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THE EFFECT OF CHLORIDE ON THE BINDING OF WARFARIN TO ALBUMIN AS A FUNCTION OF pH

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Abstract—The effect of chloride on the binding of warfarin to human serum albumin between pH 6 and 9.5 has been studied by circular dichroism and equilibrium dialysis. The molar ellipticity of the warfarin–albumin complex is hardly affected by chloride ions when the protein is in the B conformation, whereas in the N conformation this spectroscopic quantity is greatly reduced in the presence of chloride ions. At all pHs the affinity of warfarin for albumin is decreased by the chloride ion. At pH 7.4 this is primarily due to a displacement of warfarin from its high affinity site. The binding constant of chloride for this specific site is about 20 M^{-1}. At the other pHs investigated chloride affects also the N–B transition. However, the affinity of chloride for the warfarin binding site of albumin is hardly affected by the N–B transition. The physiological importance of the N–B transition in changing albumin binding parameters for drugs and endogeneous compounds caused by small changes in blood concentrations of hydrogen and other ions is discussed.

Recently it has been shown [1, 2] that the N–B transition in the albumin† molecules affects the binding of the anticoagulant warfarin. Further it can be demonstrated [1, 2] that the physiologically important ions Ca^{2+} and Cl^- affect the free concentration of warfarin (c_{free}) at a given total concentration of drug and protein. The effect of Ca^{2+} has been explained fully by a shift in the N $\rightleftarrows$ B equilibrium, but although the chloride effect on c_{free} exceeds the calcium effect, the nature of its action was not understood completely. Janssen and Nelen [3] have found using microcalorimetry, that the binding of sulfaethidole to both bovine serum albumin and human serum albumin is affected by Cl^-ions. They found that on the average one Cl^- is displaced by each sulfaethidole molecule bound. Brown and Crooks [4] have reported that the binding of tolbutamide to albumin is strongly affected by NaCl, whereas other authors [5] report a negligible effect of NaCl, KCl, $CaCl_2$ and $MgCl_2$ on the digoxin–albumin interaction, in spite of the fact that a change in pH from 6 to 9 decreased the binding constant of digoxin to albumin by a factor of more than 40.

Free blood levels of drugs are often determined by means of equilibrium dialysis of serum or plasma against a phosphate buffer, which is occasionally made isotonic using NaCl [6–12]. For such experiments an insight into the nature of possible Cl^- effects on drug-albumin binding appears as important as those caused by Ca^{2+} and H^+. A knowledge of the Cl^- effect seems to be necessary for those studies examining the effect of endogeneous compounds on drug-protein binding in plasma or serum since such studies are carried out often using a series of samples diluted with a chloride containing buffer [10, 13]. Therefore in this paper the effect of Cl^- on the binding of warfarin to albumin as a function of pH is reported in more detail.

MATERIALS AND METHODS

Albumin, crystallized and lyophilized, (sample number 76c-8145) was obtained from Sigma Chemical Company, St. Louis, MO., U.S.A., and was treated before use as described elsewhere [2]. The fatty acid content was less than 0.1 on a molar ratio basis. Concentrations of albumin solutions were determined by drying at 105° in air to achieve a constant weight. The molecular weight of albumin was taken as 66,500. In all experiments the albumin concentration was 6×10^{-5} M. Sodium warfarin (British Pharmacopoeia quality, Brocacef, Maarssen, The Netherlands) was used without further purification. All other chemicals used were of analytical grade (Merck, Darmstadt, West Germany or J. T. Baker, Deventer, Holland). Free concentrations of warfarin were obtained by means of equilibrium dialysis using a Dianorm equilibrium dialyzer (Diachema, A. G., Rüschlikon, Switzerland) with cells of 10 ml total volume. Dialysis membranes (Diachema type 10.14, molecular weight-cutoff of 5000) of hydrated cellulose were used.

The analysis of the samples containing the free warfarin were performed using a high pressure liquid chromatographic method. Full details of the equilibrium dialysis and analysis procedures have been described previously [2].

Circular dichroic studies of the warfarin–albumin interaction have been carried out using a Dichrograph III (Jobin Yvon, Long Jumeau, France) between 300 and 370 nm. The slit was programmed for a half-band width of 2 nm (sensitivity 1 or 2×10^{-6} degree mm^{-1}, scanning speed 3 nm min^{-1}, time constant 20 or 10 s). Pathlengths of 5, 10, 20 and 50 mm were used. The observed ellipticities (θ_{obs}) are the differences between the CD spectra of

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† "Albumin" is used to mean human serum albumin, unless otherwise stated.

the drug–albumin mixture and of the albumin alone at a given wavelength [14]. All experiments were carried out at 25°.

RESULTS AND DISCUSSION

In a previous paper [2] we have shown that less warfarin is bound to albumin in the presence of chloride ions compared to their absence. The precise mechanisms of this chloride effect was not understood fully at that time. In an attempt to elucidate this mechanism we have performed a series of CD and dialysis experiments so as to study the effect of chloride both on the extrinsic Cotton effect of warfarin–albumin mixtures and on c_{free} at selected pH values.

In Fig. 1 the observed ellipticity of warfarin–albumin mixtures at pH 6.1 is plotted as a function of the warfarin to albumin ratio r in the absence and presence of 0.02 M NaCl. In both cases the titration curve is sigmoidal in nature. This shape can be interpreted in terms of the existence of at least two binding sites for warfarin with considerable difference in affinity, and which contribute unequally to the ellipticity. For all r values θ_{obs} decreases in the presence of 0.02 M NaCl, although it is remarkable that the relative decrease in θ_{obs} is nearly independent of r. The chloride effect on θ_{obs} is less pronounced at pH 7.4 and 9.3 (not shown). The effect of various concentrations of Cl^- on θ_{obs} (310 nm) for warfarin–albumin mixtures with $r = 0.4$ at pH 6.1 and 9.3 is given by Fig. 2. It can be seen that at pH 6.1 the CD signal is much more suppressed by Cl^- than at pH 9.3. In the presence of 0.1 M NaCl θ_{obs} is about 15 per cent less than in the absence of Cl^- at pH 9.3. At pH 6.1 θ_{obs} is suppressed for about 90 per cent by 0.1 M Cl^-.

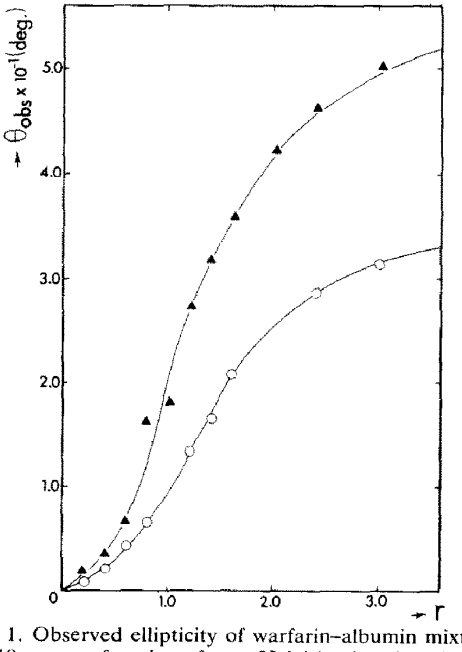

Fig. 1. Observed ellipticity of warfarin–albumin mixtures at 310 nm as a function of r at pH 6.1 in phosphate buffer, $I = 0.1$ (▲) and in phosphate + 0.02 M NaCl (○). All measurements are corrected to a 1 cm cell.

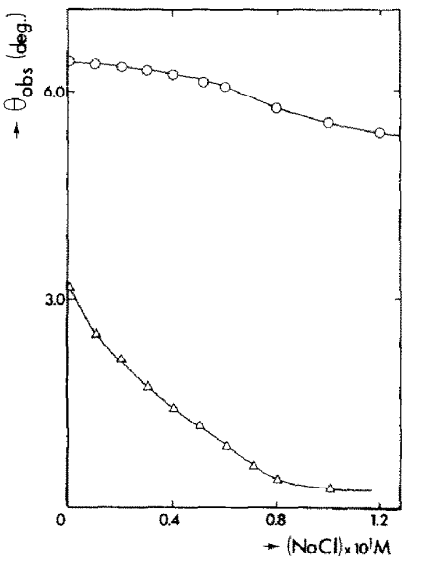

Fig. 2. Observed ellipticity of warfarin–albumin mixtures ($r = 0.4$) at 310 nm at pH 6.1, phosphate buffer (△) and pH 9.3, borate buffer (○) as a function of the sodium chloride concentration. Other conditions as in Fig. 1.

It is clear from Fig. 2 that the effect of Cl^- on θ_{obs} is pH dependent. Albumin exists in two conformational forms between pH 6 and 9, the so called N conformation (main form at pH 6), and the B conformation (main form at pH 9) [1, 2, 15–21]. This N–B transition plays an important role in the binding of warfarin to albumin [2]. The results presented in Fig. 2 suggest strongly that the chloride effect on θ_{obs} is dependent on the conformational state of the albumin. This is supported by the results given in Fig. 3, where θ_{obs} of warfarin–albumin mixtures ($r = 0.4$) is plotted as a function of pH in the absence

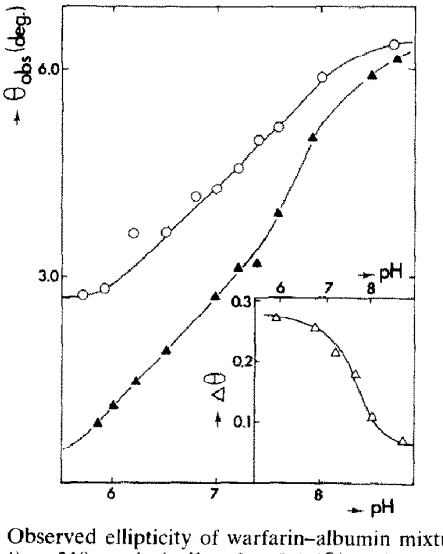

Fig. 3. Observed ellipticity of warfarin–albumin mixtures ($r = 0.4$) at 310 nm in buffer, $I = 0.1$ (○) and in buffer + 0.04 M NaCl ($I = 0.1$) (▲) as a function of pH. Other conditions as in Fig. 1. Inset: $\Delta\theta$ as a function of pH, $\Delta\theta$ being defined as $(\theta - \theta_{Cl^-})/\theta$. θ and θ_{Cl^-} are θ_{obs} in the absence and in the presence of Cl^-, respectively.

and in the presence of 0.04 M NaCl. Since Na^+ ions do not affect θ_{obs} [2] the observed changes in θ_{obs} must be due to the chloride ion. The magnitude of suppression of θ_{obs} by Cl^- decreases with increasing pH and this is shown clearly in the inset of Fig. 3, where the relative decrease in θ, ($\Delta\theta$), is plotted as a function of pH. The pH dependence of $\Delta\theta$ is very similar to that found for the ratio B to N, (see also ref. 2). The question which now arises is to what extent does this decrease in θ_{obs} reflect a decrease in the amount of warfarin bound? To answer this the free concentration of warfarin in the presence and in the absence of Cl^- for $r = 0.4$ needs to be known. Values for $r = 0.1$ have been determined by us [2] and it is found that the free concentration of warfarin in the presence of 0.1 M chloride is about 2–3 times higher than in phosphate buffer. The free concentrations for $r = 0.4$ are presented in Table 1. In the presence of Cl^- the free concentration of warfarin is almost 60 per cent higher. Since warfarin is very tightly bound to albumin this marked increase in c_{free} causes only a small decrease in the concentration of bound drug. Using the data from Table 1 it can be computed that at pH 6.1 in phosphate buffer 95.0 per cent of the warfarin is bound, whereas in the presence of 0.1 M Cl^- this decreases to 92.3 per cent. At pH 9.3 the amounts bound are 98.0 per cent and 96.8 per cent respectively. The large decrease in θ_{obs} when Cl^- is added at pH 6.1 can only be interpreted therefore in terms of a decrease in molar ellipticity, $[\theta]$, of the warfarin–albumin complex. Therefore we can conclude from Fig. 2 that at pH 6.1 $[\theta]$ is very sensitive to the Cl^- concentration and that this is not the case at pH 9.3. Since the chloride effect on θ_{obs} has to be associated with the N–B transition (Fig. 3), this means that $[\theta]$ is very sensitive to Cl^- when albumin is in the N conformation, whereas in the B form such a strong dependence is lacking.

Circular dichroism and other spectroscopic techniques are indirect methods for measuring the amount of drug bound, and are frequently used in displacement studies in which the amount of drug displaced by another drug is derived directly from the decrease in spectroscopic signal in the presence of the displacing agent. A proportionality is then assumed between the amount of drug bound and the magnitude of the signal. The finding that $[\theta]$ of such a complex can be dependent on the composition of the solution makes results of displacement studies based on spectroscopic techniques alone very questionable. Several authors [10, 22–30] have derived the amount of displaced drug directly from the decrease in spectroscopic signal in the presence of a displacing agent. In such cases the molar ellipticity of the drug–albumin complex may be affected by the antagonist. Sjöholm *et al.* [10] have found that uremic sera produces significantly smaller Cotton effects than normal sera, although in both cases the same amount of warfarin was present. They conclude from this observation an impaired binding ability of warfarin for albumin in the uremic sera. However it is not known to what extent the changed composition of the sera is responsible for a smaller molar ellipticity of the extrinsic Cotton effect.

Recently it was reported [2] that at pH 7.4 Cl^- hardly changes the N ⇄ B equilibrium, as concluded from an unchanged value of the pH_{50}. (pH_{50} being defined as the pH where 50% of the protein is in the B form). However, at other pHs between 6.1 and 9.3 the N ⇄ B equilibrium was strongly affected by Cl^-. To elucidate the nature of the interaction between Cl^- and warfarin with respect to their binding affinity for albumin the free concentration of warfarin at different warfarin to albumin ratios was determined in the absence and in the presence of 0.1 M NaCl at pH 7.4. Since the high affinity site of warfarin is mainly involved in the binding of warfarin for $r < 0.4$ [2], experiments were restricted to such r values. The results are shown in Table 2. For comparison the free concentrations in phosphate and in the presence of 0.1 M Cl^- + 2.5×10^{-3} M Ca^{2+} are given also. It can be concluded from the table that the effect of Cl^- and Ca^{2+} on the free concentration is different. This is shown clearly by Table 3, where the quantities $c_{free}(Cl^-)/c_{free}$ and $c_{free}(Ca^{2+}, Cl^-)/c_{free}(Cl^-)$ are given. The former quantity decreases with increasing drug to protein ratio, whereas the latter ratio remains nearly constant. Since for $r < 0.4$ only one site for warfarin binding is involved, the decrease in $c_{free}(Cl^-)/c_{free}$ with r suggests a direct competition between Cl^- and warfarin for the same site. The calcium effect is due to a shift in the N ⇄ B equilibrium in favour of the B conformation [2] and therefore it can be expected that $c_{free}(Ca^{2+}, Cl^-)/c_{free}(Cl^-)$ is nearly independent of r under the experimental conditions (see Appendix). To illustrate the idea of a direct competition between Cl^- and warfarin plots of $1/\mu$ versus $1/c_{free}$ [31] in the presence and in the absence of 0.1 M Cl^- have been made (Fig. 4). Two straight lines are obtained with the ordinate intercept appearing to be common to

Table 1. Dialysis data for $r = 0.4$ in phosphate or borate, $I = 0.1$ and in 0.1 M NaCl at four pHs*

	pH			
	6.1	7.4	8.2	9.3
$c_{free} \times 10^6$ (M) in phosphate or borate ($I = 0.1$)	1.18 ± 0.01	1.07 ± 0.02	0.70 ± 0.01	0.49 ± 0.01
$c_{free} \times 10^6$ (M) in 0.1 M Cl^-	1.83 ± 0.01	1.66 ± 0.01	1.09 ± 0.02	0.77 ± 0.01

* Phosphate buffers were used at pH 6.1, 7.4 and 8.2; a borate buffer was used at pH 9.3. Every value of c_{free} is based on three measurements. The standard deviations are given. [Albumin] = 6×10^{-5} M.

Table 2. Effect of Ca^{2+} and Cl^- on the free concentration of warfarin at pH 7.4*

r	$c_{free} \times 10^6$ (M)	$c_{free}(Cl^-) \times 10^6$ (M)	$c_{free}(Ca^{2+}, Cl^-) \times 10^6$ (M)
0.05	0.05 ± 0.02	0.16 ± 0.02	0.13 ± 0.02
0.1	0.11 ± 0.01	0.33 ± 0.03	0.26 ± 0.02
0.2	0.30 ± 0.01	0.80 ± 0.02	0.66 ± 0.01
0.3	0.77 ± 0.02	1.21 ± 0.10	0.92 ± 0.02
0.4	1.07 ± 0.02	1.66 ± 0.01	1.38 ± 0.02

* $[Ca^{2+}] = 2.5 \times 10^{-3}$ M, $[Cl^-] = 1.0 \times 10^{-1}$ M. c_{free}, $c_{free}(Cl^-)$ and $c_{free}(Cl^-, Ca^{2+})$ are the free concentrations of warfarin in the presence of buffer, Cl^- and $Cl^- + Ca^{2+}$ respectively. Other conditions as for Table 1.

both, suggesting a competition between Cl^- and warfarin for the same site. The slope of these lines depends on the presence of Cl^- as does the intercept on the $1/c_{free}$-axis. From this intercept the binding constant of warfarin to its high affinity site on albumin in the absence of Cl^- can be calculated ($K \approx 10^6\ M^{-1}$, pH 7.4). Assuming competition between Cl^- and warfarin, in the presence of Cl^- the apparent binding constant K_{app} and the binding constant for Cl^- on the warfarin binding site (K_{Cl^-}) can be derived from the intercept using the equation $K_{app} = K/(1 + [Cl^-]K_{Cl^-})$ where $[Cl^-]$ is the free concentration of Cl^- [32]. Using $K = 10^6\ M^{-1}$ and $[Cl^-] = 10^{-1}$ M a value for $K_{Cl} \approx 20\ M^{-1}$ can be calculated. This value for K_{Cl} corresponds reasonably with the binding constant of the third class of chloride-binding sites as found by Scatchard *et al.* [33, 34]. Janssen and Nelen [3] reported that Cl^- ions compete with sulfaethidole for the same site on bovine serum albumin. They found that the Cl^- ion has a heat of binding of -14.3 kJ/mol^{-1} on that site, which corresponds well with the value for Cl^- on the secondary class of chloride binding sites on bovine serum albumin. That $[Cl^-]$ can be taken as 0.1 M is because Cl^- is present in a large excess to albumin (molar ratio about 1600) and the affinity of chloride for albumin is relatively low. The drug to protein ratio was varied only between 0 and 0.4 since for $r > 0.4$ the contribution of sites of lower affinity to the total warfarin binding can no longer be neglected [2] and hence a linear relationship between $1/\mu$ and $1/c_{free}$ would no longer exist.

When the binding data in the absence and presence of competitor are available only at one drug to protein ratio the binding constant of competitor can be calculated using the equation [35]

$$K_{Cl} = \frac{K\,c_{free}}{[PW]} \cdot \frac{K\,c_{free}[P]_t - K\,c_{free}[PW] - [PW]}{K\,c_{free}[Cl^-]_t - K\,c_{free}[P]_t + K\,c_{free}[PW] + [PW]} \quad (1)$$

where K_{Cl} is the binding constant for Cl^-, K the binding constant for warfarin on the high affinity site, c_{free} the free concentration of warfarin, $[P]_t$ the total albumin concentration and $[PW]$ the total concentration of warfarin–albumin complex. This equation is valid only with simple competition between two compounds for the same binding site. This means that in case of competition between warfarin and Cl^- for the same site, the K_{Cl} values calculated with equation 1 must be independent of r. This was indeed found for $r \leq 0.4$, K_{Cl} being 20 ± 2 M^{-1}. At pH 6.1 and 9.3 the protein is completely in the N and B conformation respectively. This is also the case when Cl^- is present. Therefore it is reasonable to assume that at these pHs the mechanism of the chloride effect on c_{free} of warfarin is that of a direct compe-

Table 3. Effect of Ca^+ and Cl^- on the free concentration of warfarin at pH 7.4*

r	$c_{free}(Cl^-)/c_{free}$	$c_{free}(Ca^{2+}, Cl^-)/c_{free}(Cl^-)$
0.05	3.20	0.81
0.1	2.89	0.80
0.2	2.65	0.82
0.3	1.58	0.76
0.4	1.56	0.83

* $[Ca^{2+}] = 2.5 \times 10^{-3}$ M, $[Cl^-] = 1.0 \times 10^{-1}$ M. c_{free}, $c_{free}(Cl^-)$ and $c_{free}(Cl^-, Ca^{2+})$ are the free concentrations of warfarin in the presence of buffer, Cl^- and $Cl^- + Ca^{2+}$ respectively. Other conditions as for Table 1.

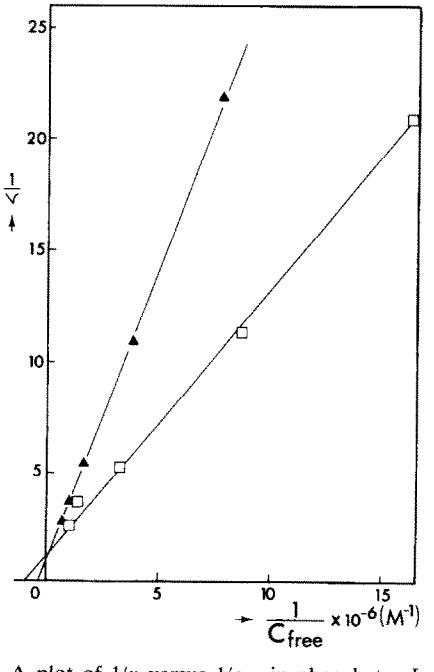

Fig. 4. A plot of $1/v$ versus $1/c_{free}$ in phosphate, $I = 0.1$ (□) and in 0.1 M Cl^-, pH adjusted with NaOH (▲) at pH 7.4; v = molar ratio of drug bound and albumin.

tition. At these pHs the binding data from Table 1 were used. Using equation 1 and taking the binding constant for warfarin at this pH ($K = 5 \times 10^5$ M^{-1} at pH 6.1 and 1.5×10^6 M^{-1} at pH 9.3, see ref. 2), values for K_{Cl} are found to be 10 M^{-1} at pH 6.1 and 25 M^{-1} at pH 9.3. At other pHs between pH 6.1 and 9.3 Cl^- also affects the N ⇆ B equilibrium. A reasonable estimation of K_{Cl} can thus be obtained by calculating the binding constant of warfarin with the equation $K = (1-\alpha)K_N + \alpha K_B$, where α is the fraction of the protein in the B form when 0.1 M Cl^- is present and K_N and K_B are the pH independent binding constants of warfarin when the protein is in the N and B form respectively (see also ref. 2). The fraction α has been derived from published θ_{obs} versus pH curves [2]. When these calculated binding constants for warfarin are substituted into equation 1 using the binding data for $r = 0.1$ [2], values for K_{Cl} are found to be between 10 and 25 M^{-1}. Taking into account the large effect of Cl^- on the N–B transition this observed pH effect on K_{Cl} is rather small. Possibly the pronounced effect of Cl^- on the N–B transition is caused by the binding of Cl^- on sites other than the warfarin binding site on albumin. The found value for K_{Cl} at the lowest pH is in excellent agreement with the reported values for the third class of chloride binding sites at pH 5.2 [33].

Implications of Ca^{2+}, Cl^- *and pH on drug–albumin binding*

It is clear that the chloride ion affects the binding of warfarin to its high affinity site on the albumin by two different mechanisms. At pH 7.4 a competition between Cl^- and warfarin is dominant, whereas at other pHs over the range 6.1 to 9.3 a marked effect of Cl^- on the N ⇆ B equilibrium also occurs, which affects the binding of warfarin in an indirect manner. Ca^{2+} affects the binding of warfarin only via the N–B transition [2]. The combined effect of Ca^{2+} and Cl^- results in a pronounced pH dependence of the free concentration of warfarin in a small pH interval around pH 7.4 ($6.8 < pH < 7.8$; see also ref. 2). Therefore it may be expected that small changes in plasma pH alter the state of the N ⇆ B equilibrium and hence the concentrations of free and bound warfarin in the plasma. Warfarin acts and is metabolised mainly in the liver. In healthy man the pH in the bulk phase of the blood is lowered about 0.1 pH unit, when passing through the liver [36]. Considering the high buffer capacity of the blood [37] a pH even lower than 7.3 is very likely in the direct vicinity of the vessel walls, where exchange of the drug and other substances with the pericellular environment of the liver occurs. It is reported [38–43] that the pH on the pericellular level in organs as the liver can be as low as 6.9–7.0. Therefore it seems possible, that c_{free} of warfarin available for metabolism and pharmacological action is not equal to the c_{free} present in the bulk phase of the blood. Preliminary stopped-flow experiments on the kinetics of the B → N transition and the dissociation of the warfarin–albumin complex showed, that the corresponding half-lives are within the transit time of albumin in the liver (~10 sec) [44]. An adaptation of the N ⇄ B equilibrium and the warfarin binding equilibrium to the new condition with respect to the pH seems therefore possible.

If the patient's condition alters the pH in the blood, in the bulk phase or locally on the pericellular level in tissues or organs such as the liver, then also changes in free concentration of warfarin can be expected. Such changes are also likely to occur when the chloride concentration in the blood is altered. Although the plasma level of Cl^- is normally about 100 meq/l, disturbance in the acid–base balance can result in a level within the range of about 75–115 meq/l [45]. Such conditions may be acute or chronic and can be of primary respiratory or metabolic origin. Chronic deviations of the chloride level may lead to an individual dosing of warfarin. It is clear that in diseased states a variety of possible shifts of pH, (a tolerated pH range 6.7 to 7.9 is possible [45]), and chloride concentration in the blood, in combination or alone, is possible. In the case of warfarin the free concentration of the drug approximately doubles as a result of a shift in pH from 7.4 to 7.0, when 100 meq/l Cl^- and 5 meq/l Ca^{2+} are present. This difference in free concentration due to a small difference in affinity of warfarin for the N and B conformations may be not so dramatic, but in other cases, such as that of oxyphenbutazone*, this difference will be larger and a pronounced sensitivity of the free concentration of a drug for small changes concentration of H^+ and other ions can be expected.

It is clear that from a pharmacokinetic point of view changes in ionic content of the blood and in N ⇄ B equilibrium should be taken into account in studies of drug–albumin interactions. For such studies a knowledge of the free concentration of a drug is necessary. As far as knowledge of free levels of drugs in patients are necessary, then those in plasma serve as a useful parameter. However, this free plasma level of drug is often determined using equilibrium dialysis. Such dialysis experiments are carried out with the sample on one site of the membrane and a buffer on the other. Due to the lower Ca^{2+} and Cl^- concentration in the protein compartment than in the plasma caused by dilution (Ca^{2+} and Cl^-) and binding to phosphate (Ca^{2+}) the binding parameters will be changed and so the results are affected by the dialysis procedure itself. In particular, when the buffer compartment is larger in volume than the plasma compartment, larger deviations from the actual free plasma level will be found. Therefore more attention should be paid to the buffer composition. Due to the strong pH dependent binding of warfarin to albumin around 7.4 and the increase in pH of the plasma as soon as it is taken from the patient, pH control during the dialysing procedure is another point of importance. On the basis of the above arguments differences in results between serum and plasma containing citrate may be expected also, since citrate will complex most of the Ca^{2+} ions. It has been reported [46] that indeed such differences exist. Although the present discussion centres around warfarin, it may be remarked that the binding of other drugs such as oxyphenbutazone* and diazepam [47] is affected also by the N–B transition.

* Will be published elsewhere.

Summarizing, we can conclude that it is likely that the (free) concentration of drugs available for pharmacological action at the receptor site is not equal to the free concentration in the plasma and that the free concentration determined by dialysis is not the one actually present in the plasma.

Finally, for studies on the role of endogeneous binding inhibitors the specific effects of endogeneous compounds should be taken into account. The chloride ion is especially important here, since it has been reported [10, 11, 48] that such studies are carried out using a series of plasma or serum samples, diluted with a buffer containing chloride. In such cases the chloride to drug ratio will be changed, while chloride itself will affect the binding parameters. Reported changes in binding of warfarin upon dilution of uremic serum [10] may therefore be partly due to the chloride ion. This can also explain the variability of the slope of the straight lines, obtained when K_{app} is plotted versus $K_{app} \times C_{sc}$, [10] where K_{app} and C_{sc} are the apparent binding constant and the fractional concentration of the serum in the diluted samples respectively. It was assumed by these authors [10] that the concentration of inhibitor would be changed in proportion to the dilution factor. However, this assumption is only partly true, since chloride itself acts also as an inhibitor, and so the slope I_oK_i of their straight lines will be changed on dilution (I_o is concentration of inhibitor in the undiluted plasma, and K_i is the inhibitor constant). It is clear from this that in inhibitor studies the choice of the composition of the plasma diluting buffer should receive far more attention than has been the case thus far.

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APPENDIX

The following equation has been derived previously [2]

$$\frac{1}{c_{free}} = \frac{1-\alpha}{c_{free,N}} + \frac{\alpha}{c_{free,B}} \qquad (1)$$

where c_{free} is the free concentration of warfarin at a pH between pH 6.1 and 9.3, $c_{free,N}$ the free concentration at pH 6.1 when all the protein is in the N conformation, $c_{free,B}$ the free concentration at pH 9.3 when all the protein is in the B conformation, α is the fraction of the protein in the B form and c_{free} the corresponding free drug concentration. When calcium is added to a warfarin–albumin mixture α will be changed to α' and so c_{free} will be changed to c'_{free}. It can easily be derived that

$$\frac{c'_{free}}{c_{free}} = \frac{(1 - \alpha)c_{free,B} + \alpha c_{free,N}}{(1 - \alpha')c_{free,B} + \alpha' c_{free,N}} \quad (2)$$

Since warfarin has only one high affinity site, the following relation between ν and c_{free} holds:

$$\nu = K\, c_{free}/(1 + K\, c_{free}) \quad (3)$$

When $K\, c_{free} << 1$, then c_{free} changes proportional to ν (molar ratio of drug bound and albumin). Since the affinity of warfarin to albumin is high, it can be approximated that $\nu \approx r$. Therefore r changes proportional to c_{free}. For larger values of r, c'_{free}/c_{free} is dependent on r. That is, in equation 2 c'_{free}/c_{free} is independent of r for small values, since α and α' under given conditions are also constant values.