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CONCENTRATION-DEPENDENT EFFECTS OF FATTY ACIDS ON WARFARIN BINDING TO ALBUMIN

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Abstract—The interrelationships between fatty acid and warfarin binding to albumin were investigated. Various molar ratios of palmitic acid and oleic acid were added to defatted human albumin in the presence of warfarin, and the warfarin binding association constants, K_a, were calculated. Warfarin association constants increased from $0.84 \times 10^5\ M^{-1}$ to $3.66 \times 10^5\ M^{-1}$ as the oleic acid concentration increased from zero to three moles per mole of albumin and from $1.19 \times 10^5\ M^{-1}$ to $3.13 \times 10^5\ M^{-1}$ as the palmitic acid concentration increased from zero to two moles per mole of albumin. Larger amounts of either fatty acid progressively decreased the amount of warfarin bound in a noncompetitive fashion. In addition, two proteolytic fragments were utilized to define the location of the warfarin binding site on albumin. The warfarin site was located between loops 5 and 6 on the albumin molecule in close proximity to the secondary and tertiary binding sites of palmitic acid.

Human albumin is a single peptide chain composed of 584 residues. The complete amino acid sequence of human albumin has been determined by Brown and co-workers [1, 2]. The secondary structure contains 50–55% α-helix, about 15% β-pleated sheet and the rest random coil [3]. Brown has proposed a tertiary structure consisting of nine major loops and 17 disulfide bonds [1, 4]. Normally, a plasma albumin has bound to it a complement of bilirubin and fatty acid molecules.

Normal fat metabolism requires that fatty acids be solubilized and transported by plasma albumin from the liver to the active metabolizing tissues. Therefore, the three most common plasma fatty acids, palmitic, oleic and stearic acids, are all highly bound to plasma albumin [5]. Spector *et al.* [6] proposed that palmitic acid binds to three classes of sites on the albumin molecule. The primary and secondary classes each contained three sites, but the tertiary class contained approximately 63 sites. Studies on several bovine albumin fragments supported the hypothesis that there are three primary binding sites on bovine albumin for palmitic acid and determined that the three strongest palmitic acid binding sites are located in the carboxyl-terminal two thirds of the albumin molecule [7].

Since only the free form of a drug is available to receptor sites, drug bound to protein is pharmacologically inactive. For a highly bound drug such as warfarin, which at therapeutic plasma concentrations is 99 per cent bound to albumin, an increase of only 1 per cent in the free fraction doubles the number of warfarin molecules available for pharmacological activity. One cause of such an increase in the free fraction described initially by Solomon *et al.* [8] was substantiated by Gugler *et al.* [9]; both groups reported that an excess of fatty acids decreased warfarin binding to albumin. In the present study, interrelationships between fatty acids and warfarin binding were investigated by the addition of various molar ratios of palmitic and oleic acids to defatted human albumin in the presence of warfarin. In addition, two proteolytic fragments of bovine albumin, which contain independent fatty acid binding sites and recombine in solution to form an albumin-like molecule [7, 10], were utilized to help locate the warfarin binding site.

MATERIALS AND METHODS

Chemicals. [^{14}C]Warfarin was purchased from Amersham/Searle Corp., Arlington Heights, IL, and had a specific activity of 23.6 mCi/m-mole. Nonradiolabeled warfarin was obtained from ICN Pharmaceuticals, Plainview, New York. The palmitic and oleic acids (Sigma Chemical Co., St. Louis, MS) were approximately 99 per cent pure. Bovine serum albumin and crystalline human serum albumin (fatty acid free) were purchased from Sigma Chemical Co. Bovine serum albumin fragments P-A and P-B were those purified and characterized by Feldhoff and Peters [10] and kindly provided by Dr. Theodore Peters, Jr.

Preparation of albumin solutions. Palmitic acid was dissolved in absolute ethanol to a concentration of one microgram per milliliter and added in the appropriate quantities to test tubes. The ethanol was allowed to evaporate at room temperature, thereby leaving a thin film of fatty acid on the wall of the test tube. Ten milliliters of phosphate buffer containing 11.4 μM fatty acid free albumin were added to the test tubes. These solutions were stored overnight at 4°.

Since oleic acid is a fluid at room temperature, it was added directly to test tubes in microliter quantities with ten milliliters of phosphate buffer containing 11.4 μM fatty acid free albumin. These solutions were also stored overnight at 4°.

Equilibrium dialysis. The binding of warfarin to albumin was measured at 37° and pH 7.4 by equilibrium dialysis through the use of microcells (Bell Arts). In each determination the albumin concentration was

11.4×10^{-6} M. The albumin concentration was determined spectrophotometrically with an extinction coefficient of $E^{1\%}_{1\,cm} = 5.3$ at 280 nm and a mol. wt of 66,000. All binding measurements were performed in an isotonic buffer (50 mM sodium phosphate, 75 mM sodium chloride, pH 7.4). Readings were taken after 5 hr of dialysis, at which time it had been determined that equilibrium had been established. Ten determinations were performed on each albumin sample over a warfarin concentration range of 0.5–10.0 μg/ml. The concentration of warfarin was determined through the use of [^{14}C]warfarin, the radiolabeled source representing 12.5 per cent of the total warfarin present during all determinations.

Warfarin binding association constants and the number of warfarin binding sites per albumin molecule were determined by the methods of Scatchard [11] and Hughes and Klotz [12]. All data were analyzed by linear regression.

The binding of warfarin to the bovine albumin fragments (P-A, P-B and P-A + P-B) and to intact bovine serum albumin was performed at 37°, pH 7.4, and in phosphate buffer. Warfarin determinations were made on each sample at one and four μg/ml of warfarin. The concentration of the fragments and the bovine serum albumin was 1×10^{-5} M in all experiments. The following extinction coefficients and mol. wt were employed: bovine albumin, $E^{1\%}_{1\,cm} = 6.6$, mol. wt 66,000; bovine fragment P-A, $E^{1\%}_{1\,cm} = 5.3$, mol. wt 31,000; and bovine fragment P-B, $E^{1\%}_{1\,cm} = 7.4$, mol. wt 35,000.

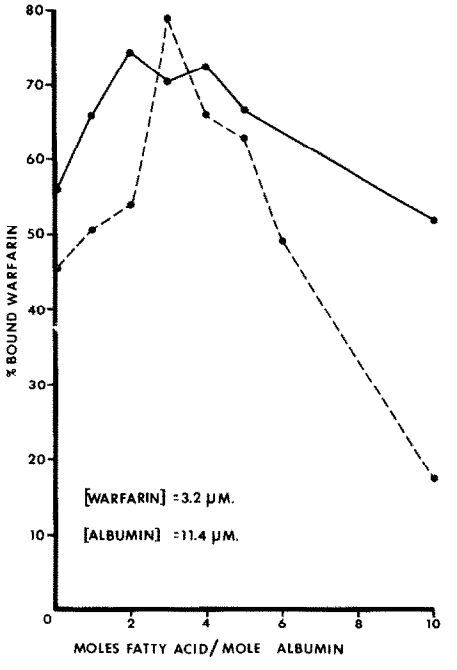

Fig. 1. Percent binding of warfarin to human serum albumin in the presence of various mole to mole ratios of fatty acids to albumin. Dashed line represents warfarin binding in the presence of oleic acid; solid line represents binding in the presence of palmitic acid.

RESULTS

Three or more moles of either palmitic or oleic acid reduced the degree of warfarin binding to albumin (Fig. 1). Scatchard plots of warfarin binding data exhibit decreasing slopes with increasing quantities of fatty acids above 3 moles per mole of albumin, and the number of primary warfarin binding sites remains one. As expected, Hughes–Klotz plots show increasing slopes as the fatty acid concentration is increased (Fig. 2 and 3). Both indicate that the warfarin binding association constants, K_a, decrease as fatty acids are added in excess of 3 moles per mole of albumin. The binding association constants, K_a, were derived from the Hughes–Klotz plots by assuming one primary warfarin binding site, n, on the albumin molecule and a slope equal to $1/K_a n$.

By increasing the concentration of palmitic or oleic acid from 0 to 3 moles per mole of albumin, the binding of warfarin to albumin was enhanced (Fig. 1). When the data were plotted by the double-reciprocal method of Hughes–Klotz (Fig. 2 and 3), the binding association constants increased when the fatty acid concentration was increased from 0 to 3 moles of fatty acids per mole of albumin (Table 1). Therefore, albumin not only binds more warfarin in the presence of 3 moles of fatty acid per mole of albumin, but the binding is of greater strength. This is supported by the thermodynamic data shown in Table 1. The magnitude of the ΔG values shown in Table 1 concur with the ΔG values for warfarin–albumin interactions reported by Rippie [13].

Results of warfarin binding experiments performed on charcoal-treated [14] crystalline albumin (Miles Laboratories) concurred with the results obtained with commercially prepared, fatty acid free albumin. Palmitic acid was used in these latter studies.

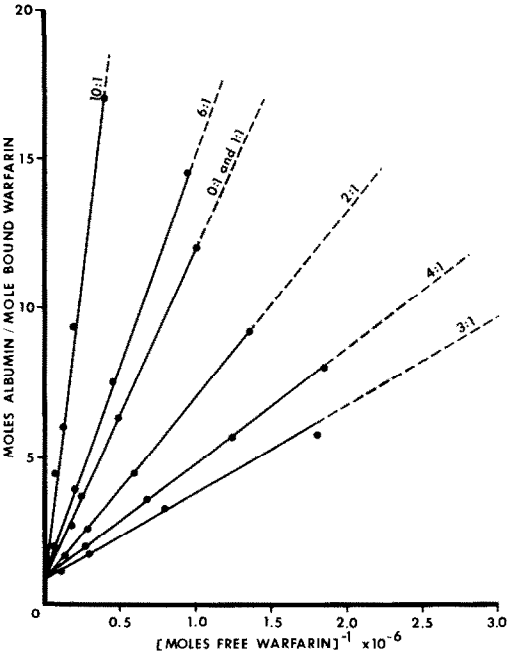

Fig. 2. Hughes–Klotz plot of the binding of warfarin to human serum albumin in the presence of varying amounts of oleic acid. The ratios indicated at the end of each line represent the ratios of the moles of oleic acid present per mole of albumin.

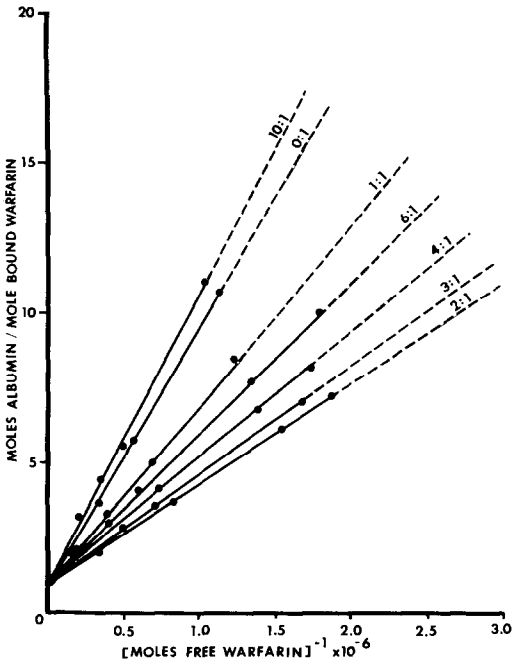

Fig. 3. Hughes–Klotz plot of the binding of warfarin to human serum albumin in the presence of varying amounts of palmitic acid. The ratios indicated at the end of each line represent the ratios of the moles of palmitic acid present per mole of albumin.

Using the P-A and P-B bovine serum albumin fragments of Feldhoff and Peters [2], warfarin bound very little to fragment P-A and P-B individually, synergistically in a solution of P-A and P-B, and to an even greater extent to intact bovine serum albumin (Table 2).

Table 1. Thermodynamic data on the effects of fatty acids on warfarin binding to human serum albumin (HSA)

Fatty acid	Ratio*	K_a† ($M^{-1} \times 10^{-5}$)	ΔG‡ (cal/mole)	ΔΔG§ (cal/mole)
None	0:1	0.84	−6984	0
Oleic	1:1	0.84	−6984	0
	2:1	1.68	−7411	−427
	3:1	3.66	−7890	−906
	4:1	2.70	−7703	−719
	5:1	1.45	−7320	−336
	6:1	0.71	−6880	104
	10:1	0.23	−6186	798
None	0:1	1.19	−7198	0
	1:1	1.75	−7436	−238
Palmitic	2:1	3.13	−7841	−643
	3:1	2.91	−7749	−551
	4:1	2.43	−7638	−440
	5:1	2.67	−7696	−498
	6:1	2.08	−7542	−344
	10:1	1.07	−7133	65

[Albumin] = 11.4 μM.
* Ratio = moles FFA/mole albumin.
† K_a = Warfarin binding association constant (assuming $n = 1$).
‡ $\Delta G = -RT \ln K_a$.
§ Variation of ΔG from fatty acid free (0:1) ΔG value.

Table 2. Warfarin binding to bovine serum albumin (BSA) fragments and intact BSA

Protein source 10×10^{-6} M	Percent of warfarin bound [Warfarin] = 1 μg/ml*	[Warfarin] = 4 μg/ml†
BSA Fragment P-A	7.1	5.0
BSA Fragment P-B	5.6	1.9
BSA Fragments P-A + P-B	17.6	15.3
BSA Intact	52.5	48.4

* 1 μg/ml warfarin = 3.23×10^{-6} M.
† 4 μg/ml warfarin = 12.92×10^{-6} M.

DISCUSSION

The present studies show that four or more moles of fatty acids reduce the degree of warfarin binding to albumin by decreasing the affinity of albumin for warfarin. Affinities of albumin for fatty acids are one or two orders of magnitude greater than the affinity of albumin for warfarin; in the present studies fatty acids were present in 35 times greater concentrations than warfarin. Therefore, no warfarin binding would be expected to occur if decreased binding of warfarin to albumin in the presence of fatty acids arose from competitive binding of fatty acids to albumin. However, as shown in Fig. 1, 52 per cent of the warfarin remains bound in the presence of 10 moles of palmitic acid per mole of albumin and 17.5 per cent is still bound in the presence of 10 moles of oleic acid. These results together with those of Rippie [13] suggest that fatty acids may displace warfarin from albumin noncompetitively, despite the fact that Hughes–Klotz plots show a common y-intercept, conventionally interpreted as indicative of competitive displacement.

In contrast to the results with 4 or more moles of fatty acids, increasing the concentration of palmitate and oleate from 0 to 3 moles per mole of albumin enhanced warfarin binding. Fatty acids stabilize albumin; conversely, the albumin molecule is more labile in the absence of fatty acids [5]. This statement is supported by thermodynamic data presented in Table 1. Increasingly negative ΔG values at low fatty acid levels suggest a strengthening of the warfarin–albumin interaction, while the trend toward more positive ΔG values at high fatty acid levels suggests a decline in the warfarin–albumin interaction strength. Fatty acids not only place the albumin molecule in a more favorable thermodynamic state in concentrations of 2–3 moles per mole of albumin but also alter the tertiary structure, thereby affecting the interaction of other ligands with albumin [5]. For example, in the present experiment fatty acids alter the interaction of albumin with warfarin, increasing warfarin binding (Table 1 and Fig. 1).

The interactions of fatty acids with warfarin were studied further through the use of albumin fragments. Since fragments P-A and P-B retain their native secondary structure and associate to form an albumin-like complex [8], the synergistic response obtained when both fragments associate suggests that

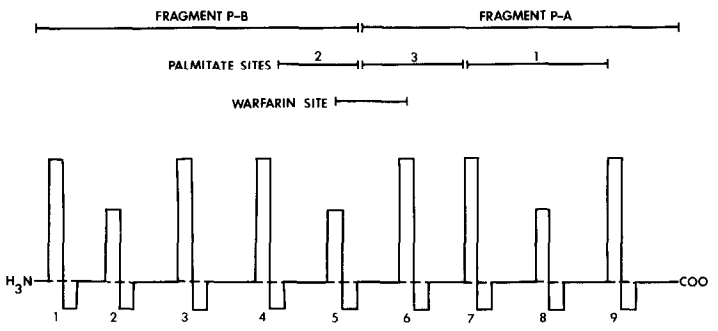

Fig. 4. Schematic diagram of BSA showing: The tertiary structure proposed by Brown consisting of nine major loops and 17 disulfide bonds [1, 3], the three strongest sites on BSA for the binding of palmitic acid as determined through the use of bovine albumin fragments by Reed *et al.* [7], the portions of the BSA molecule represented by fragments P-A and P-B [7, 10], and the proposed location of the primary warfarin binding site.

there is one primary warfarin binding site and that it may be cleaved during formation of the fragments. Various values for the number of primary warfarin binding sites have been reported: a single primary site [8, 15, 16], two primary sites [17, 18] and approximately 1.5 primary sites [19]. Also, warfarin has been reported to have a low affinity set of binding sites numbering three to four [18].

The first primary binding site for palmitate (Fig. 4) on albumin is located in the region of loops 7, 8 and 9; second primary site, loop 5; and third primary site, loop 6 [7]. The point at which the albumin molecule is cleaved to form fragments P-A and P-B is between loops 5 and 6 [10]. If the primary binding site for warfarin is located between P-A and P-B (i.e. between loops 5 and 6), as suggested by the fragment binding studies, then the biphasic effects observed in this study for fatty acids on warfarin can be explained by allosteric mechanisms as proposed by Rippie [13].

The first fatty acid molecule added would bind predominantly to the first primary fatty acid site. Although this is not the proposed location of the warfarin site, the first bound fatty acid would tend to stabilize the albumin tertiary structure and be allosterically responsible for enhanced warfarin binding. Since warfarin binding does not decrease until 3 or more moles of fatty acids are added, the presence of a fatty acid molecule at the second primary binding site must also allosterically enhance warfarin binding.

If the proposed warfarin binding site is located between the second and third primary fatty acid binding sites, small conformational changes at either site might exert significant effects on the tertiary structure of the surrounding regions including the proposed warfarin site. For example, a fatty acid binding to the second primary binding site at loop 5 may be far enough away from the warfarin site so that steric hindrance of warfarin binding does not occur. However, fatty acid may be close enough to induce conformational changes in the albumin molecule, thereby stabilizing the warfarin site and enhancing warfarin binding. Warfarin binding might also be enhanced by the presence of a fatty acid at the second primary site, if an induced conformational change led to more stable hydrophobic regions around the nearby warfarin binding site.

It is difficult to assess the role of the third primary fatty acid site in warfarin binding, since maximal warfarin binding occurs in the presence of 3 moles of oleic acid and 2 moles of palmitic acid. The implication is that the warfarin binding site is in close proximity to the third primary fatty acid binding site with warfarin binding affected by the steric conformation and/or the binding affinity of the particular fatty acid ligand.

The binding of four or more fatty acids occurs at relatively weak nonspecific secondary and tertiary class sites. This nonspecific binding adversely affects warfarin binding [5, 8, 9]. The degree to which nonspecific fatty acid binding to albumin and the binding of warfarin to low affinity sites on albumin affect the thermodynamics of warfarin–albumin interactions is not known.

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