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EFFECT OF HEPARIN AND FATTY ACIDS ON THE BINDING OF QUINIDINE AND WARFARIN IN PLASMA

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Abstract—After an intravenous injection of heparin, the plasma protein binding of warfarin was greatly increased while the binding of quinidine seemed to be less affected. The increased binding of warfarin seemed partly due to the release of plasma non-esterified fatty acids, NEFA, but the level of NEFA alone could not explain the interindividual variations of plasma warfarin binding. Addition *in vitro* of palmitic acid to serum demonstrated an increased binding of warfarin and an unaltered binding of quinidine up to a serum NEFA level of 3.0 meq/l. Albumin isolated from post heparin plasma revealed an increased binding affinity for warfarin at the warfarin high affinity binding sites, and a slightly depressed affinity for quinidine. The low affinity binding sites on the albumin molecule for both drugs did not seem to be influenced by NEFA.

Lipids as non-esterified fatty acids (NEFA), steroids and many drugs are transported in blood bound to albumin. However, the binding of various drugs and other substances by human serum albumin was reported to be decreased by an addition *in vitro* of NEFA [1–5]. In the rabbit, the binding of several drugs was depressed both in serum and in an albumin fraction of serum after an elevation *in vivo* of NEFA [6].

It is well documented that the enzyme lipoprotein lipase (LPL) is released into plasma after heparin injection [7–10], but the exact nature of this releasing prosess is not known in detail. The function of LPL is to hydrolyse the circulating triglyceride molecules which are mainly transported by chylomicrons and low density lipoproteins. A decreased amount of chylomicrons and very low density lipoproteins was observed [11] in plasma after heparin injection. Simultaneously the concentration of NEFA and glycerol increased while the concentration of triglycerides declined [12, 13].

The protein binding of quinidine and warfarin at different levels of NEFA in plasma was considered worthwhile to investigate, mainly because these drugs are bound to human serum albumin to a great extent. Concerning quinidine, a great variability in plasma protein binding was observed [14] and the total body clearance and apparent volume of distribution for this drug have been found to correlate well with the concentration of unbound quinidine in plasma [15]. Quinidine was of further interest because of the high binding affinity to all classes of lipoproteins [16–18]. Warfarin was of special interest because small variations in protein binding of this drug could produce great alterations in pharmacological effect and elimination, because warfarin possesses an extremely high degree of serum protein binding (about 99 per cent) and possesses a small volume of distribution.

MATERIALS AND METHODS

A healthy male, O.N.: 27 yr of age fasting over night, was given 10 IE heparin (A/S Apothekernes Laboratorium, Norway, containing 5000 IE heparin and 2 mg tricresol/ml) per kg body weight by an intravenous (i.v.) cannula (Venflon, Viggo AB, Sweden) over a period of 1 min. Blood was collected before heparin injection (zero time) and 15, 90, 120, 180 min after heparin injection. About 40 ml of venous blood was taken after each interval during a period of 2 min. Blood was collected in glass tubes containing 2 IE heparin per ml blood, and plasma was immediately prepared by centrifugation at 1100 *g* for 15 min at room temperature. Binding experiments were carried out on the same day on fresh unfrozen plasma. The remaining plasma was kept at −21° until further use.

An identical procedure was followed in another experiment where 70 IE heparin per kg body weight was injected into three healthy persons, L.S.: 38 yr, D.F.: 38 yr and O.N.: 27 yr of age, who had fasted overnight. Blood was collected in the same glass tubes as before but containing 8 IE heparin per ml blood. Blood was collected before heparin injections and 5, 15 and 150 min after heparin injection.

Addition of non-esterified fatty acids by celite. Palmitic acid (Applied Science Laboratories Inc. Lot 1888) was added to serum by a slightly modified method of that described by Spector and Hoak [19]. Celite (A. Tøssebro & Co., A/S, Aalesund, Norway) with particle size from 6–10 μm was washed with deionized water before use and dried at 100° overnight. Palmitic acid (51.3 mg) was dissolved in hexane and 2 g celite was added. Enough hexane was used so that the particles were immersed completely. The hexane was heated to 40° and was evaporated under a stream of nitrogen. The dry palmitate–Celite complex was shaken thoroughly on a Vortex mixer.

Eight hundred mg of the palmitate-cellite complex was added to 20 ml of fresh unfrozen serum and incubated in a stoppered flask in air for 45 min at 20° under gentle shaking. The incubate was then centrifuged for 20 min at 15000 *g* and 2°. The supernatant was then filtered through a Pond-cineris unius paper filter no 5893. The concentration of NEFA before and after addition of palmitic acid was 0.5 and 3.1 meq/l and the total concentration of protein in serum was 7.3 and 7.2 g/l respectively. Palmitic acid was also added to a serum concentration of 4.2 meq/l. The reference serum was, however, in later experiments treated with the same amount of celite without NEFA. Equal amounts of celite were added to all sera.

Addition of non-esterified fatty acids by ethanol. Palmitic acid from Applied Science Laboratories Inc. was dissolved in pure ethanol (117.5 meq/l). This solution was diluted by ethanol to give a concentration range in serum from 0.5 to 3.1 meq/l when 25 μl of the ethanol solution was added to 1 ml serum. Serum was incubated under gentle shaking for 1 hr at 20° after addition of palmitic acid.

Determination of non-esterified fatty acids. Concentration of NEFA was measured by a modified colorimetric method [20]. The standards were prepared in a 4% (w/v) human serum albumin preparation (AB Kabi, Sweden) free of NEFA by charcoal treatment [21]. Palmitic acid was added as proposed by Lorch and Gey [22]. The albumin preparation was used as a blank. The method produced a linear standard curve in the range from 0.15 to 4.0 meq/l.

Chemicals. Quinidine hydrochloride was supplied by the Norwegian Drug Monopoly, Oslo, and ^{3}H labelled quinidine with a sp. act. of 500 mCi/m-mole was supplied by Buchler & Co., Braunschweig, W. Germany [17]. Warfarin sodium from Nyegaard & Co., Oslo, and ^{14}C labelled warfarin with a sp. act. of 22 mCi/m-mole from The Radiochemical Centre, Amersham, England were used.

Protein binding. Equilibrium dialysis was used to determine the binding of drugs to serum, plasma and isolated protein fractions. Dialysis was performed in Perspex® dialysis cells with two compartments, each with a volume of 1 ml [17]. Serum and plasma were dialyzed against Krebs–Ringer bicarbonate buffer pH 7.35 in an atmosphere of 5% (v/v) CO_2 in air, and the isolated protein fractions against Krebs–Ringer phosphate buffer pH 7.35. Protein concentrations were measured before and after equilibrium dialysis and the Donnan effect was neglected [16]. Prior to equilibrium dialysis serum and plasma were gassed with 5% (v/v) CO_2 in air to obtain pH 7.35 and protein fractions of about 30–40 ml were dialyzed 24 hr at 4° against three shifts of 1000 ml Krebs–Ringer phosphate buffer pH 7.35. All equilibrium dialyses were performed at 20°.

The binding of drugs to isolated lipoprotein fractions was measured as the ratio ($B/F \times P$); the molar concentration of drug bound divided by the molar concentration of free drug multiplied by protein concentration measured after equilibrium dialysis [17]. In this expression one is able to make corrections for protein concentration which may not be equal in the different isolated lipoprotein preparations. The binding of drugs to different sera was measured as the ratio (B/F).

Protein determination. Protein concentration was determined by the method of Lowry *et al.* [23]. Bovine serum albumin (fraction V, Sigma) was used as a standard. Concentration of total lipoprotein ($d < 1.195$ g/l) was expressed in g protein per litre because of the inhomogeneity of this fraction. Albumin was, however, expressed in molar concentrations, assuming a mol. wt of 69,000. Concentration of albumin in plasma was determined by an immunologic technique at pH 8.6 using agarose in a concentration of 1% (w/v). The gel contained 2% (w/v) antialbumin serum from Dakopatts, Denmark. Ten-μl samples were applied to the gel which had a thickness of 2 mm [24].

Radioactivity. The activity of the ^{3}H- and ^{14}C-isotopes was determined in a Packard Tri-Carb liquid scintillation spectrometer model 3330 operated at 5° [17]. The counting efficiency was 28.5 and 85.1% respectively, and was the same in the buffer and the protein solution.

Thin-layer chromatography of unlabelled warfarin and of the ^{14}C-labelled isotope was performed before and after equilibrium dialysis after extraction by ethylene dichloride as described by Lewis *et al.* [48], with exception of the thin-layer chromatography sheet which was a Silica gel I B-F (J. C. Baker, No 4-4463). Location of warfarin was determined by fluorescence. The chromatograms were cut out and the radioactivity was determined in the scintillation spectrometer. The ^{14}C-labelled warfarin isotope contained 5.3% of ^{14}C-impurities before dialysis. The labelled compound was not subject to further degradation by equilibrium dialysis, as the total amount of impurities in the dialysis cell was constant. The distribution of the labelled impurities between buffer and protein was somewhat different from that of warfarin, as the impurities constituted 1.5 and 8.5 per cent of the total radioactivity in the protein and buffer solution respectively. However, this influence of labelled impurities will not influence the accuracy of the binding assay to any large extent, even though warfarin possesses an extremely high degree of serum protein binding. The serum protein binding of warfarin calculated by the specific assay of ^{14}C-warfarin radioactivity only and by the unspecific assay of total ^{14}C radioactivity demonstrated a B/F ratio of 73.2 and 68.3 respectively.

Isolation of lipoproteins. All classes of lipoproteins, very low, low and high density lipoproteins, were separated as a whole by preparative ultracentrifugation at 4° in a Spinco L2-65B ultracentrifuge. To plasma was added solid potassium bromide to achieve a density of 1.195 g/l and it was centrifuged for 45 hr at 105000 *g* in a Ti-50 rotor. The floating lipoproteins were withdrawn and dialyzed for 24 hr at 4° against four subsequent vols of 1000 ml Krebs–Ringer phosphate buffer pH 7.35. After withdrawing the lipoproteins, the waterphase separating the lipoproteins and the other serum proteins, was pipetted off and the density controlled.

Isolation of albumin. After removal of lipoproteins from plasma, the remaining serum proteins were dialyzed for 24 hr at 4° against three shifts of 1000 ml Krebs–Ringer phosphate buffer pH 7.35 and applied

on a G-200 Sephadex column at 4°. Albumin was eluted in the last protein peak and concentrated as described earlier [25].

Buffers. Krebs–Ringer phosphate, bicarbonate or sodium phosphate buffer [26, 27] was used as noted in the different experiments.

RESULTS

Plasma protein binding after heparin injection (10 IE/kg). Figure 1 gives the plasma binding expressed by the ratio [bound]/[free] for quinidine and warfarin and the plasma concentration of NEFA after equilibrium dialysis for one person (O.N.). As observed, the concentration of NEFA was highest in the plasma obtained 15 min after the heparin injection and this change was then reversed and at 180 min approaching the starting level of NEFA. The plasma binding of warfarin is clearly influenced by the heparin injection, the binding ratio was increased by 94 per cent after 15 min, and the binding returned to the original 180 min after the injection. The change in binding of warfarin does seem to be related to the plasma concentration of NEFA. For quinidine, however, the binding seems to be unaffected by the heparin injection and the great variation in plasma NEFA.

Plasma protein binding after heparin injection (70 IE/kg). Three healthy persons were given an injection of 70 IE/kg heparin. The plasma protein binding

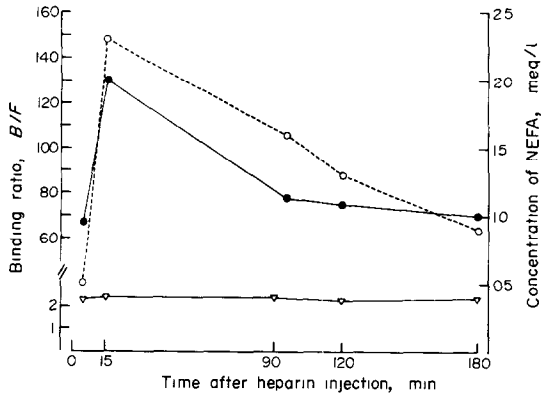

Fig. 1. Binding of quinidine (▽——▽) and warfarin (●——●) to human plasma after an elevation of NEFA level (O---O) by injection of 10 IE/kg heparin. 2×10^{-8} and 1×10^{-7} moles of quinidine and warfarin respectively were added to the chambers. Each point is the mean value of two determinations from one person.

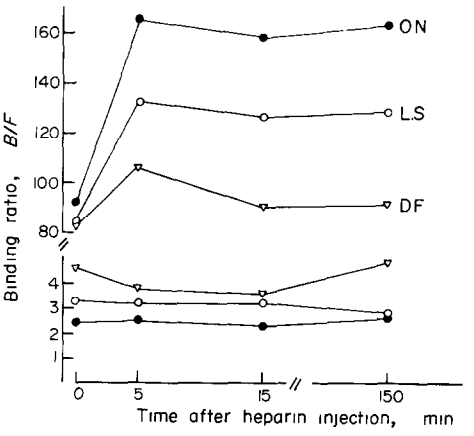

Fig. 2. Binding of quinidine and warfarin to human plasma after an elevation of NEFA level by injection of 70 IE/kg heparin. The lower binding ratios are referred to quinidine while the higher to warfarin. Concentration of drugs was equal to those used in Fig. 1. Each point is the mean value of two determinations.

of quinidine and warfarin at different time intervals after injection is given in Fig. 2. Table 1 gives the corresponding levels of NEFA measured before and after equilibrium dialysis. The level of albumin in plasma for O.N., L.S. and D.F. were 3.4, 3.9 and 3.8 g/100 ml respectively, measured after equilibrium dialysis. This increased amount of heparin injected produced a higher level of plasma NEFA and an increased binding ratio of warfarin for O.N. after 15 min compared with the results obtained in Fig. 1. The values before injection of heparin were also higher both with respect to NEFA and binding ratio of warfarin. Figure 2 demonstrates a simultaneous increase of plasma NEFA and plasma binding of warfarin while the binding of quinidine was approximately unaltered for O.N. and L.S. The third person D.F., however, demonstrates a more irregular binding of quinidine and an unexpected low increase in binding ratio of warfarin considered the high level of plasma NEFA.

In vitro *addition of palmitic acid to serum.* The increased binding of warfarin observed after heparin injection showed a relatively good correlation with the increase in plasma NEFA level. Figure 3 shows the binding of quinidine and warfarin to sera to which were added palmitic acid *in vitro* by the celite method. The range of NEFA in these sera included the range of NEFA obtained after heparin injection. The

Table 1. Plasma level of NEFA* after injection of 70 IE/kg heparin

	Before equilibrium dialysis				After equilibrium dialysis			
Persons	0 min	5 min	15 min	180 min†	0 min	5 min	15 min	180 min
L.S.	0.9	2.3	1.7	1.3	0.9	2.0	1.9	2.0
D.F.	0.4	2.8	1.8	1.1	1.0	3.5	3.0	3.1
O.N.	0.5	2.1	1.2	1.1	0.9	2.5	2.5	2.4

* Measured in meq/l.
† Time after heparin injection.

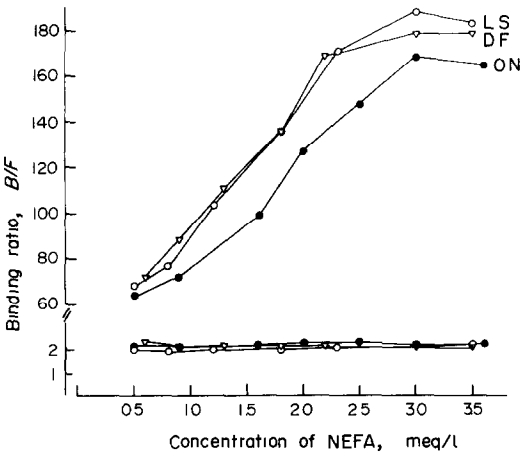

Fig. 3. Serum protein binding of quinidine and warfarin after an *in vitro* addition of palmitic acid. Concentration of drugs was equal to those in Fig. 1. The lower binding ratios are referred to quinidine while the higher to warfarin. NEFA was determined after equilibrium dialysis which was performed with Krebs–Ringer bicarbonate buffer pH 7.35. Each point is the mean value of two determinations.

already existing NEFA in serum was not removed before addition of palmitic acid and the values given for NEFA are consequently the sum of the added palmitic acid and the natural occurring NEFA in serum. Figure 3 demonstrates an increased binding ratio for warfarin with increasing concentrations of palmitic acid in serum for all persons up to a NEFA level of 3.0 meq/l. Serum levels of NEFA higher than 3.0 meq/l, seem to slightly depress the binding of warfarin. This indicates that the level of NEFA or palmitic acid are decisive for the degree of serum protein binding of warfarin. However, the extent of warfarin binding in plasma (Figs. 1 and 2) and in serum (Fig. 3) compared at the same levels of NEFA are not quite identical, especially with respect to D.F. For quinidine no regular alteration in serum protein binding was observed.

An additional experiment was performed where palmitic acid dissolved in pure ethanol was added directly to serum. This experiment demonstrated an increased binding ratio of warfarin with increasing levels of NEFA to the same extent as earlier, while the binding of quinidine was not affected. NEFA serum level was stepwise increased to 3.1 meq/l.

Binding to lipoproteins. Plasma obtained before and after injection of 70 IE/kg heparin from the three healthy individuals and which were frozen immediately after separation were melted at 20° and pooled in the ratio 1:1:1 at each time interval. From these different pooled sera the lipoproteins were separated as described in Methods. Table 2 demonstrates an extensive binding of quinidine to the lipoproteins while the binding of warfarin was almost negligible. However, none of the drugs showed any alteration in binding after heparin injection indicating that the increased binding of warfarin must be due to proteins other than lipoproteins.

Binding to albumin. Albumin was separated from the sedimentation products after ultracentrifugation

Table 2. Binding* of quinidine† and warfarin† to lipoproteins‡ before and after injection of heparin (70 IE/kg)

Drug	Time after injection			
	0 min	5 min	15 min	150 min
Quinidine	0.24	0.26	0.23	0.22
Warfarin	0.075	0.077	0.076	0.075

* Given as mean value of 2 experiments as the ratio B/F × P.

† 2×10^{-8} and 1×10^{-7} moles of quinidine and warfarin respectively were added to the chambers.

‡ Pool (1:1:1) of lipoproteins from L.S., D.F. and O.N. Concentration after equilibrium dialysis which was performed in Krebs–Ringer phosphate buffer pH = 7.35, was about 4.1 mg/ml.

of the pooled plasma obtained before and 5 min after heparin injection as described in Methods. The content of NEFA in the two albumin fractions was 1.25 and 3.57 moles per mole albumin. The concentration of NEFA in the original pooled plasma was 0.6 and 2.3 meq/l respectively, with an albumin concentration of 5.3×10^{-4} M. This demonstrates that all of the NEFA hydrolyzed from the triglycerides in plasma after heparin injection cannot be recovered on the separated albumin molecule.

The binding of warfarin to the pooled albumin fraction obtained before injection of heparin revealed quite different binding characteristics compared to the albumin fraction isolated after 5 min as can be seen from Fig. 4. This Scatchard plot [28] was obtained by adding warfarin to the chambers in the range from 2×10^{-9} moles to 4×10^{-7} moles. Figure 4 clearly demonstrates at least two different classes of binding sites for warfarin and an increased binding to the albumin fraction isolated after 5 min due to a much higher binding capacity for warfarin at the high affinity binding sites. The low affinity binding sites seemed to be about equal for both albumin fractions. Binding parameters [25, 29] obtained for warfarin are presented in Table 3.

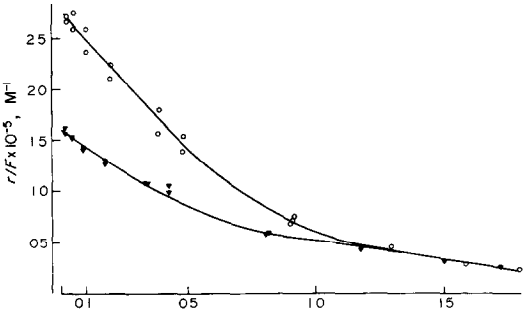

Fig. 4. Binding of warfarin to human plasma albumin plotted according to the method of Scatchard. (▼——▼) and (○——○) demonstrate the binding of warfarin to albumin isolated from plasma which was obtained before and 5 min after the 70 IE/kg heparin injection respectively. r = moles of warfarin bound per mole of albumin, F = free molar concentration of warfarin in Krebs–Ringer phosphate buffer pH 7.35. Concentration of albumin measured after equilibrium dialysis was 2.1×10^{-4} M for both preparations.

Table 3. Binding parameters for warfarin and albumin isolated from plasma before and 5 min after heparin* injection

Time after heparin injection	First binding class		Second binding class		moles NEFA
	n	K_D	n	K_D	mole albumin
0 min†	0.30	3.2×10^{-6}	1.9	3.0×10^{-5}	1.25
5 min	0.43	2.0×10^{-6}	1.9	3.0×10^{-5}	3.57

* 70 IE/kg body wt.
† Before heparin injection.

For quinidine the Scatchard plot in Fig. 5 demonstrates almost identical curves for the two albumin fractions when quinidine was added to the chambers in amounts ranging from 1×10^{-9} moles to 4×10^{-7} moles. A slightly lower binding to the albumin fraction obtained 5 min after heparin injection can, however, be observed, due to a slightly lower binding capacity for quinidine at the high affinity binding sites. The difference was, however, so negligible that the binding parameters evaluated did not differ significantly.

DISCUSSION

After injection of 10 and 70 IE/kg heparin, the plasma binding of warfarin quickly raised at the same time as a marked increase in plasma NEFA level was observed, and the plasma binding followed later roughly the changes in plasma NEFA level. However, it can be seen from Fig. 2 that the plasma binding of warfarin by D.F. showed the smallest increase after heparin injection although the level of NEFA clearly was the highest among the three persons. This demonstrates that injection of the same amount of heparin per kg body weight to different persons produces an interindividual difference in the plasma level of NEFA and that the interindividual degree of warfarin binding could not be directly related to the plasma NEFA level alone. For one and the same person, however, it seemed that increased amounts of NEFA produced a higher degree of warfarin binding.

Figure 3 demonstrates an almost linear relationship between binding ratio of warfarin and serum level of palmitic acid up to about 2.5 meq/l. If the binding ratios of warfarin in Fig. 3 are compared with the binding in Figs. 1 and 2 at corresponding plasma levels of NEFA, these are approximately in accord when compared to Fig. 1 where only small amounts of heparin were injected while they differ greatly when being compared with Fig. 2, where greater amounts of heparin were used. This indicates that heparin in greater amounts may also interfere with the plasma protein binding of warfarin by effects different from those caused by liberation of NEFA [30].

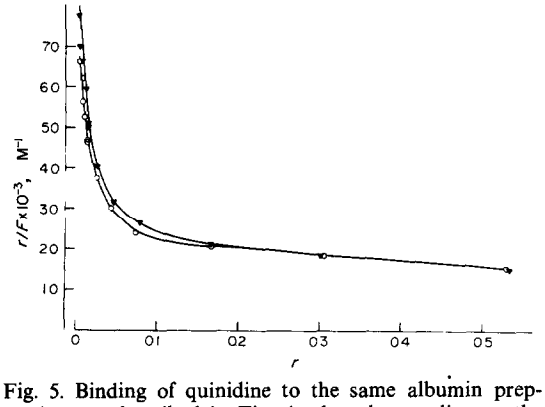

Fig. 5. Binding of quinidine to the same albumin preparations as described in Fig. 4, plotted according to the method of Scatchard. Symbols and conditions are equal to those in Fig. 4.

Figure 4 demonstrates a distinct increase in binding of warfarin to its high affinity binding sites on the albumin molecule after heparin injection. The increased binding capacity of the high affinity binding sites on the albumin molecule for warfarin seemed to be due to increased affinity for this site while number of binding sites seemed to be constant when this binding class was investigated in a double reciprocal plot in a concentration range of warfarin where the influence of other binding sites were nearly negligible. This effect is probably caused by NEFA progressively, altering the conformation of the albumin molecule [31], with an increased affinity for warfarin on the high affinity sites as a result.

Some work has been performed in evaluating binding parameters for the albumin-warfarin interaction. These have been occupied mainly with the high affinity sites probably because the low affinity sites in the albumin molecule only deal with interactions that occur with concentrations of warfarin higher than those clinically found [32, 33]. If only the high affinity binding sites are taken into consideration, our data demonstrate a dissociation constant of $(6.2 \pm 0.3) \times 10^{-6}$ and $(3.6 \pm 0.2) \times 10^{-6}$ for albumin separated before and 5 min after heparin injection. The number of high affinity binding sites per albumin molecule was 0.99 ± 0.05 and 1.00 ± 0.06 respectively. The S.D. values are given. These results were consistent with those obtained for normal albumin by Aggeler [34], Chignell [35], Solomon [5] and Tillement [36] but differed from those of others [37, 38]. The range of data obtained by the four formerly mentioned, were from 0.95 to 1.00 high affinity binding sites per albumin molecule and a dissociation constant ranging from 3.8 to 6.4×10^{-6} M. As can be seen, the variation in dissociation constants in these four investigations varied exactly between the same limits found in our experiments before and after 70 IE/kg heparin injections, which could indicate that the differences in dissociation constants obtained for the four formerly mentioned could be due to differences in amount of NEFA in their albumin preparations.

The binding parameters K_D and n, dissociation constant for the complex and number of binding sites, obtained by evaluating two classes of binding sites from the Scatchard plot are not very well suited for discussion of inhibition patterns or characteristics of one special class of binding sites, because these obtained binding parameters possess great variability, and more than one solution can fit the experimental points almost equally well [39, 40]. For this reason the binding parameters evaluated from the Scatchard plot (Table 3) may only be used to calculate the binding capacity of albumin in solution or in serum.

The decrease in plasma concentration of free unbound warfarin under influence of increased amounts of NEFA might be of great importance for both the pharmacokinetic and pharmacodynamic behavior of warfarin because it is generally assumed that the free drug is responsible for the pharmacological effect and is available for distribution and elimination [33, 41–44]. In addition the daily variation in NEFA may produce a very undesired variability in steady-state concentration of free warfarin even though the total concentration of warfarin in plasma is approximately constant because of the high degree of protein binding.

Our results with increased binding of warfarin to albumin and plasma after elevation of NEFA were not in accord with Gugler *et al.* [4] and Solomon *et al.* [5], who found that NEFA depressed the binding of warfarin. Our experiments differed from these investigations in binding assay, the method to increase NEFA content *in vivo* and *in vitro*, the choice of buffer and the ratio warfarin:NEFA:albumin. The last two matters were in our opinion the most likely explanation for the different results. However, control experiments performed with buffer and warfarin:NEFA:albumin ratio equal to the experiments performed by Gugler and Solomon, still demonstrated an increased serum binding at increased serum levels of NEFA.

With respect to the plasma binding of quinidine it seemed to be very slightly affected by small doses of heparin (Fig. 1), while at higher doses it produced a more irregular binding pattern (Fig. 2). This irregularity could not be related to the plasma level of NEFA and is most likely caused by the large dose of heparin.

The unaltered binding of quinidine to the lipoprotein fractions after heparin injection in Table 2 was rather unexpected because the level of cholesterol and triglycerides and consequently of lipoproteins, should be decreased. This indicates that the binding of quinidine to lipoproteins during degradation of glycerides and phospholipids yielding NEFA, cannot be related to alterations in lipids alone.

The binding ratios of quinidine for L.S. and D.F. are somewhat higher when NEFA was raised by heparin compared to the *in vitro* addition of palmitic acid. This phenomenon could be caused by the heparin itself, but may also be caused by an intraindividual variability in binding of quinidine by these persons. The last suggestion because the binding ratio at time zero, where neither heparin nor palmitic acid were added, also demonstrated different levels. Intraindividual variability in binding of quinidine is also demonstrated by others [15].

The decreased binding of quinidine to the high affinity sites in albumin due to increased amounts of NEFA after heparin injection (Fig. 5) seems to be of theoretical interest only, because NEFA seemed to have little influence on the total serum binding as shown in Fig. 3. However, if the binding data for the high affinity binding sites for quinidine were plotted in a double reciprocal plot, the inhibition effect produced by NEFA appeared to be competitive. Three different classes of binding sites are mentioned for NEFA on the albumin molecule ($n_1 = 2$, $n_2 = 5$ and $n_3 = 20$) [45], while the high affinity binding class for quinidine has less than one binding site per albumin molecule [25]. The small number of binding sites available for quinidine on the albumin molecule was, however, previously suggested to be due to such endogenous substances as NEFA partly blocking the binding sites for quinidine [18, 25]. These observations may indicate that at a normal level of NEFA on the albumin molecule some of the high affinity binding sites for quinidine are permanently blocked by NEFA while a minor competitive inhibition may occur at higher concentrations.

Compared to plasma, a decreased recovery of NEFA from about 105 to 78 per cent, was observed in the isolated albumin fraction after heparin injection. This may be due to loss of NEFA from highly occupied low affinity binding sites on the albumin molecule during the separation procedure or by binding of NEFA to other proteins than albumin in plasma, as for instance the lipoproteins, at elevated levels of plasma NEFA.

When NEFA level in plasma is raised *in vivo* by use of heparin given i.v., the enzyme lipoprotein lipase is released into plasma. This enzyme is active when it has a cofactor, probably specific glycoproteins [46] which are present in very low and high density lipoproteins. In separated plasma the lipase and cofactors are present in the triglycerides in plasma can be further hydrolyzed yielding an increase in plasma NEFA during equilibrium dialysis which is demonstrated in Table 1. This hydrolysis was extensive although a relatively large amount of heparin was added to the glass tubes where blood was collected. Heparin added *in vitro* was reported to partly inhibit the lipase activity [47]. The hydrolysis of triglycerides in plasma during equilibrium dialysis at 20° was, however, terminated after 3–4 hr and the binding of warfarin and quinidine was consequently determined in a system at equilibrium. This is because the equilibrium dialysis was run for 18 hr and the equilibrium for the mentioned drugs is attained after only 5–6 hr.

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