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HEPATIC EXTRACTION OF ENDOGENOUS INHIBITORS OF DRUG BINDING TO SERUM PROTEIN IN THE PREGNANT RAT*

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Abstract—Significant decreases in the serum protein binding of a fluorescent dye, 1-anilino-8-naphthalenesulfonate (ANS), and salicylic acid (SA) were observed in pregnant rats compared to that in nonpregnant (control) rats. A significant difference in the serum protein binding of ANS and SA between serum samples taken at the hepatic vein and portal vein or femoral artery was also observed in the pregnant rats, while such a sampling site difference in the serum protein binding was not observed in the control rats. In the pregnant rats the affinity of ANS binding to the primary binding site in the serum from the hepatic vein was approximately 70% higher than that in the case of serum from the portal vein. The hepatic extraction of nonesterified fatty acids (NEFA) was also determined, and the extraction ratios in the control and pregnant rats were 0.55 and 0.31 respectively. We concluded from these findings and other evidence that certain endogenous inhibitors of drug binding to serum protein (such as NEFA), which increase during pregnancy, were extracted efficiently by the liver and that the difference in the serum protein binding of ANS and SA between serum samples taken at the hepatic and portal veins or femoral artery in the pregnant rats may be explained by the hepatic extraction of endogenous inhibitors. Our present results support the previous finding by Chou *et al.* [*Int. J. Pharm.* **18**, 217 (1984)] that in pregnant rats the serum protein binding of phenytoin is greater in the hepatic vein than that in the femoral vein.

During pregnancy, a progressive reduction in the serum protein binding of salicylic acid, sulfisoxazole and phenytoin in rats and humans has been reported [1–4]. These drugs are known to bind primarily to albumin in serum. Stock *et al.* [2] proposed that the decreased protein binding of drugs in the serum of pregnant rats was due, at least in part, to the accumulation of endogenous displacing agents. Charcoal treatment suggested that serum nonesterified fatty acids (NEFA) could be one of the endogenous inhibitors of the serum protein binding, since the serum concentration of NEFA significantly increased during pregnancy [2]. Furthermore, in humans, four or five times higher serum NEFA concentration was observed at late pregnancy than in nonpregnant women [5]. The change in plasma protein binding of diazepam was also attributed to the parallel increase in the total NEFA concentration during pregnancy [6]. However, Chou and Levy [7] recently reported that plasma from pregnant rats was subject to rapid *in vitro* lipolysis and that the serum protein binding of phenytoin and salicylate decreased during incubation or dialysis for 4 hr at 37° compared to that obtained by immediate ultrafiltration. In addition, they found a significant difference in the unbound fraction of phenytoin between control and pregnant rats, although there was only a small difference in the serum NEFA concentration between the two groups [8]. Therefore, the difference between the control and pregnant rats with respect to the plasma binding of phenytoin measured immediately after separation of plasma has not been considered to be due to the difference in the NEFA concentration between the control and pregnant rats [7–9]. The nature of the binding inhibitors thus remains unsolved.

The hepatic extraction of endogenous inhibitors of drug binding to serum protein during pregnancy could be one of the factors which affect the blood levels of binding inhibitors. If the binding inhibitors are extracted efficiently by the liver, a difference between the serum protein binding of drugs in the femoral artery and hepatic vein may be observed [10]. In fact, Chou *et al.* [11] observed a difference in the unbound fraction of phenytoin between serum samples from the femoral and hepatic veins in pregnant rats, although this difference was small (approximately 20%).

In the present study, the serum protein binding of 1-anilino-8-naphthalenesulfonate (ANS), an anionic fluorescent probe, and salicylic acid (SA) was determined in systemic, portal and hepatic venous blood from both control and pregnant rats in order to test the above possibility. Application of a fluorescence method using ANS permitted us to determine both the binding affinity and binding capacity with a small amount of serum. Furthermore, the hepatic extraction of NEFA was also determined.

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MATERIALS AND METHODS

Animals. Adult female rats (Wistar ST strain, Shizuoka Jikkendobutsu Co., Hamamatsu, Japan), weighing 230–260 g, were mated with male rats overnight. The day when the spermatozoa were first found in the vaginal smear was counted as day 1 of pregnancy. The animals were housed under conditions of controlled temperature and lighting with access to food and water *ad lib*. Twenty-day-pregnant rats were used in the present study and all non-pregnant rats, which were of the same age as the pregnant rats, were used as controls.

Materials. ANS (sodium salt) and SA (sodium salt) were purchased from the Tokyo Kasei Co. (Tokyo, Japan) and the Koso Chemical Co. (Tokyo, Japan) respectively. Oleic acid (sodium salt) and rat serum albumin were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{14}C-carbonyl]Salicylic acid (51.7 mCi/mmole) was purchased from the New England Nuclear Corp. (Boston, MA, U.S.A.) and was found to be at least 97% pure by TLC.

Experimental procedure. Under light ether anesthesia, the femoral artery was cannulated with polyethylene tubing (PE-50), and the hepatic vein of the left lobe [12] and the portal vein were cannulated with PE-50 tubing attached to a 25 gauge needle. All these procedures were completed within 10 min. Blood samples (1 ml each) were then obtained simultaneously through the three cannulae by pulling the syringe very gently for 10 min, so that the backward flow of venous blood was minimized. The concentrations of albumin and NEFA in the serum were determined using commercial kits (Albumin B-Test and NEFA C-Test, Wako Pure Chemical Co., Ltd., Osaka, Japan).

The binding of ANS to serum was determined at room temperature (19–22°) in a Hitachi MPF-4 fluorospectrometer (Hitachi Ltd., Tokyo, Japan) as reported previously [13]. The serum was diluted to 0.3% with sodium-potassium-phosphate buffer (130 mM, pH 7.4) for use in the measurement of ANS binding. To determine the inhibition pattern of ANS binding by NEFA, the titration with oleic acid of a mixture of ANS (0.3, 0.7 and 1 μM) and defatted rat serum albumin (1 μM) was carried out. The change of fluorescence intensity of ANS with the addition of oleic acid was measured as described above. The binding of SA to serum was determined by an ultrafiltration method using a membrane cone [14]. The serum concentration of total SA was adjusted to 30 μg/ml.

In other groups of control and pregnant rats, serum was obtained from the femoral artery. The serum samples from both the control and pregnant rats were treated with activated charcoal (Sigma Chemical Company) as reported by Chen [15]. After activated charcoal (50 mg/ml) had been added, the serum was adjusted to pH 3 with 2 N HCl, and the suspension was stirred for 1 hr in a cold room (5°). The charcoal was separated by centrifugation for 2 min in a table-top microfuge (Beckman Instruments, Fullerton, CA, U.S.A.), and the pH was raised to 7.4 with 2 N NaOH. This charcoal-treated serum was used for the protein binding study.

Data analysis. The data on ANS binding to serum protein were analyzed on the assumption of two classes of binding sites using the equation

$$C_b = \frac{n_1 \cdot P_T \cdot C}{K_{d1} + C} + \frac{n_2 \cdot P_T \cdot C}{K_{d2} + C} \qquad (1)$$

where C_b and C are the bound and unbound concentrations of ANS, respectively, n_1 and n_2 are the numbers of binding sites on albumin, K_{d_1} and K_{d_2} are the dissociation constants, and P_T is the concentration of albumin in the diluted serum. The binding parameters were obtained by a non-linear least squares method [16] using a Hitachi M-200/280H digital computer. The binding parameters thus obtained for the plasma, which contains various proteins and several possible inhibitors, are only apparent parameters, and cannot be compared with its intrinsic parameters for purified albumin.

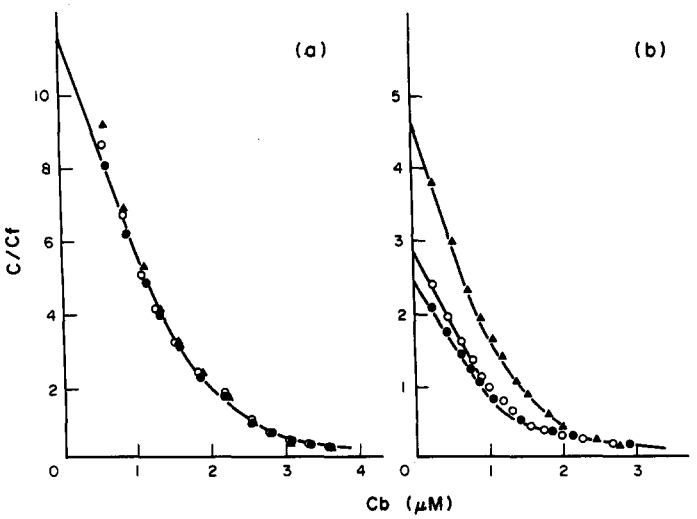

Fig. 1. Scatchard plots of ANS bindings to serum from the three different sampling sites in the control (a) and pregnant (b) rats. Key: (▲) hepatic vein, (○) femoral artery, and (●) portal vein.

Table 1. Binding parameters of ANS, SA to rat serum, and the serum concentrations of albumin and NEFA in control and pregnant rats*

		ANS					SA		
		K_{d_1} (μM)	$n_1 \cdot P$† (mM)	n_1	K_{d_2} (μM)	$n_2 \cdot P_T$† (mM)	f_p	Albumin (mM)	NEFA (m-equiv./l)
Control									
	F.A.‡	0.11	0.55	0.99	3.06	2.94	0.15	0.56	0.44
		(0.02)	(0.06)	(0.09)	(0.58)	(0.48)	(0.02)	(0.01)	(0.10)
	P.V.§	0.10	0.55	0.93	2.72	2.54	0.16	0.58	0.54
		(0.03)	(0.05)	(0.07)	(0.63)	(0.19)	(0.02)	(0.02)	(0.11)
	H.V.‖	0.10	0.52	0.98	2.44	2.47	0.14	0.57	0.25
		(0.02)	(0.06)	(0.07)	(0.33)	(0.17)	(0.02)	(0.01)	(0.06)
Pregnant									
	F.A.	0.44¶	0.40**	0.86	13.93**	2.50	0.53**	0.47¶	1.26**
		(0.10)	(0.03)	(0.06)	(2.04)	(0.22)	(0.07)	(0.03)	(0.12)
	P.V.	0.46**	0.36**	0.78	14.62**	2.59	0.57**	0.48¶	1.45**
		(0.07)	(0.04)	(0.04)	(2.00)	(0.25)	(0.06)	(0.02)	(0.10)
	H.V.	0.27¶	0.45	0.92	8.61**	2.24	0.41**	0.49¶	0.99**
		(0.05)	(0.06)	(0.07)	(1.03)	(0.08)	(0.06)	(0.02)	(0.09)

* Results are given as the means (±S.E.) of five control or seven pregnant rats.
† Corrected for the dilution of the sample serum.
‡ Femoral artery.
§ Portal vein.
‖ Hepatic vein.
¶ Significantly different ($P < 0.10$) from the control.
** Significantly different ($P < 0.05$) from the control.

Statistical analysis. All means are presented with their standard errors (mean ± S.E.). Student's *t*-test was utilized to estimate the significance of differences between the control and the pregnant rats.

RESULTS

Representative Scatchard plots of ANS binding to serum protein in the control (a) and pregnant (b) rats are shown in Fig. 1. A significant decrease in the ANS binding to serum was observed during pregnancy. An increase in ANS binding in serum from the hepatic vein was observed in the pregnant rats as compared to that in serum from the femoral artery or portal vein, while in the control rats no difference was observed among the three serum samples from different blood vessels. All these data are summarized in Table 1. The serum protein binding of salicylic acid was also determined in the same samples of serum from the control and pregnant rats. A sampling site difference in the serum protein binding was also observed in the case of SA (Table 1). The calculated binding parameters of ANS, the unbound fraction (f_p) of SA, and the serum concentrations of albumin and NEFA determined are also listed in Table 1. The ratios of the binding parameters of ANS, the unbound fraction of SA, and the concentrations of albumin and NEFA in the hepatic venous serum to those in the femoral arterial or the portal venous serum are listed in Table 2. Sampling site differences in the values of K_{d_1} and K_{d_2} for ANS were observed in all pregnant rats, and the hepatic venous serum showed the highest affinity for both primary binding sites and secondary binding sites. The number of primary binding sites (n_1) corrected for the albumin concentration was approximately 1, suggesting that the primary binding probably represents the binding to serum albumin. Compatible results were obtained for the serum unbound fraction of SA. No significant difference was observed among albumin concentrations in the serum samples from the three different sampling sites in either the control or pregnant rats. On the other hand, the serum concentration of NEFA was increased significantly in the pregnant rats and was lowest in the hepatic venous serum among the three sampling sites in both the control and pregnant rats, suggesting that NEFA may be extracted efficiently by the liver.

The relation between the value of K_{d_1} for ANS or the serum unbound fraction of SA and the serum concentration of NEFA in the hepatic and portal veins for both the control and pregnant rats is shown in Fig. 2. The value of K_{d_1} for ANS or the unbound fraction of SA does not seem to be related to the NEFA concentration when the serum NEFA concentration is in the lower range (approximately below 0.8 m-equiv./liter). However, at NEFA levels higher than 0.8 m-equiv./liter, an increase in the value of K_{d_1} for ANS or the unbound fraction of SA with increase in the serum concentration of NEFA was found.

The effect of oleic acid on the ANS binding to defatted rat serum albumin is shown in Fig. 3. The decrease in the fluorescence intensity of ANS was slower up to an oleic acid/albumin value of around 2, but it became steeper after that.

Pretreatment of serum with activated charcoal had little effect on the value of K_{d_1} for ANS or on the unbound fraction of SA in the control rats. The mean pre- and post-treatment of K_{d_1} values of ANS in the

Table 2. Ratios of the binding parameters of ANS, the unbound fraction of SA and serum concentrations of albumin and NEFA in the hepatic vein to those in the femoral artery and portal vein*

		Control rats	Pregnant rats
K_{d_1}	H.V.†/F.A.‡	1.00 ± 0.10	0.64 ± 0.05‖
	H.V./P.V.§	1.06 ± 0.12	0.60 ± 0.06‖
$n_1 \cdot p_T$	H.V./F.A.	1.02 ± 0.04	1.11 ± 0.05
	H.V./P.V.	1.02 ± 0.04	1.22 ± 0.06
K_{d_2}	H.V./F.A.	0.85 ± 0.09	0.66 ± 0.08¶
	H.V./P.V.	0.95 ± 0.07	0.62 ± 0.07‖
$n_2 \cdot p_T$	H.V./F.A.	1.02 ± 0.06	0.93 ± 0.06
	H.V./P.V.	0.98 ± 0.05	0.93 ± 0.12
f_p for SA	H.V./F.A.	0.89 ± 0.06	0.78 ± 0.05¶
	H.V./P.V.	0.86 ± 0.06	0.73 ± 0.05¶
Serum albumin concn	H.V./F.A.	1.02 ± 0.02	1.04 ± 0.02
	H.V./P.V.	0.98 ± 0.03	1.02 ± 0.02
Serum NEFA concn	H.V./F.A.	0.56 ± 0.03	0.79 ± 0.05‖
	H.V./P.V.	0.45 ± 0.04	0.69 ± 0.07‖

* Results are given as the means ± S.E. of five control or seven pregnant rats.
† Hepatic vein.
‡ Femoral artery.
§ Portal vein.
‖ Significantly different ($P < 0.05$) from the control.
¶ Significantly different ($P < 0.10$) from the control.

control serum were 0.188 ± 0.009 (mean ± S.E., N = 5) and 0.138 ± 0.009 (N = 5), respectively, and the corresponding values of the unbound fraction of SA were 0.131 ± 0.016 (N = 5) and 0.106 ± 0.018 (N = 5), respectively. On the other hand, charcoal treatment of serum from pregnant rats caused a remarkable decrease in both the values of K_{d_1} for ANS and the unbound fraction of SA, but not to the control levels. The mean pre- and post-treatment values of K_{d_1} for ANS in the serum of pregnant rats were 0.778 ± 0.180 (S.E., N = 5) and 0.271 ± 0.027 (N = 5), respectively, and the corresponding values of unbound fraction of SA were 0.677 ± 0.041 and 0.214 ± 0.021, respectively (Fig. 4). Under these conditions, charcoal treatment removed 84.0 ± 2.1% (mean ± S.E., N = 5) of serum NEFA from both the control and pregnant rats, whereas the concentrations of albumin changed little (less than 5%).

DISCUSSION

The serum protein binding of ANS showed a decrease during pregnancy. Previously, we reported a correlation of the plasma protein bindings of ANS and several acidic drugs [17]. The decrease in the serum protein binding of ANS which occurred during pregnancy was due mainly to a decrease in the binding affinity rather than to a decrease in the binding capacity (Table 1). Stock *et al.* [2] suggested that an increase in endogenous inhibitors in the serum of pregnant rats may be the cause of the reduced serum protein binding of drugs during pregnancy. If inhibitors are extracted efficiently by the liver, a sampling site difference in the protein binding of drugs may occur between the hepatic venous serum and portal venous or femoral arterial serum. In fact, Chou *et al.* [11] found that the unbound fraction of phenytoin was significantly lower in the hepatic vein serum than in the femoral vein serum in 20-day-pregnant rats, though the difference was small. However, in their study it was not clarified whether the change in the binding affinity or binding capacity is related to this sampling site difference in the serum protein binding

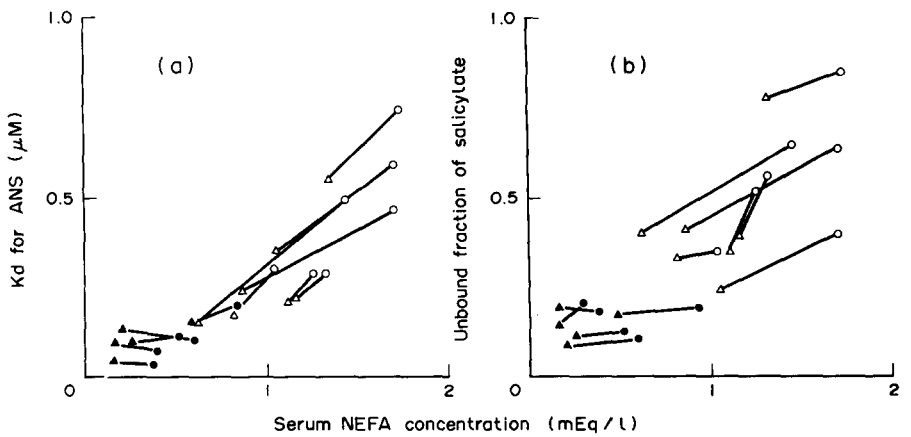

Fig. 2. Effect of hepatic extraction on the relationship between serum NEFA concentration and dissociation constant (K_{d_1}) for ANS or unbound fraction of SA. Key: (▲) hepatic vein and (●) portal vein in control rats, and (△) hepatic vein and (○) portal vein in pregnant rats.

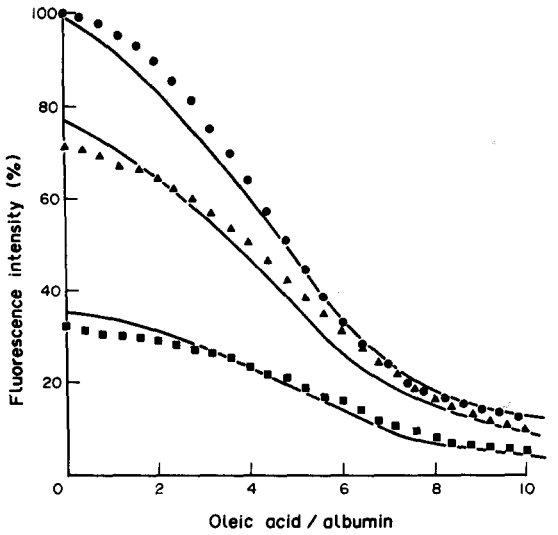

Fig. 3. Effect of oleic acid on ANS fluorescence intensity in a solution of defatted rat serum albumin. Measurements were performed in phosphate buffer (pH 7.4) containing 1 μM albumin and 0.3 (■), 0.7 (▲) and 1.0 (●) μM ANS. The fluorescence intensity in the presence of 1 μM ANS and the absence of oleic acid was fixed at 100 units. The solid lines represent the calculated curves obtained by the use of equations 2 and 3. The parameters used in this calculation were: $C_T = 0.3$, 0.7 and 1.0 μM, $P_T = 1$ μM, $n_1 = 1$, $n_2 = 1$, $n_3 = 4$, $K_{d1,A} = 0.0772$ μM, $K_{d1,I} = 0.039$ μM, $K_{d2,I} = 0.039$ μM and $K_{d3,I} = 0.245$ μM.

of phenytoin. In the present study, the affinity of ANS binding in the serum from the hepatic vein was higher than in the serum from the other sampling sites in the pregnant rats, while no difference was observed among the three sampling sites in the control rats. Furthermore, the serum protein binding of SA was also higher in the hepatic vein than in other sampling sites only in the pregnant rats, suggesting the possibility of a sampling site difference in the serum protein binding of other acidic drugs.

NEFA is known to reduce ANS binding to human serum albumin [18]. In the present study, the concentrations of serum NEFA were approximately two or three times higher in the pregnant rats than in the control rats. Similar findings were reported previously in humans [6, 19, 20]. On the other hand, Chou *et al.* [9] reported that the plasma concentration of NEFA in pregnant rats was similar to that in non-pregnant rats under conditions where the *in vitro* lipolysis was minimized. Therefore, the following experiment was performed to test whether the decrease in the serum protein bindings of ANS and SA observed in the present study was due to artifacts arising from *in vitro* lypolysis. Immediately after blood collection from pregnant rats from the jugular vein without the use of heparin, both the serum concentration of NEFA and the serum protein binding of ANS were measured (Table 3). Even under these conditions, an increased serum concentration of NEFA was observed in the pregnant rats compared to that in the control rats. In addition, the serum concentration of NEFA did not change during incubation of serum for 15 min at room temperature (19–22°). This time period corresponded to that of the ANS binding experiment. Therefore, the effect of *in vitro* lipolysis on the decreases in the serum protein bindings of ANS and SA in pregnant rats observed in the present study might be small, if any.

The hepatic extraction of each individual NEFA (oleic acid, palmitic acid, stearic acid, linoleic acid) in the pregnant rat was also reported by Chou *et al.* [9]. They suggested that each NEFA was extracted efficiently by the liver, but that the hepatic extraction of each NEFA did not change during pregnancy. In the present study, the hepatic extraction of NEFA was somewhat higher in the control rats than in the pregnant rats (Table 2). Nevertheless, the sampling site difference in the serum binding of ANS was observed only in the pregnant rats. This may be explained as follows. Gugler *et al.* [21] reported that the inhibition of serum protein bindings of warfarin and phenytoin by increased serum NEFA after heparin administration is observed only when the serum concentration of NEFA is higher than 0.8 m-equiv./

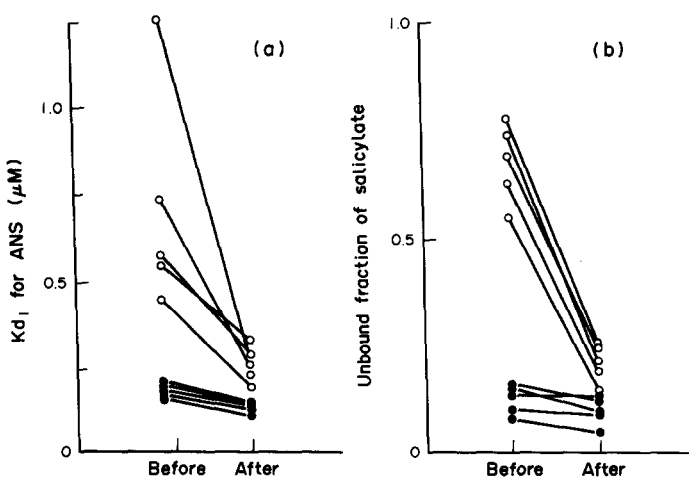

Fig. 4. Effect of treatment with activated charcoal on the dissociation constant of ANS and the unbound fraction of SA (see text for details). Key: (●) control, and (○) pregnant rats.

Table 3. NEFA concentrations in serum obtained from pregnant rats

Rat. no.	Conditions		
	Immediately measured (m-equiv./liter)	After 2 hr on ice (m-equiv./liter)	After 15 min at room temperature (m-equiv./liter)
1	1.31	1.33	1.37
2	0.78	0.80	0.78
3	1.17	1.13	1.17
4	1.36	1.24	1.24
Mean ± S.E.	1.16 ± 0.13	1.13 ± 0.12	1.14 ± 0.13

liter and that little binding inhibition is observed below this concentration. In the present study, the concentration ranges of serum NEFA in both the femoral artery and the portal vein were approximately 0.3 to 0.9 m-equiv./liter in the control rats and 0.9 to 1.7 m-equiv./liter in the pregnant rats (Table 1). Increases in the value of K_{d_1} for ANS and the unbound fraction of SA were also observed when the serum concentration of NEFA increased above approximately 0.8 m-equiv./liter (Fig. 2). Considering both the finding of Gugler *et al.* [21] and our present results, the lower concentrations of serum NEFA in the control rats may explain the small difference in ANS binding among the three sampling sites, even though NEFA was extracted efficiently by the liver. On the other hand, if the higher serum concentration of NEFA (0.9 to 1.7 m-equiv./liter) observed in the pregnant rats, which is high enough to inhibit the serum protein bindings of ANS and SA, was decreased by hepatic extraction, the protein binding would be affected to a great extent.

The inhibition of ANS binding to rat serum albumin by a typical NEFA, oleic acid, was examined using defatted albumin (Fig. 3). As we previously reported [22], oleic acid showed a somewhat peculiar pattern of inhibition. The assumption of simple competition by ANS and oleic acid for a single binding site could not explain this inhibition pattern [22]. However, NEFA is known to bind very strongly to albumin at two primary binding sites and several secondary binding sites [23]. If simple competition by two ligands (ANS and NEFA in this case) for one of the primary binding sites of NEFA is assumed, the following equations hold (see Appendix for details):

$$I_T = I + \frac{n_1 \cdot P_T \cdot I}{K_{d_{1,I}} + I} + \frac{n_3 \cdot P_T \cdot I}{K_{d_{3,I}} + I} + \left(n_2 \cdot P_T - C_b - \frac{K_{d_{1,A}} \cdot C_b}{C_T - C_b}\right) \quad (2)$$

and

$$I = \frac{K_{d_{2,I}} \cdot (C_T - C_b)}{K_{d_{1,A}} \cdot C_b} \times \left(n_2 \cdot P_T - C_b - \frac{K_{d_{1,A}} \cdot C_b}{C_T - C_b}\right) \quad (3)$$

where C_T and I_T are the total concentrations of ANS and NEFA, respectively, P_T is the albumin concentration, I is the unbound concentration of NEFA, n_1, n_2 and n_3 are the number of each class of binding sites on albumin, and $K_{d_{1,I}}$, $K_{d_{2,I}}$ and $K_{d_{3,I}}$ are the dissociation constants for NEFA (relating to each class of binding site).

The experimental data shown in Fig. 3 were fitted to these equations by use of a non-linear least-squares method [16]. In this fitting, C_B and C_T were considered as independent variables and I_T was considered as a dependent variable. In the present case, the five parameters used in this calculation were fixed as follows: $P_T = 1\ \mu M$, $n_1 = 1$, $n_2 = 1$ and $n_3 = 4$ [23], and $K_{d_{1,A}} = 0.077\ \mu M$. In the present study, the value of $K_{d_{1,A}}$ was independently determined as described previously [22]. On the assumption that the value of $K_{d_{1,I}}$ was equal to that of $K_{d_{2,I}}$ [23], $K_{d_{1,I}}$ and $K_{d_{3,I}}$ were calculated by this fitting. The converged values for $K_{d_{1,I}}$ and $K_{d_{3,I}}$ were 0.039 and 0.245 μM respectively. The fitting curve shown in Fig. 3 well reproduced the anomalous pattern obtained in the titration with oleic acid. Therefore, the slow fluorescence decrease up to a serum NEFA/albumin concentration ratio of approximately 2 and the steep decrease after that could be well explained by this model.

In the control rats, the serum NEFA/albumin concentration ratios in the femoral artery and portal vein were 1–2. Therefore, the serum protein binding was inhibited little by NEFA and might not change across the liver. On the other hand, in the pregnant rats, the serum NEFA/albumin concentration ratios in the femoral artery and portal vein were 2–4, whereas the values were 2–2.25 in the hepatic venous serum. The serum protein binding of ANS decreased with increase in the concentration of NEFA in this range. Consequently, the increase in the serum protein binding of ANS and SA across the liver in the pregnant rats might be (at least partly) explained by the hepatic extraction of NEFA.

Pretreatment of serum with activated charcoal is known to remove certain endogenous inhibitors of drug binding to serum proteins, including NEFA [15], and to increase the protein binding of drugs in plasma or serum from uremic patients [24, 25] and from heparin-treated rats [10] to essentially normal levels. Stock *et al.* [2] suggested that such treatment also increased the serum protein binding of SA in pregnant rats, but not to the control levels. In the present study, pretreatment of serum from the pregnant rats with charcoal decreased the dissociation constant for ANS and the unbound fraction of SA, but not to the control levels (Fig. 4). Therefore, the

decrease in the serum protein binding of ANS and SA in the pregnant rats may be due not only to the increase in the NEFA level but also to an increase in other endogenous inhibitors. Our present results thus confirmed the previous finding by Stock *et al.* [2].

In summary, a difference in the serum protein binding of ANS and SA between the hepatic vein and the portal vein or femoral artery was observed in pregnant rats, while such a sampling site difference in the serum protein binding of the drugs was not observed in the control rats. Furthermore, it was found that the affinity of ANS binding in the serum from the hepatic vein was approximately 70% higher than that in serum from the portal vein in pregnant rats. Our present results also support the previous finding by Chou *et al.* [11] that in pregnant rats the serum protein binding of phenytoin was increased in the hepatic vein as compared to that in the femoral vein.

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APPENDIX

The model describing the inhibition of ANS binding to rat serum albumin by NEFA is as follows. It is well known that NEFA has two primary binding sites and four secondary binding sites on human serum albumin [23, 26], but the number of primary binding sites of albumin for ANS is only one [13]. Further, NEFA inhibits the serum protein binding of ANS [18]. Consequently, the binding sites of NEFA might be classified into three categories as follows:

(1) The primary binding site for NEFA (site 1, $n_1 = 1$).

(2) The primary binding site for both NEFA and ANS; both compounds compete for this site (site 2, $n_2 = 1$).

(3) The secondary binding sites for NEFA (site 3, $n_3 = 4$) [23].

where n_1, n_2 and n_3 represent the number of binding sites in class i. According to this model, the following equations hold:

For site 1:

$$P_1 + I \overset{K_{d_{1,I}}}{\rightleftharpoons} P_1I = I_{1,b} \qquad K_{d_{1,I}} = \frac{P_1 \cdot I}{I_{1,b}} \tag{4}$$

For site 2:

$$P_2 + I \overset{K_{d_{2,I}}}{\rightleftharpoons} P_2I = I_{2,b} \qquad K_{d_{2,I}} = \frac{P_2 \cdot I}{I_{2,b}} \tag{5}$$

$$P_2 + C \overset{K_{d_{1,A}}}{\rightleftharpoons} P_2C = C_b \qquad K_{d_{1,A}} = \frac{P_2 \cdot C}{C_b} \tag{6}$$

For site 3:

$$P_3 + I \overset{K_{d_{3,I}}}{\rightleftharpoons} P_3I = I_{3,b} \qquad K_{d_{3,I}} = \frac{P_3 \cdot I}{I_{3,b}} \tag{7}$$

where P_1, P_2 and P_3 are the concentrations of unoccupied binding sites, and $I_{1,b}$, $I_{2,b}$ and $I_{3,b}$ are the concentrations of NEFA bound to each class of binding site.

Furthermore, $n_1 \cdot P_T$, $n_2 \cdot P_T$, $n_3 \cdot P_T$, C_T and I_T are given as follows:

$$n_1 \cdot P_T = P_1 + I_{1,b} \tag{8}$$

$$n_2 \cdot P_T = P_2 + I_{2,b} + C_b \tag{9}$$

$$n_3 \cdot P_T = P_3 + I_{3,b} \tag{10}$$

$$C_T = C + C_b \tag{11}$$

$$I_T = I + I_{1,b} + I_{2,b} + I_{3,b} \tag{12}$$

Substitution of equations 6 and 11 into equation 9 gives the following equation

$$I_{2,b} = n_2 \cdot P_T - C_b - \frac{K_{d_{1,A}} \cdot C_b}{C_T - C_b} \tag{13}$$

and substitution of equations 6 and 13 into equation 5 gives equation 3 in the text.

Substitution of equation 4 into equation 8 gives the following equation

$$I_{1,b} = \frac{I \cdot n_1 \cdot P_T}{K_{d_{1,I}} + I} \tag{14}$$

Also, substitution of equation 7 into equation 10 gives the following equation

$$I_{3,b} = \frac{I \cdot n_3 \cdot P_T}{K_{d_{3,I}} + I} \tag{15}$$

Finally, we obtain equation 2 in the text by substitution of equations 3, 13, 14 and 15 into equation 12.