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INFLUENCE OF pH ON THE BINDING OF SURAMIN TO HUMAN SERUM ALBUMIN

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Abstract—The pH dependence of the binding of suramin to albumin has been studied by means of equilibrium dialysis and circular dichroism. Dialysis experiments have revealed that the association constants of the high and low affinity binding sites are strongly influenced by the pH. At pH 6.0 $K_1 = 1.4 \times 10^6\ M^{-1}/n_1 = 2.0$ and $K_2 = 1.3 \times 10^5\ M^{-1}/n_2 = 1.0$; at pH 9.2 $K_1 = 2.0 \times 10^5\ M^{-1}/n_1 = 2.0$. At the high pH no low affinity sites could be demonstrated any more. The pH dependence of the induced ellipticity of the suramin–albumin complex at low molar drug-to-protein ratio $r = 0.1$ can be superimposed upon the neutral-to-base (N–B) transition of albumin alone. By means of the Linderstrøm–Lang equation for electrostatic interaction and a two-state model for the N–B transition of albumin, evidence is obtained of a link of the pH dependent binding behaviour of suramin to albumin and the neutral-to-base transition of albumin. The possible correlation of this link with transport processes of suramin in the body and with selective uptake of suramin in cells and parasites is discussed.

Suramin (hexasodium salt of 8,8′(carbonylbis(imino-3,1 - phenylenecarbonylimino))bis - 1,3,5 - naphthalenetrisulphonic acid, see Fig. 1) was introduced in 1920 and has been applied ever since in the treatment of human trypanosomiasis (sleeping sickness); since 1950 it has also been used to treat onchocersiasis [1]. In 1979 De Clercq [2] reported that suramin acts as a potent inhibitor of the reverse transcriptase of a number of animal retroviruses. Mitsuya *et al.* [3] demonstrated that suramin also inhibits the reverse transcriptase of HTLV-III/LAV (human T-cell lymphotropic virus type III/lymphadenopathy associated virus), the causative agent of the acquired immune deficiency syndrome (AIDS).

Suramin is an organic polyanion with six sulphonic groups, which are negatively charged under physiological circumstances.† It is well known that the drug forms complexes with a large variety of plasma-proteins, including globulins, fibrinogen and histones [4]. Furthermore, suramin inhibits numerous enzymes [5], including proteases like trypsin, chymotrypsin and papain.

Albumin is known to strongly influence the uptake of several compounds in liver cells, for instance, the polyanion dibromosulphtalein [6]. Some researchers believe that the drug–albumin complex undergoes a transient interaction with the hepatocytic cell membrane, thereby promoting release of the drug and making it available for uptake by the liver cell [7, 8]. Earlier, the binding of suramin to albumin was studied at pH 7.4 by Müller and Wollert [9]. In healthy man the pH in the bulk phase of the blood is lowered about 0.1 pH unit, when passing through the liver. Considering the buffer capacity of the blood [10], a pH even lower than 7.3 is very likely in the direct vicinity of the vessel walls, where exchange of the substances with the pericellular environment of the liver occurs. It is reported that the pH on the pericellular level in organs as the liver can be as low as 6.9–7.0 [11–14]. In general, the pH influences the binding parameters of drug-plasma protein complexes [15]. Distribution and clearance of acidic drugs are processes governed largely by their degree of binding to plasma proteins [16]. Since, for instance, alkalosis and acidosis could produce alterations in the duration and the intensity of the action of the drug, knowledge of the pH dependence of the suramin–albumin binding may improve the treatment of patients. It is possible that phenomena such as the long half-life of the drug (44–45 days, according to Collins *et al.* [17]) and the minor role of hepatic clearance [17] with respect to the total body clearance of suramin can be explained in terms of albumin binding.

Albumin undergoes a conformational change in the pH range 6–9; this can be monitored by means of circular dichroism [18–24]. This so-called neutral-to-base or N–B transition is strongly influenced by physiologically important ions as Ca^{2+} and Cl^-, as well as by fatty acids [23–25]. Furthermore, it has been demonstrated that the pH dependent binding of the drugs warfarin and diazepam is linked to the conformational state of albumin [19, 26]. These drugs are accepted as representatives of compounds that bind to site I and site II: the two major binding sites of albumin, as denoted by Sudlow *et al.* [27]. It is conceivable that the N–B transition plays a distinct role in the uptake mechanism of suramin. In this paper the pH dependence of the binding of suramin to albumin and the possible link between the N–B transition and the suramin–albumin binding is discussed.

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† Unpublished results.

Abbreviations used: By albumin we mean human serum albumin, unless otherwise stated; CD, circular dichroism, $[\Theta]$, molar ellipticity; Θ_{obs}, observed ellipticity; N, neutral; B, basic.

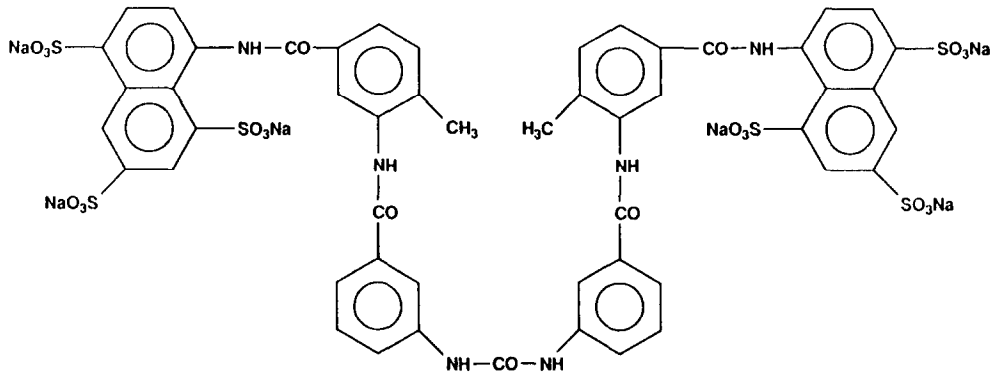

Fig. 1. Structure of suramin.

MATERIALS AND METHODS

Materials. Albumin (M_r 66,500) was isolated from outdated pooled plasma according to the method described by Kremer [28]. Solutions of albumin were deionized and albumin concentration was determined as described by Wilting *et al.* [18]. Suramin obtained from Bayer (Leverkusen, F.R.G.) was used without further purification. ^{14}C-Labelled suramin (specific activity 1.18 MBq/mg) was kindly provided by the UNDP/World Bank/WHO-Special programme for Research and Training in Tropical Diseases. All other chemicals were of analytical grade (J.T. Baker Chemicals, Deventer, The Netherlands; Merck, Darmstadt, F.R.G.).

Equilibrium dialysis. The affinity of suramin for albumin was studied by means of equilibrium dialysis as described by Wilting *et al.* [19]. A Dianorm equilibrium dialyser (Diachema AG, Ruschlikon, Switzerland) was used. The cells contained a total volume of 2 ml. Adsorption on the membranes (Diachema AG, type 1014, molecular weight cut-off 5000) was found to be negligible. Dialysis was continued for 15 hr at 25°. Control experiments revealed that after this period equilibrium was achieved for all suramin concentrations. No correction was made for the Donnan effect, since the ionic strength of the phosphate and borate buffers ($I = 0.1$) was sufficiently high to suppress this effect. In blanco experiments no effect of phosphate or borate on the affinity of suramin for albumin could be demonstrated.

On the basis of UV-spectrophotometric criteria, solutions of suramin appeared to be stable for at least 48 hr. The concentration of free suramin was determined by liquid scintillation counting (Packard, TriCarb Liquid Scintillation Spectrometer model 2425). The dialysis experiments were performed using 6.0×10^{-5} M albumin solutions and a molar drug-to-protein ratio (before dialysis) of 0.00 to 3.00.

In order to determine the pH dependence of the free fraction, measurements were performed at the constant molar drug-to-protein ratio $r = 0.1$. All data were obtained in duplo. The counting time of the samples containing the ^{14}C-suramin was chosen sufficiently long to minimize the counting error; this error never exceeded 1%. The results of the dialysis experiments were also used later in Fig. 6, to calculate the molar ellipticity of the suramin–albumin complex.

Circular dichroism. The circular dichroism spectra of the suramin–albumin complexes between 300 and 400 nm were obtained by means of a Dichrograph III (Jobin Yvon, Longjumeau, France). The slit was programmed for a half-band width of 2 mm (sensitivity 1×10^{-6} degree/mm, scanning speed 3 nm/min, time constant 10 sec). A rectangular cell of a path-length of 2 cm was used. The observed ellipticities (Θ_{obs}) are the differences between the circular dichroism spectra of the suramin–albumin complex and of albumin alone at a given wavelength. Molar ellipticities [Θ] were calculated with the equation [26]

$$[\Theta] = \frac{100\Theta_{obs}}{lc} \text{ (degree} \times \text{cm}^2/\text{dmol)} \qquad (1)$$

where l is the path-length in cm, and c is the molar concentration of the suramin–albumin complex, as determined by equilibrium dialysis.

To determine the pH dependence of the molar ellipticity of the suramin–albumin complex, we used a molar drug-to-protein ratio of 0.1; the albumin concentration was 6.0×10^{-5} M. To monitor the N–B transition of albumin alone, a concentration of 1.2×10^{-3} M was used. The induced extrinsic Cotton effects were recorded at 340 nm; at this wavelength an acceptable signal-to-noise ratio is achieved. All measurements were performed in a borate- or phosphate-buffered solution ($I = 0.1$).

Treatment of binding data. The experimental data on the binding are presented in terms of free suramin concentration and the molar ratio of suramin bound to albumin at equilibrium. For the treatment of the data we choose the following model, in which the concentration of free suramin is denoted as c_f and the molar ratio of suramin bound to albumin is denoted as ν

$$\nu = \frac{n_1 K_1 c_f}{1 + K_1 c_f} + \frac{n_2 K_2 c_f}{1 + K_2 c_f}; \qquad (2)$$

n_1 and n_2 are interpreted as the number of molecules of suramin bound to albumin, with the corresponding affinity constants K_1 and K_2.

Equation (2), which is derived from the law of mass action, implies the existence of two sets of independent, non-cooperative binding sites. The binding parameters n_1, n_2, K_1 and K_2 were estimated

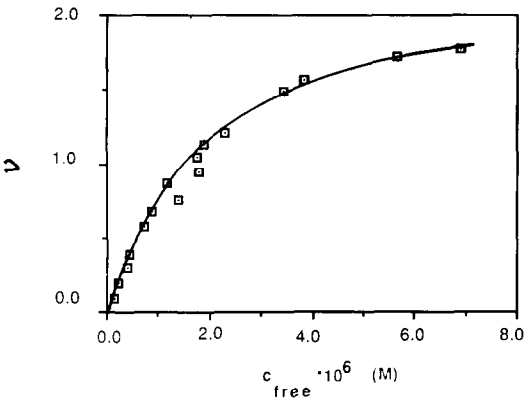

Fig. 2. Binding isotherm for the interaction of suramin with albumin at pH 7.4 and 25° in a phosphate buffer ($I = 0.1$). The albumin concentration is 6.0×10^{-5} M. In the figure is presented the direct plot of the molar ratio of suramin bound to albumin (ν) vs the free suramin concentration (c_{free}). The curve is drawn according to the parameters estimated by the non-linear least-squares analysis: $K_1 = 5.0 \times 10^6\,M^{-1}/n_1 = 2.0$ and $K_2 = 5.6 \times 10^5\,M^{-1}/n_2 = 1.0$.

Table 1. The effect of pH on the binding parameters n_1, n_2, K_1 and K_2 of the binding of suramin to albumin. The parameters were estimated by means of non-linear least-squares analysis, according to equation (2). For each pH the parameters were obtained by analysis of a complete binding isotherm consisting of 12–16 experimental points. SE represents the standard error of ν on c_{free} from the best fit to equation (2)

pH	n	K (M^{-1})	SE
6.0	$n_1 = 2.0$ $n_2 = 1.0$	$K_1 = (1.4 \pm 0.1) \times 10^6$ $K_2 = (1.3 \pm 0.1) \times 10^5$	0.13
7.4	$n_1 = 2.0$ $n_2 = 1.0$	$K_1 = (5.0 \pm 0.3) \times 10^5$ $K_2 = (5.6 \pm 0.3) \times 10^4$	0.08
9.2	$n_1 = 2.0$	$K_1 = (2.0 \pm 0.1) \times 10^5$	0.08

with a non-linear least-squares computer program (Hewlett Packard, HP98820A-Statistical Library) on a Hewlett Packard computer (Hewlett Packard, model 9153). To obtain optimum fits, it appeared to be desirable to start the parameter fit with a fixed set of values for n_1 and n_2. The program was started with the best possible estimate of the association constants K_1 and K_2. Values for n_1 and n_2 were varied between 0.0 and 5.0. The best combination of n_1, n_2, K_1 and K_2 is selected, on the basis of the minimum of standard error of ν on c_f.

RESULTS

Equilibrium dialysis

The first topic of interest is the association constant at physiological pH. In the concentration range studied, the bound fraction of the drug varies from 99% at a drug-to-protein-ratio $r = 0.05$ to 89% at $r = 2.00$. In Fig. 2 the curve fit of the experimental data is presented. From the figure it is evident that binding reaches saturation. The model with two independent binding classes fitted the experimental data adequately within the concentration range studied. The estimated binding parameters are summarized in Table 1. It appeared that at pH 7.4 albumin is able to bind two molecules of suramin with high affinity, and one molecule with lower affinity.

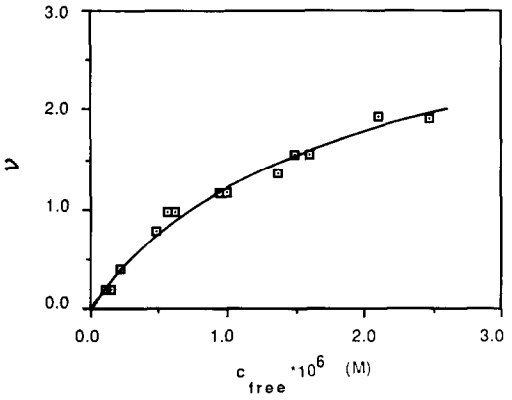

Fig. 3. Binding isotherm for the interaction of suramin with albumin at pH 6.0 and 25° in a phosphate buffer ($I = 0.1$). In the figure is plotted ν vs c_{free}. The drawn curve is calculated with $K_1 = 1.4 \times 10^6\,M^{-1}/n_1 = 2.0$ and $K_2 = 1.3 \times 10^5\,M^{-1}/n_2 = 1.0$.

To investigate the binding of suramin to albumin in the N- and B-conformation, binding experiments were also performed at pH 6.0 and pH 9.2. The experimental data are presented in Figs 3 and 4. Again, at both pHs binding is saturable. The estimate of the binding parameters revealed some remarkable differences. The degree of binding was found to decrease markedly at increasing pH. At pH 9.2 one binding site even completely vanished under these experimental conditions. However, with higher molar drug-to-protein ratios it might be possible to demonstrate some low affinity sites. The results of the estimates are shown in Table 1. It can be concluded that the binding constants of the suramin–albumin complex decrease drastically with increasing pH. The change of n_1, however, is only small and may, within experimental error, be regarded as being independent of the pH.

Figure 5 illustrates how the value for n_1 for the

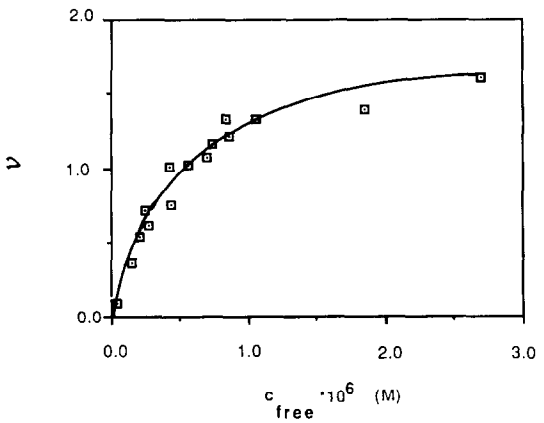

Fig. 4. Binding isotherm for the interaction of suramin with albumin at pH 9.2 and 25° in a borate buffer ($I = 0.1$). In the figure is plotted ν vs c_{free}. The drawn curve is calculated with $K_1 = 2.0 \times 10^5\ M^{-1}$ and $n_1 = 2.0$.

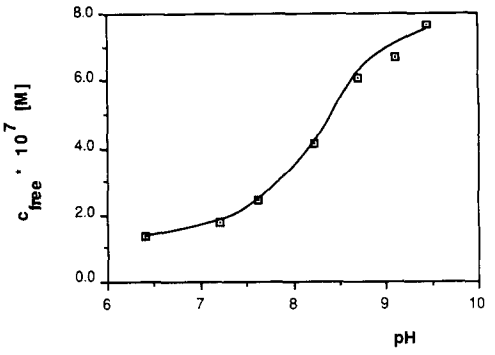

Fig. 6. The free concentration of suramin (c_{free}) is plotted vs the pH. Data were obtained by dialysis at 25°. Conditions: albumin concentration = 6.0×10^{-5} M, molar drug-to-protein ratio = 0.1, phosphate buffers ($I = 0.1$) were used below pH 8.2 and borate buffers of the same ionic strength were used above pH 8.2.

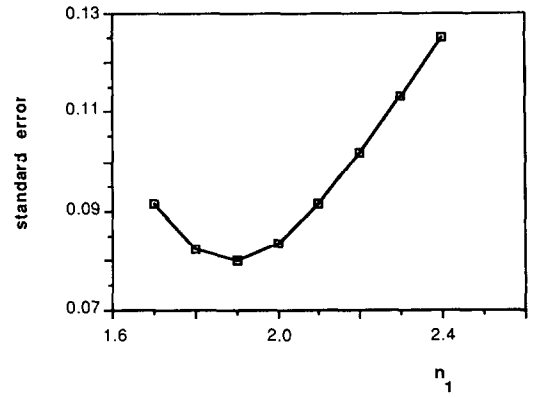

Fig. 5. A plot of the standard error of ν on c_{free} vs n_1. The experimental data are from the binding experiment of suramin to albumin at pH 9.2.

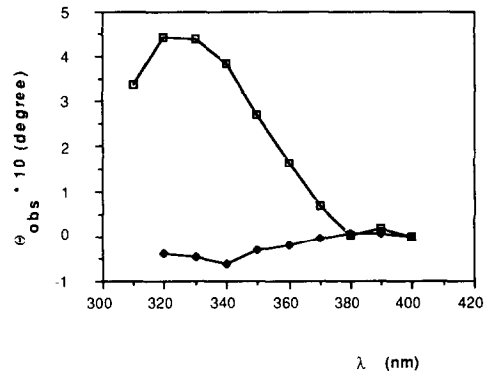

Fig. 7. CD spectra of the suramin–albumin complex at pH 6.0 (◆) and pH 9.2 (□). All conditions are the same as in Fig. 6. Ordinate: the observed ellipticity (Θ_{obs}) of the complex calculated with reference to Θ_{obs} of albumin alone. Abscissa: wavelength (λ).

complexation at pH 9.2 was selected from an assembly of curve fits with varying n_1. It shows that the best fit of the experimental data is obtained at $n_1 = 1.9$. Fits for $n_1 = 1.8$ and $n_1 = 2.0$ also turned out to be reasonable. For further calculations we, therefore, used the value $n_1 = 2.0$.

The effect of the pH on the concentration of the free drug is the next topic of interest. From the data in Table 1 it can be calculated that at the drug-to-protein ratio $r = 0.1$ the contribution of the low affinity sites to the number of suramin molecules bound to albumin is approximately 5% at pH 6.0, and even less at other pHs. This justifies the interpretation that pH-induced changes in the non-bound drug concentration can be attributed mainly to changes in K_1. The pH dependence of the free suramin concentration at molar drug-to-protein ratio 0.1 was determined by means of equilibrium dialysis. The results are shown in Fig. 6. From this figure it can be seen that the free concentration of suramin increases drastically between pH 7.5 and pH 8.5. Since no changes in the physico-chemical properties of suramin are known to occur in this pH region, the observed pH dependence must be due to changes in the albumin molecule.

Circular dichroism

The binding of suramin to albumin was found to give rise to induced extrinsic Cotton effects between 300 and 370 nm. The CD difference spectra (i.e. the differences between the CD spectra of the suramin–albumin complex and of albumin alone) are shown in Fig. 7. It is evident from the figure that the difference spectra at pH 6.0 and 9.2 are not identical. At 340 nm, the difference in Θ_{obs} as measured at the two pHs is at a maximum so this wavelength is suitable for monitoring the pH dependence of the molar ellipticity of the complex.

The extrinsic Cotton effects generated by the binding of suramin at various molar drug-to-protein ratios are shown in Fig. 8. It follows from the figure that at pH 9.2 Θ_{obs} increases linearly with r for $r < 0.4$. At pH 6.0 Θ_{obs} is found to decrease with r.

The influence of the pH on the molar ellipticity of the suramin–albumin complex was measured next.

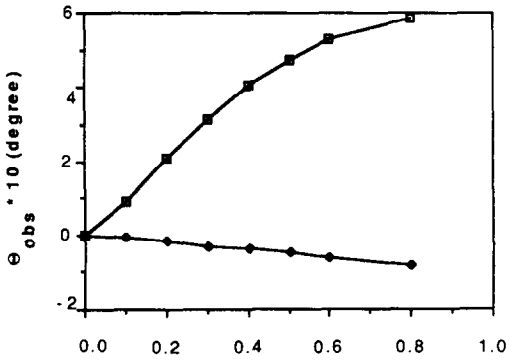

Fig. 8. Θ_{obs} at 340 nm as a function of the molar drug-to-protein ratio (r) at pH 6.0 (◆) and pH 9.2 (□). Conditions: albumin concentration = 6.0×10^{-5} M. Phosphate resp. borate buffers were used ($I = 0.1$). Temperature, 25°. Values of Θ_{obs} of the complex were calculated with reference to Θ_{obs} of albumin alone at this wavelength.

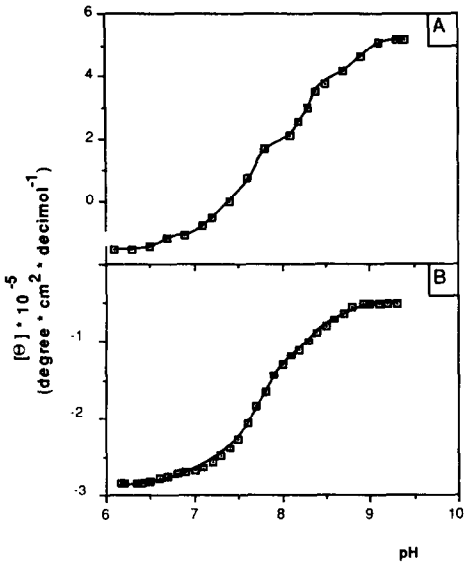

Fig. 9. Panel A: [Θ] of the suramin–albumin complex as function of pH. Conditions as in Fig. 6. [Θ] is calculated with equation (1), see text. Panel B: [Θ] of albumin alone as a function of pH. Albumin concentration = 1.2×10^{-3} M. Other conditions as above.

At the ratio used, the observed effects arise only from the binding of suramin to the high affinity binding site. From equation (1) it follows that the changes in molar ellipticity cannot be attributed to changes in the amount of the bound fraction. The results are depicted in Fig. 9A. It is evident from the figure that between pH 7.5 and pH 8.5 the molar ellipticity increases drastically. Furthermore, it can be seen that at pH 7.4 the sign of the molar ellipticity changes. In order to check whether the observed pH dependence can be explained in terms of the N–B transition of albumin, the influence of pH on the molar ellipticity of albumin alone was measured. The results are depicted in Fig. 9B. From the figure it is clear that the pH influence on the molar ellipticity of the suramin–albumin complex parallels the pH influence on the molar ellipicity of albumin alone. Therefore, we are justified in concluding that the change in molar ellipticity of the suramin–albumin complex arises from the N–B transition of albumin.

Another interesting effect occurs around pH 8.0. In Fig. 9A and B it can be seen that around this pH the dependence of [Θ] of the complex is no longer purely sigmoidal. This may be due to the existence of an intermediate conformation state of albumin at this pH. The occurrence of such an intermediate conformation state was indeed demonstrated by 't Hart *et al.* [29] for the nitrazepam–albumin complex. Our results indicate that this effect also appears in albumin alone, as is shown in Fig. 9B.

DISCUSSION

In the present work an attempt has been made to elucidate the characteristics of the binding of suramin to albumin. At pH 6.0 the existence of two high affinity binding sites and one low affinity binding site was demonstrated. At pH 7.4 two classes of binding sites still exist, although with lower affinities. At pH 9.2 the complexation is different: one binding site has vanished. The results differ somewhat from the findings of Müller and Wollert [9]. This may be caused by the fact that in our experiments we used fatty acid-free albumin. In addition, we used different methods for determining the degree of binding and for calculating the binding parameters. The effect of the pH on binding of the suramin to albumin is found to be completely the reverse of the effect of the pH on warfarin–albumin and diazepam–albumin equilibria; in the two latter complexation processes an increase in affinity constants at increasingly pH was found [19, 26]. It is likely that the large negative charge of the suramin molecule plays a role in the observed pH dependence. In the pH region studied all six sulphonic acid groups are deprotonated, so the charge on the molecule is −6. The influence of the pH on the binding of suramin to albumin is possibly due to pH dependent changes in the electrostatic interaction of the negatively charged suramin with the net negative charge on the albumin molecule, which decreases with increasing pH. The net charge of the albumin, due to bound or dissociated protons, as a function of the pH was determined by means of acid–base protein titrations, as described earlier [30].

The electrostatic effect on association constants is usually described by the well-known Linderstrøm–Lang equation

$$K_{app} = K_{int}\, e^{-2\omega Z_p Z_w} \qquad (3)$$

where K_{app} is the observed association constant, K_{int} is the intrinsic association constant (i.e. the association constant when $Z_p = 0$), Z_p is the net charge on the albumin (due to bound or dissociated protons), Z_w denotes the charge on the ligand and ω is the electrostatic interaction factor, which is a characteristic of the conformation of the ligand–albumin complex. According to equation (3) a plot of log K_1

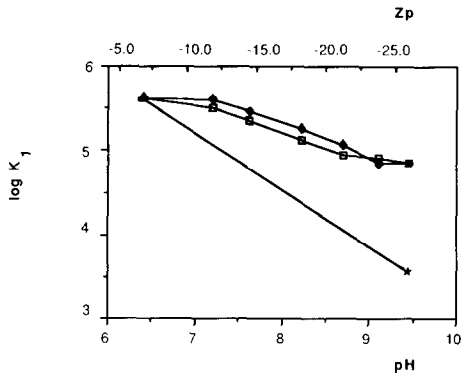

Fig. 10. The pH dependence of log K_1: (1) calculated from the dialysis data from Fig. 6 (□) and (2) calculated with the two-state model (equation 4) (◆). The Z_p (i.e. the net charge on the protein due to bound or dissociated protons) dependence of log K_1 as calculated with the Linderstrøm–Lang equation (equation 3) is shown in the same figure (★). Z_p is the abscissa.

vs Z_p should yield a straight line with a slope of $-0.868\omega Z_w$. In an attempt to explain the pH dependence within the Linderstrøm–Lang concept, we calculated the values of log K_1 as a function of Z_p (and pH) from the free suramin concentrations, as determined with equilibrium dialysis. For this purpose the experimental data from Fig. 6 were used. The following assumptions were made: (a) at low drug-to-protein ratio less than 5% of the total amount of suramin bound is bound to the low affinity binding sites, (b) the value of n_1 is assumed to be constant in the pH region 6.0–9.0. The results are shown in Fig. 10. It is evident that log K_1 does not vary linearly with Z_p (and pH). Further, it is shown in the figure that if the interaction between suramin and albumin is assumed to be merely electrostatic at constant ω—any influence of conformational changes on log K_1 is thereby neglected—then the simulated log K_1 vs Z_p curve would have quite a different shape from the experimental curve. Consequently, at pH 9.2 equation (3) predicts a value for log K_1 which is much lower than the observed value. In this simulation ω was taken 0.022, according to Tanford [31]. The experimental results suggest that ω is not a constant at all, but actually changes with the Z_p (and pH). Apparently, the high affinity constant K_1 is affected both by an alteration in electrostatic interaction and by a pH induced change of the conformation of the suramin–albumin complex. At this point it is interesting to investigate whether this conformational change is local and influences only the direct environment of the suramin binding site, or whether it can be correlated to the pH induced N–B transition of albumin. According to Wilting *et al.*, the N–B transition can be represented by a two-state model [19]. In this model with two pH-independent high affinity constants for albumin in the N and B conformation (K_N, K_B), the value of K at any intermediate pH can be calculated with the help of a circular dichroism experiment: K is related in the following way to the fraction of the albumin which is in the B-form (α) [19]:

$$\frac{K}{1 + Kc_f} = \frac{\alpha K_B}{1 + K_B c_f} + \frac{(1 - \alpha)K_N}{1 + K_N c_f}. \quad (4)$$

Assuming that albumin is entirely in the N-conformation at pH 6.4 ($Z_p = -7.0$) and in the B-conformation at pH 9.2 ($Z_p = -25.0$), we can calculate K_1 for all intermediate pH and Z_p values (see Fig. 10). From the figure, it follows that the theoretical pH dependence of log K_1, as calculated with the two-state model parallels the experimental pH dependence of log K_1 as determined by equilibrium dialysis. This might be evidence to support the idea that the conformational change influencing the high affinity binding constant K_1 is indeed the N–B transition of albumin; in other words, the results suggest that the binding of suramin to albumin is linked to the N–B transition of albumin.

In therapy, suramin is administered up to steady-state levels of 100–150 μg/ml plasma [17], which is equivalent to a molar drug-to-albumin ratio of 0.1–0.2. According to Collins [17] plasma binding of suramin is at least 99.7%. Our results suggest that albumin is in all probability the prime cause of this high degree of plasma binding. In addition, since suramin tends to bind to a variety of proteins and enzymes, it is difficult to imagine that there would in fact be any free suramin at all in plasma. This again suggests that the uptake of suramin by cells and parasites is a protein-mediated process.

In this paper it is demonstrated that the conformation of albumin governs the degree of suramin binding. As stated in the introduction, some researchers believe that drug–albumin complexes undergo a transient interaction with the hepatocytic cell membrane, which promotes release of the drug. It is possible that this transient interaction resembles the N–B transition of albumin. Furthermore, it is known that albumin stimulates hepatic uptake of compounds such as oleate, rose bengal and warfarin [7, 8, 32]. Collins *et al.* [17] demonstrated that for suramin the hepatic clearance plays only a minor role in the total body clearance. Apparently, the degree to which albumin enhances or decreases hepatic uptake varies with the nature of the drug. At this point, it is worth noting that the influence of the albumin conformation on warfarin binding is entirely opposite to its influence on suramin binding: warfarin preferably binds the B-conformation of albumin [19], whereas suramin appears to have the highest affinity for the N-conformation. It is therefore postulated, somewhat tentatively, that whenever a tightly bound drug with binding characteristics dependent on the albumin conformation has higher affinity for the B-conformation, clearance by the liver is facilitated by albumin binding; in the case of drugs with a preference for the N-conformation however, clearance is hampered by albumin binding. This seems to hold for warfarin and suramin. The actual situation might be far more complex, but the hypothesis may function as a starting point for new experiments and ideas.

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