affinity to the two albumins. Taking this together with the present results leads to the conclusion that the albumin preparation used in this work contains two proteins, a main valproate-binding component and another, about one third of the albumin, not binding valproate or binding with considerably less affinity.

Binding constants. In Fig. 2, the right ordinate scale shows r/f, the average number of valproate molecules bound to each molecule of binding albumin. The fraction of binding albumin, f in Eq. 4, was given the value 0.67, which gave the best fit to model 4. It is interesting to note that the curve has a slight step at the binding of three molecules of valproate to each molecule of binding albumin. The stoichiometric binding constants (Table 1), in agreement with this, show that three molecules are bound with high affinities and seven additional molecules with low affinities.

Table 1, in addition to the best fit set of binding constants in model 4, shows the range of each K when 30 sets of binding constants were generated that were acceptable within the probability limit of 0.75, according to an F test. It is noted that K_1, K_2, and K_3 are fairly well defined with limited, although increasing, variation; K_4 shows a variation approaching 1 order of magnitude, and the following binding constants are highly variable indeed.

The 30 sets of K values are pictured as Klotz' affinity profiles (iK_i versus i) in Fig. 4, including K_1 to K_4 only. Homogeneous binding of the first three molecules of valproate and weak binding of the following would be signified by points located on a straight line intersecting the abscissa axis at $i = 4$. The present results are at variance with this mechanism and indicate that binding of the first three molecules is anticooperative in the stoichiometric sense. For a full discussion of Klotz' affinity profiles, see Ref. 13.

Site models. Until this point, we have used a stoichiometric description of binding equilibria, not considering localization of the bound molecules to sites in the albumin. It is usually not possible from binding equilibrium studies alone to draw any conclusions about the presence of binding sites or interaction between sites. We can, however, exclude certain site models and leave other possibilities open. A model with three equal and independent high affinity sites and several weaker sites can be excluded, because this would give a straight-line affinity profile, intersecting the abscissa axis at $i = 4$. The experimental findings, on the other hand, are compatible with the presence of three independent sites with different affinities for valproate. A model with three equal high affinity sites with negative interaction is equally probable. Also, the three first valproate molecules may be bound to one flexible site with decreasing affinities. For each of these models, it is possible to calculate site binding constants and constants of interaction. These site constants are different for each model and different from the stoichiometric binding constants reported in Table 1.

Scatchard plot. Fig. 5 shows the 356 observed points, depicted as r/c versus r. The first part of this graph is almost linear until r approaches 2, when the curve bends acutely. Little valproate appears to be bound beyond 4 molecules/molecule of albumin. A conclusion of saturation at $r = 4$ would, however, be erroneous, as seen from the Bjerrum plot (Fig. 2). It is further noted that the step, visible in the Bjerrum plot, is not apparent in Fig. 5. In spite of a good experimental precision, it is somewhat difficult to draw a tangent to the curve at $r = 0$ and obtain accurate abscissa and ordinate intercepts. The line drawn in the figure corresponds to an abscissa intercept $x_0 = 1.42$ and an ordinate intercept $y_0 = 1.9 \times 10^5$ M^{-1}. Let us investigate whether these values can be used for determination of f and binding constants in Eq. 4.

The ordinate intercept, derived from Eq. 4, is

$$y_0 = \lim_{r \to 0} (r/c) = fK_1$$

and the slope of the line at $r = 0$ is

$$\alpha = \left(\frac{d(r/c)}{dr}\right)_{r=0} = -(K_1 - 2K_2)$$

The abscissa intercept is then

$$x_0 = -\frac{y_0}{\alpha} = \frac{fK_1}{K_1 - 2K_2}$$

It is, thus, possible to calculate fK_1 and $K_1 - 2K_2$ from the Scatchard plot. The numerical values listed above give $fK_1 = 1.9 \times 10^5$ M^{-1} and $K_1 - K_2 = 1.3 \times 10^5$ M^{-1}, in good agreement with the values listed in Table 1. It is, on the other hand, not possible to obtain f or individual binding constants from Scatchard plots in the present case.

It is concluded that a Scatchard analysis would be inferior to a Bjerrum plot, combined with a computerized analysis, as a means of determination of binding parameters according to model 4.

Competition with Palmitate

Fig. 6 presents the results of valproate binding studies with albumin having 0.5, 1, and 1.5 mol of palmitate attached per mol of protein. The curves were obtained by computerized fitting to the observed points. It is seen that increasing amounts of palmitate cause a continuous decrease of valproate binding.

Discussion

Valproate binding. The valproate-human serum albumin binding isotherm in Fig. 2 has a slight step at two bound molecules of valproate/molecule of total albumin. A similar step was previously found for the binding isotherm of *n*-hexanoate to human serum albumin, using commercial albumin from the same manufacturer and the same experimental conditions as in the present work. Binding isotherms for *n*-octanoate and *n*-decanoate showed similar steps but at less than one molecule of the fatty acid bound/molecule of albumin. With all three ligands, the results clearly indicated the presence of two albumin components, one binding the acids with high affinity and the other with low affinity or not at all. The binding albumin component is capable of binding two molecules of *n*-hexanoate or one molecule of *n*-octanoate or *n*-decanoate with high affinity, plus several additional molecules that are bound more loosely. The molecular ratio of the two albumin components was 2:1. It was, accordingly, expected that valproate as well would bind differently to the two albumins. This expectation has been experimentally confirmed in the present work, because a better fit of the experimental points was found to a model with heterogeneous albumin.

It is remarkable how difficult it is to obtain conclusive evidence for one model versus another. Even with 330 experimental points, measured with good precision, the superiority of model 4 could only be demonstrated by a detailed examination of weighted residuals. This is in agreement with previous findings of Nimmo *et al.* (14).

Chromatographic evidence has recently been obtained for the presence of two components in human serum albumin with different binding affinity for decanoate.[1]

A similar analysis of the relative binding isotherm for palmitate has been tried, with a negative result. It is not possible to distinguish whether palmitate binds equally or differently to the two albumin components. However, as concluded from the results presented in Fig. 6, palmitate decreases the binding of valproate and it can, thus, be concluded that palmitate at least binds to the albumin component that binds valproate. We do not know whether palmitate is bound to the other component.

These findings constitute clear evidence for displacement of valproate upon the binding of palmitate. The reverse displacement of valproate by palmitate follows from this, as a thermodynamic necessity. Previous work has further shown that valproate is capable of displacing palmitate (5). Competition of binding of these two ligands to human serum albumin is evident.

Competition is here understood in the stoichiometric sense; binding of one ligand inhibits binding of the other and vice versa. We do not consider it possible to conclude that palmitate and valproate compete for binding to one or several distinct sites in the albumin molecule. This is immaterial for practical pharmacological purposes.

Pathogenesis of obesity in valproate-treated patients. An increased availability of fatty acids is likely to cause obesity through the following hormonal mechanism (Fig. 7). A persistent high concentration of valproate increases the availability of fatty acids to the insulin-producing β cells of the pancreas. The increased availability of fatty acids gives an extra stimulus to the secretion of insulin (15, 16), relative to the secretion in patients not treated with valproate. The extra amount of insulin causes decreased lipolysis, increased lipogenesis, and stimulation of appetite through lowered blood glucose concentration. The effects of insulin combine to cause an increase of the amount of fat in adipose tissues.

[1] H. Vorum, A. O. Pedersen, and R. Brodersen, unpublished observations.

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