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DIFFERENTIATED EFFECTS OF LIVER CIRRHOSIS ON THE ALBUMIN BINDING SITES FOR DIAZEPAM, SALICYLIC ACID AND WARFARIN

ANITA KOBER, ÅSA JENNER and INGVAR SJÖHOLM*
Department of Pharmaceutical Biochemistry, Biomedical Center, University of Uppsala, Box 578, S-751 23 Uppsala, Sweden

and

OLOF BORGÅ
Department of Clinical Pharmacology, Huddinge University Hospital, S-141 86 Huddinge, Sweden

and

INGEGERD ODAR-CEDERLÖF
Department of Internal Medicine, Karolinska Hospital, S-104 01 Stockholm, Sweden

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Abstract—The protein binding of diazepam, salicylic acid and warfarin in serum of patients with liver cirrhosis has been studied by equilibrium dialysis and circular dichroism measurements and compared with that in normal serum. Diazepam and warfarin are mainly bound to two separate sites on the serum albumin molecule and salicylic acid to both sites. The binding of diazepam and salicylic acid was impaired in serum from the patients when compared with the binding in serum from healthy individuals, while the binding of warfarin was unchanged. The results were the same when the binding was studied with albumin isolated from patient serum and normal serum. After treatment of the albumin with charcoal at pH 3, however, the binding was improved.

The apparent binding constants of the drugs in serum both from patients with liver cirrhosis and from healthy persons were increased by dilution of the sera in buffer. This effect is due to the presence of competitive inhibitors in the sera. The decreased binding of diazepam and salicylic acid in the serum from the patients with liver cirrhosis can in addition be related to the presence of allosteric inhibitors specifically affecting the diazepam binding site on albumin, while the warfarin site apparently is unaffected.

The possible influence of disease on the serum protein binding of drugs has been the subject of several studies in recent years, as reviewed by Reidenberg [1]. For many drugs a decreased protein binding has been observed in patients with uremia [2–5] or with certain liver diseases [6–16]. On the other hand an increased binding of some steroids [17, 18] is detected in patients with liver cirrhosis, which is probably due to an increased concentration of steroid-binding globulins in the serum.

Table 1 summarizes studies on the binding of some drugs to proteins in plasma or serum from patients with liver diseases. Albumin is the principal binding protein for the acidic drugs mentioned in the table. It is generally accepted that albumin has binding sites specific for different drugs and indubitably also for endogenous substances [25]. The decreased binding of drugs in uremia, as studied with salicylic acid and warfarin, has earlier been shown to be due to the presence of metabolites inhibiting the binding [4]. Charcoal treatment of the albumin or sera

Table 1. The serum or plasma binding of various drugs in liver diseases*

Drug	Binding compared to normal	References
Amylobarbitone	Decreased	22
Clindamycin	Unchanged	19
Dapsone	Decreased	6
Diazepam	Decreased	7, 8
Fluorescein	Decreased	6
Morphine	Decreased	10
Phenylbutazone	Decreased	11
Phenytoin	Unchanged	6
Phenytoin	Decreased	9, 10
Propranolol	Decreased	23
Quinidine	Decreased	6, 12
Salicylic acid	Decreased	11, 12
Sulphadiazine	Decreased	11
Thiopentone	Decreased	13
Tolbutamide	Decreased	8, 24
Triamterene	Decreased	6
d-Tubocurarine	Unchanged	20
Warfarin	Unchanged	21

*The diagnosis includes alcoholics, acute hepatitis, hepatic failure, liver disease or liver cirrhosis.

* To whom correspondence should be addressed.

removes the inhibitors and restores the binding capacity [4, 5]. It has recently been shown that warfarin binds with high affinity mainly to one site on the albumin molecule, to which also bilirubin is bound [25, 26]. The inhibitors in uremic serum thus affect the warfarin binding site. Diazepam binds to a site not coinciding with that of bilirubin and warfarin [27, 28]. Since the serum binding of diazepam is also impaired in uremia [4, 29], the diazepam binding site should also be affected. Salicylic acid binds to two sites, one of which is identical with the warfarin site and one with the diazepam site [26].

The present study was undertaken to examine how the binding of drugs to these two sites on albumin was influenced in patients with liver cirrhosis, using diazepam, salicylic acid and warfarin as model drugs.

MATERIALS AND METHODS

Patients. Twenty-one patients, 12 men and 9 women with liver cirrhosis took part in the study. Clinical data and drug treatment are given in Table 2. Their serum bilirubin concentrations were within normal limits the three exceptions. When possible, blood samples were collected on admission to hospital before drug treatment was started. As far as possible such patients were selected who were not treated with drugs known to act as inhibitors of drug protein binding.

Normal subjects. Serum was also collected from 10 drug-free healthy volunteers, 4 men and 6 women, age range 25 to 45 years, to give a serum-pool for reference purposes.

Serum. Blood samples were drawn under resting conditions from an antecubital vein. The blood was centrifuged and the separated serum was stored frozen at $-20°$. When thawed for analysis the patient sera were pooled to give two pools. From one albumin was separated, and the other was used as whole serum.

Human serum albumin. Albumin (HSA) was prepared from normal blood bank plasma by $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography, mainly according to McMenamy *et al.* [30]. The albumin was treated with activated charcoal at pH 3.0 according to Chen [31]. The albumin monomer was then isolated by gel-filtration on Sephadex G-100. The concentration was determined from the extinction at 279 nm ($E^{1\%}_{1cm} = 5.80$). Albumin from

Table 2. Clinical data of the cirrhotic patients

Patient	Age	Sex	Diagnosis	Bilirubin	ASAT	ALAT	ALP	Gamma-GT	Proteins g/1				Drugs (g daily)
				μmol/1	μkat/1	μkat/1	μkat/1	μkat/1	Alb	IgG	IgA	IgM	
KN	60	M	Liver cirrhosis (alcohol) Op porta cava shunt	15.4	0.62	0.53	3.02	0.92	41	17.2	2.1	1.8	Spironolactone 0.100 Furosemide 0.120 KCl 4.0
JZ	34	M	Liver cirrhosis Ulcerative colitis	13.0	0.29	0.33	2.89		46				Salicylazosulfapyridin 2.0
KU	65	F	Liver cirrhosis	4.0	0.29	0.46	2.15		40	21.6	0.9	0.6	0
GJ	67	M	Liver cirrhosis (alcohol)	3.0	0.99	1.04	2.38		44	12.6	0.3	0.06	Vitamins
NP	73	M	Liver cirrhosis Psoriasis Epilepsy	2.0	0.71	0.44	7.30	2.88	35	10.5	0.3	0.02	Phenytoin 0.200
MB	64	F	Liver cirrhosis	13.0	0.53	0.66	10.5	3.5	39	10.7	0.2	0.06	Conjugated estrogens 0.000625
KN	73	M	Liver cirrhosis (alcohol)	20.0	0.53	0.40	2.8		37				0
FN	50	M	Liver cirrhosis (alcohol) Chronic bronchitis	0.9	1.08	0.68	2.83		48				Vitamin B
IA	58	F	Liver cirrhosis (biliary)	20.0	2.61	3.61	34	8.8	42	13.3	0.9	0.38	Prednisolone 0.010, Azathioprine 0.075, Cholestyramine 12.0
NA	62	M	Liver cirrhosis	23.0	0.70	0.76	2.4						Digoxin 0.00025, Lactulose 60.0
HN	42	M	Liver cirrhosis	13.7	normal	normal	4.1						Vitamin K, Methylscopolamine 0.0015, Amobarbital 0.150
NR	55	M	Liver cirrhosis (portal)	59.0	2.10	0.40	4.9						Furosemide 0.080, Spironolactone 0.100 Vitamins K, B, Clomethiazole 2.0
KD	39	F	Liver cirrhosis	10.0	3.04	1.04	2.7						Spironolactone 0.075, Furosemide 0.040, Mepenzolate 0.075
UB		F	Liver cirrhosis		normal	normal							Prednisolone 0.10 KCl 3.0 Spironolactone 0.100 Lactulose 20.0
GA	63	F	Liver cirrhosis	20.0	1.88	1.44	3.9						Prednisolone 0.010, KCl 8.0, Lactulose 60, Carbidopa 0.037 Levodopa 0.370
BH	43	F	Liver cirrhosis	18.0	1.62	1.85	26	8.9	42	17.0	1.1	1.1	Cholestyramine 12.0
SJ	57	M	Liver cirrhosis	14.0	1.06	0.75	4.0		40	12	3.2	0.4	Vitamins
HL	53	M	Liver cirrhosis (alcohol)	16.0	4.57	2.54	3.2		48				Clomethiazole 2.0, Spironolactone 0.100, KCl 4.0
MH	62	F	Liver cirrhosis	15.0	1.25	0.25	9.8	5.1	45	21.5	4.8	2.1	Spironolactone 0.100, Furosemide 0.40, KCl 1.0
OS	65	M	Liver cirrhosis	16.0	1.06	0.90	7.0	2.5	36	11.7	3.3	16	Neomycin sulfate 2.0, Furosemide 1.60, Digoxin 0.00025
SL	33	F	Liver cirrhosis (portal)	33.0	1.85	1.70							0

Normal serum values: Bilirubin 4–21 μmol/1; ASAT 0.20–0.70 μkal/1; ALAT 0.10–0.70 μkat/1; ALP (alkaline phosphatase) 0.8–4.0 μkat/1; Gamma GT (gamma-glutamyl transpeptidas) 0.1–1.0 μkat/1; albumin 37–52 g/1; IgG 7–11 g/1; IgA 0.8–3.8 g/1; IgM 0.4–2.0 g/1.

pooled patient serum was prepared by the same method.

Drugs. [^{14}C] Salicylic acid (52.0 mCi/mmole) and [^{14}C] warfarin (51 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England. [^{14}C] Diazepam (59.4 mCi/mmole) was synthesized from nordiazepam by methylation with [^{14}C] methyl iodide obtained from the Radiochemical Centre and was purified by chromatography (in heptan-chloroform-ethanol 50:50:5) on Silica Gel 60 ready-to-use column from E. Merck, Darmstadt, West Germany. The radiochemical purity (>98 per cent in all cases) was checked by thin layer chromatography. The corresponding unlabelled drugs were added to the isotope solutions to achieve suitable drug concentrations. Unlabelled drugs were obtained as gifts from the different manufacturers.

Equilibrium dialysis. The serum protein binding was determined by equilibrium dialysis at 37° as described earlier [4]. The time used for equilibration was 6 hr for salicylic acid, 17 hr for warfarin and 5 hr for diazepam. Diazepam was dissolved in serum, while salicylic acid and warfarin were dissolved in buffer. Serum albumin concentration was determined by immunochemical quantitation according to Mancini *et al.* [32] using M-Partigen^{R} plates obtained from Hoechst Behringwerke AG, Marburg-Laten, West Germany. The determinations were made at the end of the dialysis.

Circular dichroism (CD) measurements. These were made on a JASCO J-41 polarimeter, Japan Spectroscopic Co., Tokyo. The temperature was kept at 25°. The samples were dissolved in a 0.005 M phosphate buffer with 0.1 M KCl, pH 7.4. When diazepam was studied, the albumin concentration was 0.8 mg/ml and the drug was present in equimolar concentration. A rectangular cell with a path-length of 10 mm was used. In studies on warfarin the albumin concentration was 5.0 mg/ml and the drug was present in a 1.5 molar excess. A rectangular cell with a path-length of 5 mm was used.

The results are expressed as the difference molar ellipticity of the albumin–drug complex obtained after compensation for the ellipticity of the albumin alone. The molar ellipticity $\{\theta\}$, in degrees.cm^2.dmole^{-1}, was calculated with reference to the albumin concentration and a molecular weight of 66,500 [33] according to the equation:

$$\{\theta\} = \frac{\theta . M}{c.l.10}$$

where θ is the observed ellipticity in degrees, M is the molecular weight, c the concentration in g/ml and l the path-length in cm.

Mathematical analysis. The serum protein binding data were analyzed in the same way as described earlier [4]. K_{app}, the apparent association constant, obtained with the different samples, was determined from the Scatchard equation [34]:

$$\frac{r}{(D)} = n.K_{app} - r.K_{app}. \qquad (1)$$

In this equation r = moles of bound drug/mole of albumin, (D) = the concentration of unbound drug and n = the number of sites on albumin.

The possible effects of inhibitors present in the samples can be studied in diluted samples, as earlier advanced (4), with the following equation

$$K_{app} = K_a^* - K_{app}.C_{se}.I_0.K_i. \qquad (2)$$

C_{se} is the fractional concentration of the serum in the different diluted samples and I_0 the initial unbound concentration of the inhibitor in the serum. When K_{app} is plotted against $K_{app}.C_{se}$, a straight line is obtained when the binding is competitively inhibited, and K_a^*, characterizing the particular drug–protein binding equilibrium, can be obtained from the intercept on the y-axis. The slope of the line $(-I_0.K_i)$ will be characteristic for the particular inhibitor–serum system.

RESULTS

Equilibrium dialysis studies. In Table 3 the percent binding obtained for the undiluted sera with the lowest drug concentrations of salicylic acid, warfarin and diazepam are given. The binding of salicylic acid and diazepam was decreased while the binding of warfarin was unchanged. The Scatchard plots, as exemplified with diazepam in Figs. 1 and 2, gave the same results. The values obtained from these plots are summarized in Table 4, in the first two lines. K_{app} and $n.K_{app}$ for both diazepam and salicylic acid were significantly lower for patient serum than for the normal serum pool, while the values obtained with warfarin appear to be the same. For salicylic acid the intercept on the r-axis (n) gave values around 2.5 (see Table 4). In this case the estimated K_{app} is a composed constant with contributions from at least two binding sites. This means that equation (2) can only be used for a comparative study of the binding of salicylic acid in different sera and HSA solutions.

Table 3. Serum protein binding in per cent of total drug concentration

Serum	Salicylic acid 0.05 mM	Warfarin 0.02 mM	Diazepam 0.02 mM	Serum albumin mg/ml
Normal	94.3	99.1	98.9	38.7
Patient	88.5	99.0	98.0	32.0

The binding of the drugs was also studied at different dilutions in buffer of the patient and normal sera. Examples of the Scatchard plots for diazepam at different dilutions of the sera are shown in Figs. 1 and 2. The intercepts on the $r/(D)$-axis ($n.K_{app}$) increased with increasing dilution of serum, while the intercept on the r-axis (n) remained constant. The effects of the Donnan equilibrium are small and will not significantly influence the results [4].

Figures 3 and 4 show the plots of $K_{app}.C_{se}$ for the binding of diazepam, salicyclic acid and warfarin to normal serum and patient serum. The K_{app} values for the binding to charcoal-treated HSA are also marked in the figures. The experimental data fell on straight lines. The slopes of the lines obtained with

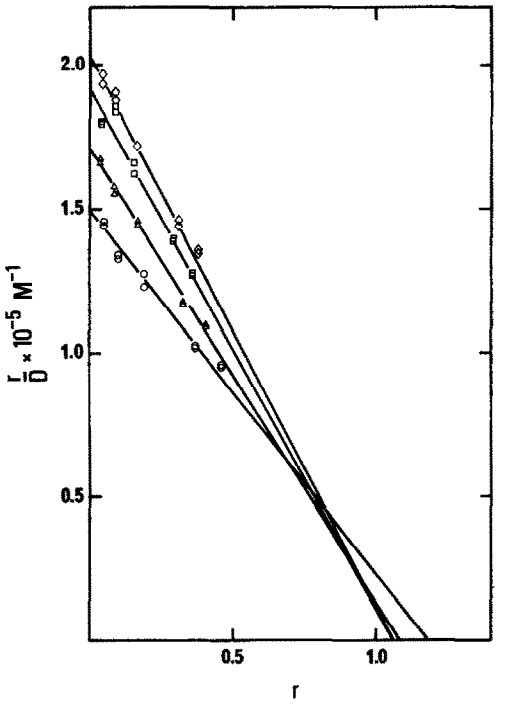

Fig. 1. Scatchard plots of the binding of diazepam to normal serum (○—○ undiluted, △—△ diluted 1 + 1, □—□ diluted 1 + 4, ◇—◇ diluted 1 + 9) studied by equilibrium dialysis. The sera were diluted with an isotonic phosphate buffer, pH 7.4.

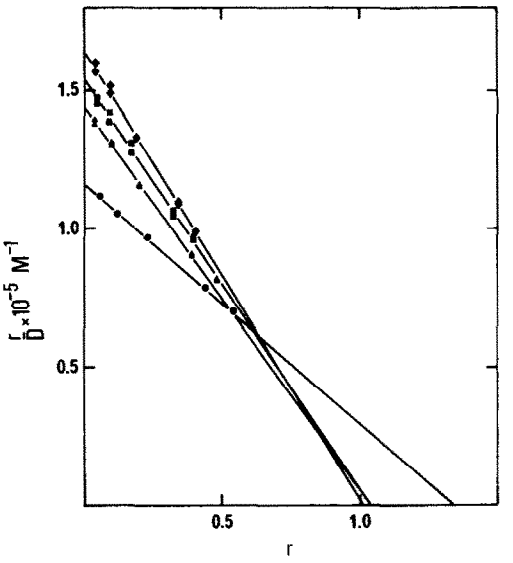

Fig. 2. Scatchard plots of the binding of diazepam to cirrhotic serum (●—● undiluted, ▲—▲ diluted 1 + 1, ■—■ diluted 1 + 4, ◆—◆ diluted 1 + 9) studied by equilibrium dialysis. The sera were diluted with an isotonic phosphate buffer, pH 7.4.

the two sera are similar considering the experimental errors involved, indicating that the product $K_i.I_0$ was not changed in the patient serum. The intercepts on the ordinate, K_a^*, for the normal serum pool coincided with the K_{app} value for normal HSA thus demonstrating that the serum protein binding of the three drugs can be entirely explained by the albumin content. The intercepts giving the K_a^* values for the patient serum pool were however lower than normal for diazepam and salicylic acid. In contrast, the warfarin data from the patient serum coincided with those from the normal serum (Fig. 4).

The binding of salicylic acid to albumin isolated from patient serum was lower than that to normal HSA (Table 4). The binding was, however, improved after charcoal treatment.

Circular dichroism studies. The binding of diazepam and warfarin to HSA isolated from patient and normal sera was studied in the wavelength region 250–350 nm. When drugs are bound to albumin new extrinsic Cotton effects are created at the wavelengths where the drugs have absorption maxima. The magnitude of the Cotton effects are directly proportional to the concentration of the drug–protein complexes. In Figs. 5 and 6 the difference CD spectra for the drug–albumin complexes are shown. As seen in Fig. 5 the binding of diazepam to patient albumin produced significantly smaller Cotton effects

Table 4. Protein binding data obtained from Scatchard plots with undiluted sera and isolated albumin

	Diazepam			Salicylic acid			Warfarin		
	$n.K_{app}$ $M^{-1}.10^{-5}$	n	K_{app} $M^{-1}.10^{-5}$	$n.K_{app}$ $M^{-1}.10^{-4}$	n	K_{app} $M^{-1}.10^{-4}$	$n.K_{app}$ $M^{-1}.10^{-5}$	n	K_{app} $M^{-1}.10^{-5}$
Normal serum pool	1.5	1.2	1.3	2.6	2.5	1.1	1.9	1.2	1.6
Cirrhotic serum pool	1.2	1.3	0.9	1.7	2.2	0.75	1.9	1.1	1.7
Charcoal-treated HSA from normal serum	2.4	1.1	2.2	4.8	2.7	1.8	3.1	1.0	3.1
Untreated HSA from cirrhotic serum		*		3.1	2.3	1.4	3.0	1.1	2.7
Charcoal-treated HSA from cirrhotic serum		*		3.5	2.3	1.6	3.1	1.0	3.1

*Not determined.

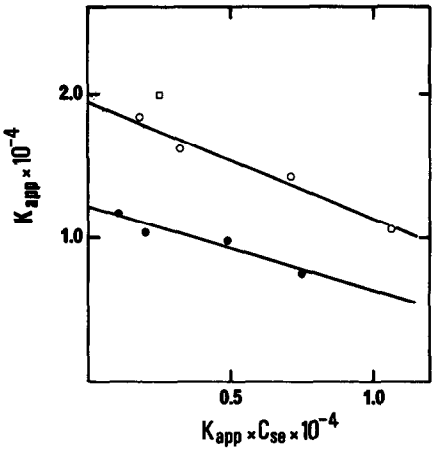

Fig. 3. Graphs of K_{app} versus $K_{app} \cdot C_{se}$ for salicylic acid in normal serum (○—○) and cirrhotic serum (●—●). K_a value for the binding of salicylic acid to charcoal-treated normal HSA (□).

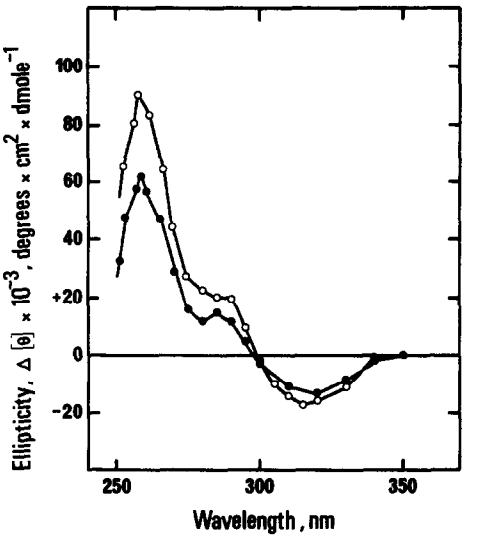

Fig. 5. Circular dichroism difference spectra of the drug protein complexes at pH 7.4 between diazepam and albumin from cirrhotic serum (●—●) and albumin from normal serum (○—○) obtained after correction for the albumin spectra. The HSA concentration was 0.8 mg/ml and the drug was present in equimolar concentration.

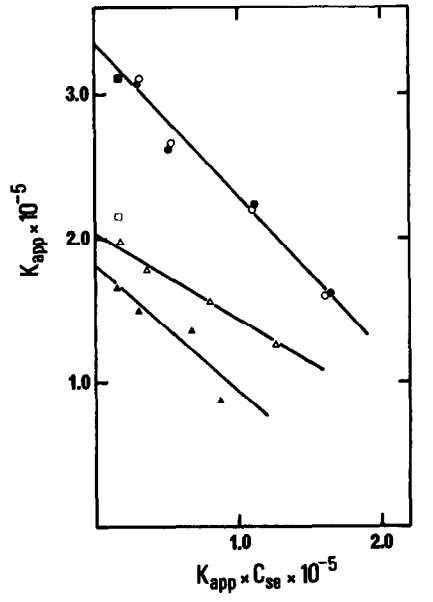

Fig. 4. Graphs of $K_{app} \cdot C_{se}$ for diazepam in normal serum (△—△) and cirrhotic serum (▲—▲). K_a value for the binding of diazepam to charcoal-treated normal HSA (□). Graphs of K_{app} versus $K_{app} \cdot C_{se}$ for warfarin in normal serum (○—○) and in cirrhotic serum (●—●). K_a value for the binding of warfarin to charcoal-treated normal HSA (■).

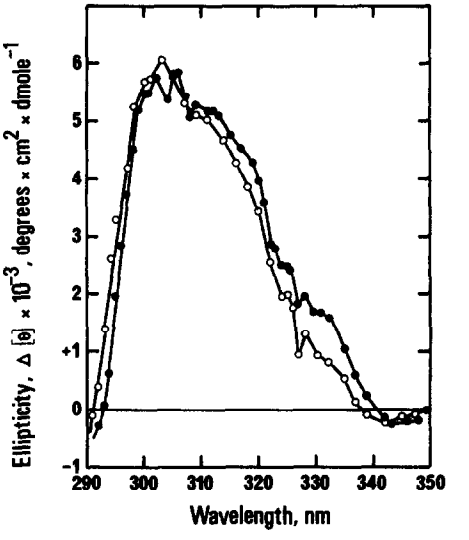

Fig. 6. Circular dichroism difference spectra of the drug protein complexes at pH 7.4 between warfarin and albumin from cirrhotic serum (●—●) and albumin from normal serum (○—○) obtained after correction for the albumin spectra. The HSA concentration was 5.0 mg/ml and the drug was present in a 1.5 molar excess.

than the binding to normal HSA. This means that the binding ability of the albumin from patients with liver cirrhosis is decreased when compared to normal albumin. On the other hand, the binding of warfarin was the same for patient and normal albumin (Fig. 6). The HSA and drug concentrations were identical in the patient and normal samples.

DISCUSSION

The serum protein binding of a drug is of great importance for its kinetics. It affects the apparent volume of distribution, V_D, of the drug, and may as well affect the hepatic [16, 35] and renal clearances [36]. If a drug is essentially metabolized in the liver prior to elimination, the rate of uptake in the liver is

of fundamental importance for the blood concentration/time profile. The factors, which in turn determine the biological half-life of a drug are the hepatic blood flow, the intrinsic clearance of the liver, the V_D and the drug binding in blood. All of these factors may be affected differently by different liver diseases. These may e.g. directly result in an impaired intrinsic clearance and indirectly in a decreased protein binding, through the accumulation of binding inhibitors in the blood. As has recently been advanced by Wilkinson and Shand [35] these basic parameters can have different significance for the hepatic clearance of different drugs. This will in turn affect the plasma levels of drugs obtained during treatment of patients. It is therefore essential that the effects of the disease states on the protein binding of the individual drugs are clarified.

Liver disease is a wide term [14]. In the present investigation we chose to study patients with liver cirrhosis. The disease was of moderate severity in all the patients since the serum bilirubin values were within normal limits. The patient material was thus considered to be fairly homogeneous. Still there are naturally inter-individual variations in the severity of the disease, in age and sex. Furthermore, some of the patients were treated with drugs, most of which, however, were known not to interact with the binding of those studied. The problems are partly overcome by the use of pooled serum. Even with the inter-individual variations described the patients' serum pool showed binding characteristics clearly different from those of the normal serum pool. Since warfarin is displaced by bilirubin and the bilirubin concentration often is elevated in patients with liver diseases, one might assume that bilirubin could be responsible for the decreased binding observed in several cases (Table 1). However, in the present investigation only patient sera with normal bilirubin concentrations were used, in order to allow studies on the possible influence of other endogenous substances.

In serum, albumin is the main drug-binding component. Decreased protein binding has been observed in patients with liver diseases even in the absence of detectable changes in albumin concentration [11, 16]. Thus, the most important factor seems to be a decrease of the apparent affinity of albumin for the drugs. The concept of several separate binding sites on albumin is now generally accepted and at least three specific sites have been described recently [26, 27, 37]. Warfarin is known to be displaced by bilirubin [28, 38]. Since bilirubin is not interfering with the primary binding site of diazepam [27], this drug must be primarily bound to a different site on the albumin than warfarin. Moreover, since salicylic acid is known to interact with the binding of both diazepam and warfarin to HSA [22, 26, 39], it apparently utilizes two different sites on the albumin. In this work it was thus possible to study and compare the influence of liver cirrhosis on two different drug binding sites on the albumin molecule.

The results show that the binding of diazepam and salicylic acid is decreased, while the binding of warfarin is unchanged. The binding of warfarin is also unchanged in acute hepatitis as shown by Williams *et al.* [21] and the results obtained with diazepam and salicylic acid are also consistent with other findings [7, 8, 11, 12].

Liver cirrhosis will thus differently affect the diazepam and warfarin binding sites on HSA. In order to study the mechanisms of the impaired binding of the drugs, a series of dilution experiments were performed. As can be seen in Figs. 1–4, the K_{app} values increased with increasing dilution both with normal and cirrhotic serum, while the n values were essentially unchanged. This indicates the presence of competitive inhibitors, the effects of which are decreased with dilution, resulting in higher K_{app} values.

In uremia the concentration of competitive binding inhibitors is increased [4], which is demonstrated by a much steeper slope in the K_{app} versus $K_{app} \cdot C_{se}$ plot. As is shown in Figs. 3 and 4 this is not the case in liver cirrhosis, since the slopes obtained with patient serum are not steeper than those obtained with normal serum. On the other hand, the intercepts on the y-axis for salicylic acid and diazepam are lower than with normal serum. This fact indicates that there are other mechanisms responsible for the decreased binding of these drugs in liver cirrhosis in addition to those effective in normal serum. If the binding sites are blocked by irreversible competitive inhibitors (i.e. the sites are inactivated), the apparent binding constants, K_{app}, will not be changed, while n is decreased. In this study n with all drugs was essentially the same as obtained with isolated albumin. Inactivation of the active sites of albumin therefore does not play any significant role. On the other hand, the active sites may be allosterically changed and if such a change results in an impaired binding, K_{app} will decrease. It can then be shown that a plot of K_{app} vs $K_{app} \times C_{se}$ (as in Figs. 3 and 4) will give straight lines with intercepts on the y-axis lower than K_a^*, if the inhibitor concentration and its binding constant are high enough. Such a mechanism seems not to be effective on the warfarin site, while the decreased binding of diazepam and salicylic acid to the diazepam binding site seems to be due to the presence of allosteric inhibitors in the serum obtained from the patients with liver cirrhosis.

Another explanation of the decreased binding is, that there might be a qualitative alteration in the albumin molecule. The concept of microheterogeneity has been discussed by several authors [40–42] and it is not unreasonable to assume that there would be a structural change of the albumin causing changed drug-binding properties in patients with liver cirrhosis. However, this theory is contradicted by the fact that charcoal treatment, which is known to remove impurities from albumin [31], considerably improved the binding capacity of the albumin isolated from the patients, causing it to approach normal values (Table 4). Moreover, the binding of warfarin was unaltered in the patient serum compared to normal serum which suggests the presence of specific inhibitors.

The present study clearly shows that liver cirrhosis has different effects on the protein binding of diazepam and warfarin, representing two separate sites on HSA. The disease state had no effect on the warfarin site, while the diazepam site was inhibited. Other diseases may have similar differentiated effects on the binding of drugs. The results demonstrate the

necessity of identifying the respective binding sites for protein-bound drugs.

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