[Chem. Pharm. Bull. 31(3) 971—978 (1983)]

N-Bromosuccinimide-oxidized Human Serum Albumin as a Tool for the Determination of Drug Binding Sites of Human Serum Albumin

Hiroko Sakamoto,* Izumi Nagata, Kiyomi Kikuchi, Masako Aida and Masachika Irie

Hoshi College of Pharmacy, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142, Japan

(Received August 5, 1982)

In order to investigate the role of the tryptophan residue of human serum albumin (HSA) in drug binding, HSA oxidized with 8 molar equivalents of *N*-bromosuccinimide (NBS) was prepared at pH 4.1. The NBS-oxidized HSA had lost the lone tryptophan residue without change in tyrosine content, and the modified protein retained the gross polypeptide conformation of native HSA as far as could be judged from the circular dichroism (CD) spectrum. The binding ability of NBS-oxidized HSA for chlordiazepoxide was decreased by about 60% from that of HSA. However, the binding of phenylbutazone and warfarin at the most reactive site of the modified HSA was not very different from that to HSA. These results suggest that the lone tryptophan residue in HSA lies in or near the indole and benzodiazepine binding site and not in the primary binding site of warfarin and phenylbutazone.

Keywords——human serum albumin; drug binding; NBS oxidation; chlordiazepoxide; phenylbutazone; warfarin; tryptophan

Serum albumin is the most important carrier for drugs, fatty acids and other small molecules. Therefore, many investigators have studied the interaction of many drugs with human serum albumin (HSA). It has become clear that several sites are involved in the binding of drugs in HSA; they are the indole and benzodiazepine binding site, the warfarin and azapropazone binding site, the digitoxin binding site, the bilirubin binding site and the fatty acids binding site.[1] On the other hand, since the primary structure of HSA has been elucidated,[2,3] studies have been carried out to clarify the functional groups involved in the individual drug binding sites. It was reported that acetylsalicylate specifically modified the Lys_{199} residue of HSA,[4] increasing the binding ability towards acetriozate and decreasing the affinity for flufenamic acid.[5,6] The modification, however, did not affect the binding of HSA with dicoumarol.[5] Fehske *et al.* carried out modification studies of the lone tryptophan residue of HSA with 2-hydroxyl-5-nitrobenzyl bromide[7] and *o*-nitrophenylsulfenyl chloride,[8] and concluded that the tryptophan residue is directly involved in the warfarin binding site and indirectly affects the indole and benzodiazepine binding site.[7-9] Another study dealing with *o*-nitrophenylsulfenyl chloride-modified HSA suggested that the lone tryptophan residue was not located in the bilirubin site.[10] The reaction of very reactive tyrosine residues with tetranitromethane effectively modified the binding ability of the benzodiazepine binding site, indicating the involvement of these tyrosine residues in this binding site.[11] The modification of arginine residues in cyanogen bromide fragment C of HSA containing amino acid residues 124—298 greatly diminished the binding of diazepam.[12] These experiments confirmed the usefulness of such chemical modifications of HSA for the elucidation of the location and the nature of individual binding sites. However, the introduction of very bulky substituents onto amino acid side chains may cause changes in the tertiary structure of HSA or steric hindrance at one or more sites. These two factors may affect the binding ability for specific drugs in various ways. Therefore, for clarification of the nature of the binding sites, it seems to be necessary to choose mild modification conditions which will not affect the tertiary structure and to use many kinds of modified HSA derivatives with appropriate sizes of modifiers.

In this work, in order to study the role of the lone tryptophan residue in HSA, we studied

NII-Electronic Library Service

the modification of HSA with *N*-bromosuccinimide (NBS).[13] The reaction proceeds very easily in aqueous solution and it is not necessary to react HSA with reagents in 8 M urea, as was employed in the cases of *o*-nitrophenylsulfenyl chloride and 2-hydroxy-5-nitrobenzyl bromide modifications of HSA. The NBS-oxidized HSA thus obtained retained the native HSA conformation as far as could be judged from CD spectra, and the substituent on the tryptophan residue was expected to be small. The binding of the NBS-oxidized HSA with chlordiazepoxide, phenylbutazone and warfarin was also studied.

Experimental

Materials——Human serum albumin (HSA) obtained from Sigma Chem. Co. (crystalized) was treated with charcoal according to the procedure of Chen.[14] The fatty acid-free HSA (about 400 mg) was applied to a column of Sephadex G-200 (3×146 cm) equilibrated with 50 mM phosphate buffer (pH 7.0) and eluted with the same buffer. Fractions of 4 ml each were collected. The monomer fractions (Tube No. 150—175) were used for further experiments. NBS was purchased from Wako Pure Chemicals and used after recrystallization from hot water. Chlordiazepoxide (CDO) was obtained from Yamanouchi Pharm. Co., Ltd. Phenylbutazone (PB) and warfarin (WA) were obtained from Sigma Chem. Co.

Protein Concentration——Protein concentration was determined spectrophotometrically based on $E^{1\%}_{1cm}$, 280 nm: 5.3.[15]

NBS Oxidation——HSA in 50 mM phosphate buffer (pH 7.0) or 50 mM acetate buffer (pH 4.1 or 3.0) was mixed with various concentrations of NBS at 4°C. The final HSA concentration was 20 μM and that of NBS was 1—40 molar equivalents to HSA. The reaction was followed in terms of the decrease in absorbancy at 280 nm. Tryptophan oxidation was calculated according to the method of Spande and Witkop.[13] The NBS-oxidized HSA was dialyzed against 50 mM phosphate buffer.

Amino Acid Analysis——Samples of *ca.* 0.3 mg protein were hydrolyzed in evacuated tubes with 6 N HCl at 110°C for 24 h. Amino acid analyses were performed by the method of Spackman *et al.*[16] with a Nihon Denshi JEOL 6AH amino acid analyzer. The tryptophan residue in protein was determined fluorometrically by measuring the fluorescence intensity at 350 nm (excited at 292 nm) with native HSA as a standard. The tryptophan content thus estimated was consistent with that measured by the procedure of Dalby and Tsai.[17]

Circular Dichroism Spectra——CD spectra were measured with a JASCO J-40 spectropolarimeter at room temperature in cells of 0.2 and 0.5 cm light path for the wavelength regions 200—250 and 250—350 nm, respectively. The protein concentrations used were 2.0 and 20 μM, respectively. The estimation of α-helix content was performed according to the method of Chen and Yang[18] from $[\theta]_{222nm}$.

SDS-Disc Electrophoresis——To check the formation of aggregates or cross-links, SDS-disc electrophoresis of NBS-oxidized HSA was performed according to the procedure of Shapiro *et al.*[19]

Interaction of NBS-oxidized HSA with Drugs——(a) Difference ultraviolet absorption (UV) spectrum: Difference UV spectra of HSA and NBS-oxidized HSA induced by addition of various amounts of drugs (0.2—8 molar equivalents to HSA) were measured at pH 7.0 (50 mM phosphate buffer) and 20°C with a Shimadzu UV 200S spectrophotometer using tandem cells. The protein concentration was 20.0 μM.

(b) Difference circular dichroism (CD) spectrum: The CD spectra of HSA or NBS-oxidized HSA, and the mixtures of the protein and drugs were measured separately at pH 7.0 and 20°C with a JASCO J-40 spectropolarimeter. The difference CD spectra were calculated from the charts. The protein concentration was 20.0 μM. The formation of drug-protein complex was estimated by using Rosen's method[20] and expressed as a Scatchard plot.[21]

Results

NBS Oxidation of HSA

The modification of the lone tryptophan residue with 2-hydroxy-5-nitrobenzyl bromide and *o*-nitrophenylsulfenyl chloride[7-9] has been performed in 8 M urea. To find milder modification conditions, oxidation of HSA with NBS was investigated in aqueous solution at pH's 3.0, 4.1 and 7.0 and in 8 M urea at pH 4.1 at 20°C. As shown in Fig. 1, addition of 8 molar equivalents of NBS to HSA solution at pH 3.0 and 4.1 produced a typical difference spectrum due to tryptophan residue. However, the reaction at pH 7.0 resulted in an increase in absorbancy at 280 nm and a different UV spectrum as compared to that at pH 4.1. The difference spectrum was very similar to that caused by addition of NBS to free tyrosine (not shown).

NII-Electronic Library Service

The results indicated that for tryptophan oxidation of HSA, pH 4.1 or 3.0 should be used. The results of analyses of tyrosine and tryptophan contents for HSA modified with various molar ratios of NBS are shown in Table I. When HSA was oxidized with increasing amounts of NBS, the decrease in fluorescence intensity at 350 nm (excited at 292 nm) paralleled the decrease in tryptophan content estimated by Dalby and Tsai's procedure.[17] Thus, tryptophan content was estimated routinely in terms of the decrease in fluorescence emission intensity (Fig. 2). As shown in Table I, the rate of tryptophan oxidation was largest at pH 4.1 among the pH's tested here, and oxidation of tryptophan was completed by the addition of 7—8 molar equivalents of NBS without any decrease in tyrosine residues. At pH 7.0, the rate of tryptophan oxidation was lower than that at pH 4.1 and a decrease in tyrosine content was

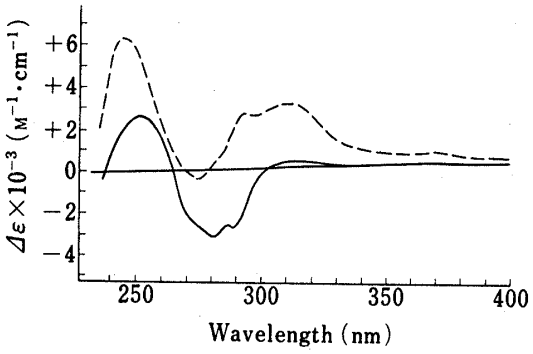

Fig. 1. Difference Spectra induced by Addition of 8 Molar Equivalents of NBS to HSA (19.1 μM) at pH's 4.1 (——) and 7.0 (----)

The experimental conditions were the same as described in the text.

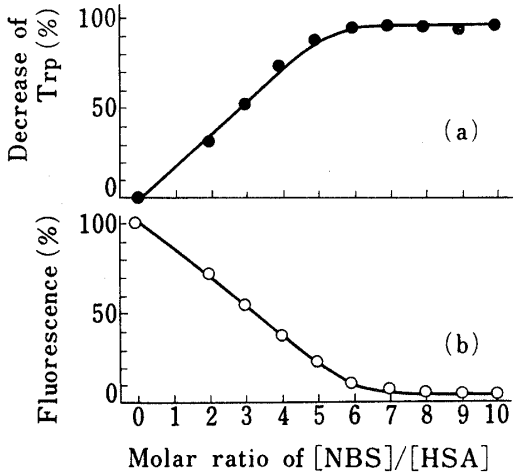

Fig. 2a. Change in Tryptophan Content of HSA (Estimated by the Method of Dalby and Tsai[17]) induced by Addition of Various Amounts of NBS at pH 4.1

Fig. 2b. Change in Fluorescence Intensity of HSA at 350 nm (excited at 292 nm) induced by Addition of Various Amounts of NBS at pH 4.1

TABLE I. Tryptophan and Tyrosine Contents of NBS-oxidized HSA's at Various Concentrations of NBS and α-Helix Contents of the Oxidized HSA's

NBS concentration ([NBS]/[HSA])	Tryptophan and tyrosine residues modified								α-Helix content[d] (%)			
	Tryptophan				Tyrosine[c]							
	pH 3.0[a]	pH 4.1[a]	pH 4.1[b] (8 M urea)	pH 7.0[a]	pH 3.0	pH 4.1	pH 4.1 (8 M urea)	pH 7.0	pH 3.0	pH 4.1	pH 4.1 (8 M urea)	pH 7.0
0	1.0	1.0	1.0	1.0	18.3	18.3	18.3	18.3	58.1	59.4	54.2	58.5
3		0.55	0.56			18.4	16.9			58.6	53.1	
5	0.36	0.22	0.50			18.2						
6	0.16	0.10	0.56		17.2	18.3	15.0			57.3	53.5	
7	0.07	0.04		0.68	17.8	18.1		15.8	59.1	59.2		
8	0.03	0	0.30		17.4	17.9	16.2			57.3	53.6	
9	0	0	0			17.8				56.2	50.4	
10	0	0	0	0.50	0.50	17.7	15.9		55.1	58.5		57.8

a) Determined fluorometrically.
b) Determined by Dalby and Tsai's method.[17]
c) Obtained from amino acid analysis.
d) Estimated from $[\theta]_{222nm}$.

NII-Electronic Library Service

observed even at the initial stage of oxidation. The results are consistent with those in Fig. 1.

The molar ellipticities of the albumin oxidized at the levels of NBS mentioned above were practically identical with that of native HSA in the wavelength region between 200—250 nm (Fig. 3), indicating the maintenance of the gross polypeptide conformation of native HSA during NBS oxidation. However, on further oxidation of HSA, the negative trough at this wavelength region decreased somewhat. The α-helix contents of NBS-modified HSA under various conditions estimated from $[\theta]_{222nm}$ according to Chen and Yang's procedure[18] are listed in Table I. The α-helix content of NBS-oxidized HSA at an [NBS]/[HSA] ratio of 7—8 at pH 4.1 in 8 M urea was slightly lower than that in the absence of 8 M urea. Thus, the presence of 8 M urea in the reaction mixture seemed to be inappropriate for the modification of HSA. The SDS-electrophoresis patterns of HSA modified at pH 4.1 with various NBS ratios are shown in Fig. 4. The results indicated that no detectable aggregation or cross-linking of NBS-oxidized HSA occurred when HSA was modified by up to 15 molar equivalents of NBS. From the results described above, HSA modified at pH 4.1 with 8 molar equivalents of NBS was selected as a suitable tryptophan-modified HSA for binding studies with various drugs.

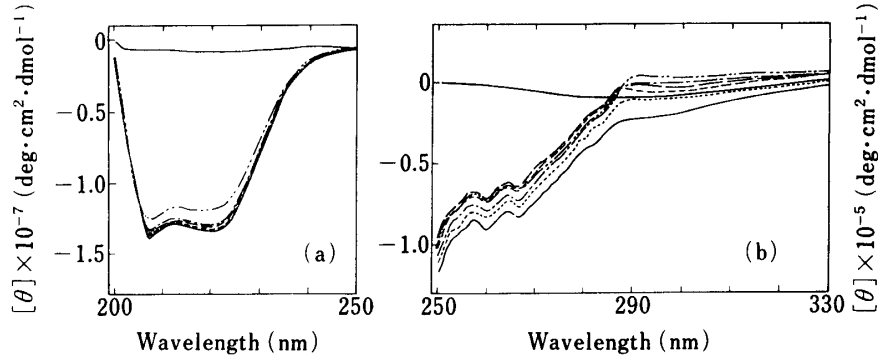

Fig. 3. The CD Spectra of HSA and HSA oxidized with Various Amounts of NBS at pH 4.1

The spectra were measured in 50 mM phosphate buffer (pH 7.0). ——, native HSA; ——, HSA oxidized with 4 molar equivalents of NBS; — —, HSA oxidized with 8 molar equivalents of NBS; ····, HSA oxidized with 10 molar equivale nts of NBS; -·-·-, HSA oxidized with 15 molar equivalents of NBS; -···-···-, HSA oxidized with 30 molar equivalents of NBS. The other experimental conditions were as described in the text.

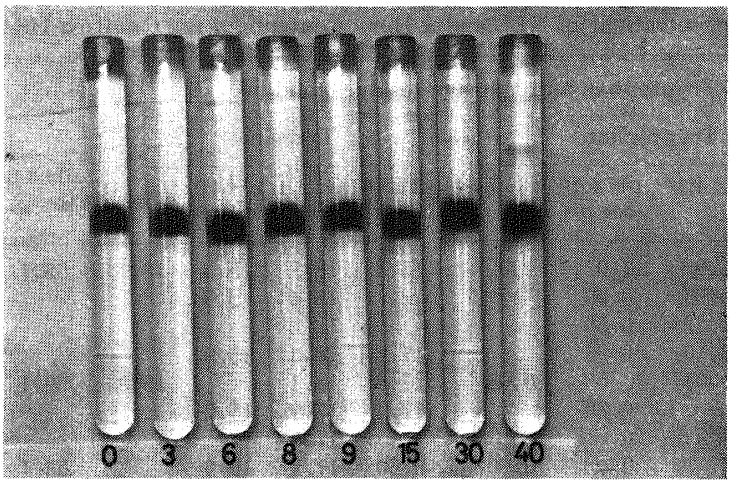

Fig. 4. SDS-Disc Electrophoresis of HSA oxidized with Various Amounts of NBS

The experimental conditions were as described in the text. The numbers in the figure represent the [NBS]/[HSA] ratios.

NII-Electronic Library Service

The HSA oxidized with NBS under the above conditions is designated as NBS-oxidized HSA hereafter in this paper.

Binding Studies of NBS-oxidized HSA with CDO, PB and WA

Bindings of NBS-oxidized HSA with CDO (a typical drug binding to the indole and benzodiazepine binding site), PB (a typical drug binding to the azapropazone binding site) and WA (a typical drug binding to the warfarin binding site) were measured by difference UV or difference CD spectroscopy.

(a) Binding of NBS-oxidized HSA with CDO. The binding of CDO induced a difference UV spectrum having positive bands at 282, 322 and 370—380 nm, and negative bands at 258 and 345 nm. Fig. 5 shows the titration of HSA and NBS-oxidized

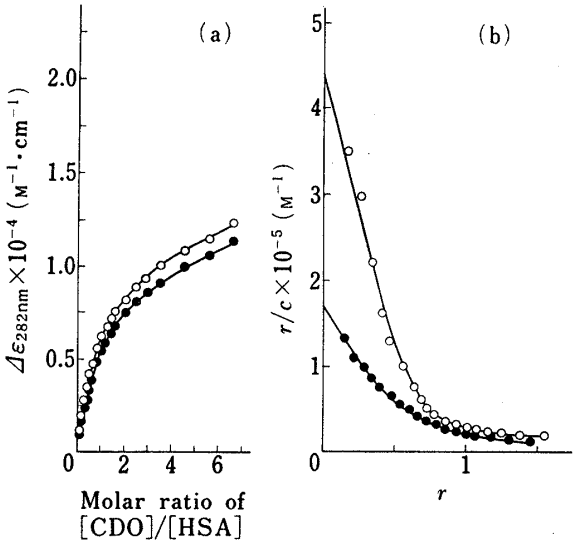

Fig. 5a. The Effect of Increasing Amounts of CDO on the Difference UV Spectra of HSA and NBS-oxidized HSA (20 μM) at 282 nm

Fig. 5b. Scatchard Plots of the Binding of CDO to HSA and NBS-oxidized HSA (determined from the Difference UV Spectra at 282 nm Shown in Fig. 5a)

—○—, native HSA; —●—, NBS-oxidized HSA.

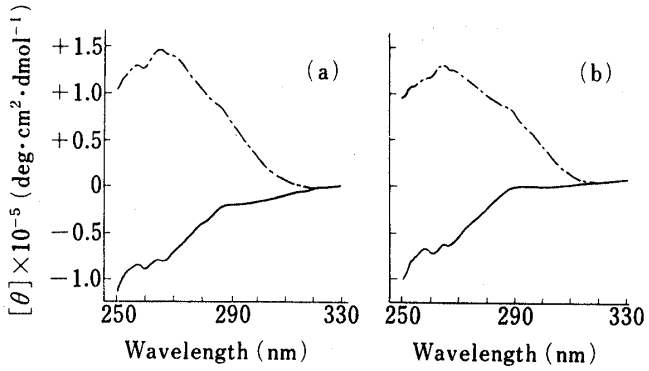

Fig. 6. The Difference CD Spectra of HSA and NBS-oxidized HSA induced by Addition of 5 Molar Equivalents of CDO (—·—).

Heavy lines represent CD spectra of HSA and NBS-oxidized HSA alone. Protein concentrations were the same as in Fig. 5. (a) HSA; (b) NBS-oxidized HSA.

HSA with various amounts of CDO by following the increase in absorbancy at 282 nm. The binding of CDO to HSA induced a positive Cotton effect a round 260 nm (Fig. 6) due to perturbation of the electronic transition in the CDO molecule. The dependence of the extrinsic Cotton effect of CDO bound to HSA or NBS-oxidized HSA on the CDO concentration is shown in Fig. 7. The results are very similar to those obtained from the difference UV spectra. The formation of CDO–HSA complex was estimated from the results in Fig. 5a and Fig. 7a by Rosen's procedure[20] and expressed as Scatchard plots (Fig. 5b and Fig. 7b). The binding constants (K) and the numbers (n) of high affinity sites of CDO–HSA and CDO–NBS-oxidized HSA were determined from the difference UV spectra and difference CD spectra and the results are shown in Table II. From these data it is evident that the binding constant of CDO–HSA complex was decreased by about 60% by NBS oxidation. The results are very similar to those obtained for HSA's modified by *o*-nitrophenylsulfenyl chloride and 2-hydroxy-5-nitrobenzyl bromide with diazepam and tryptophan.[7,10]

(b) Binding of NBS-oxidized HSA with PB. Fig. 8 shows the results of PB binding to NBS-oxidized HSA measured from the difference in absorbancy at 286 nm induced by the formation

NII-Electronic Library Service

Table II. Binding Constants and n Values of CDO, PB and WA with HSA and NBS-oxidized HSA

Compd.	Protein	Method	High affinity binding site $K \times 10^{-5}$ (M^{-1})	n	Low affinity binding site $K \times 10^{-5}$ (M^{-1})	n
CDO	Native HSA	UV	6.1	0.7		
		CD	5.8	0.7		
	NBS-oxidized HSA	UV	2.3	0.8		
		CD	2.0	0.8		
PB	Native HSA	UV	23	0.8	0.95	1.6
	NBS-oxidized HSA	UV	23	0.8	0.3	1.6
WA	Native HSA	UV	20	0.7	1.2	2.4
	NBS-oxidized HSA	UV	19	0.7	0.28	1.8

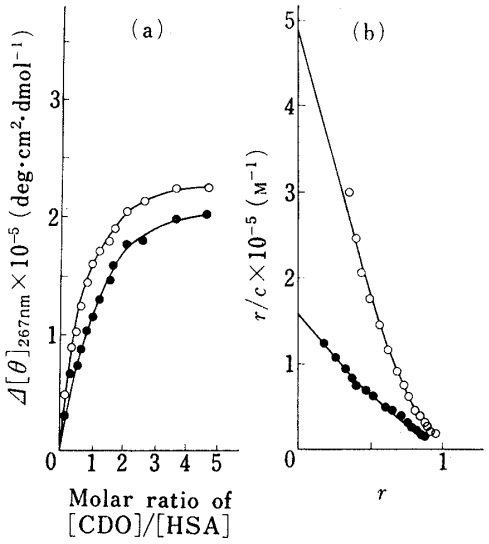

Fig. 7a. Dependence of the Extrinsic Cotton Effect of CDO Bound to HSA and NBS-oxidized HSA on the CDO Concentration

Protein concentrations were 20 μM. The wavelength used was 267 nm.

Fig. 7b. Scatchard Plots of the Binding of CDO to HSA and NBS-oxidized HSA (determined from the Difference CD Spectra Shown in Fig. 7a)

—○—, native HSA; —●—, NBS-oxidized HSA.

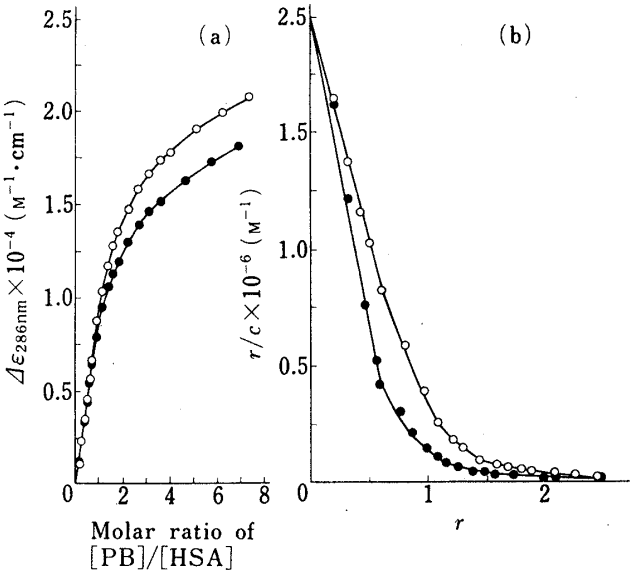

Fig. 8a. The Effect of Increasing Amounts of PB on the Difference UV Absorption of HSA and NBS-oxidized HSA at 286 nm

Protein concentrations were 20 μM.

Fig. 8b. Scatchard Plots of the Binding of PB to HSA and NBS-oxidized HSA (determined from the Difference UV Spectra Shown in Fig. 8a)

—○—, native HSA; —●—, NBS-oxidized HSA.

of PB–HSA complex. The difference in absorbancy was not affected by NBS modification up to a [PB]/[HSA] ratio of *ca.* 1. However, at higher [PB]/[HSA] ratios a decrease in PB–NBS-oxidized HSA complex formation was observed as compared to that of HSA (Fig. 8a). Scatchard plots obtained from the results described above are shown in Fig. 8b. The K value of the first high affinity binding site was not affected by NBS oxidation of the tryptophan residue (Table II). The n values of both HSA and NBS-oxidized HSA were *ca.* 0.8. The results are consistent with those obtained by another modification method reported by Fehske *et al.*[9] The estimated K and n values of low affinity binding sites for HSA and NBS-oxidized HSA are also shown in Table II. The results suggest that the binding of PB with the low affinity sites was also affected by NBS oxidation. Thus, these sites probably

NII-Electronic Library Service

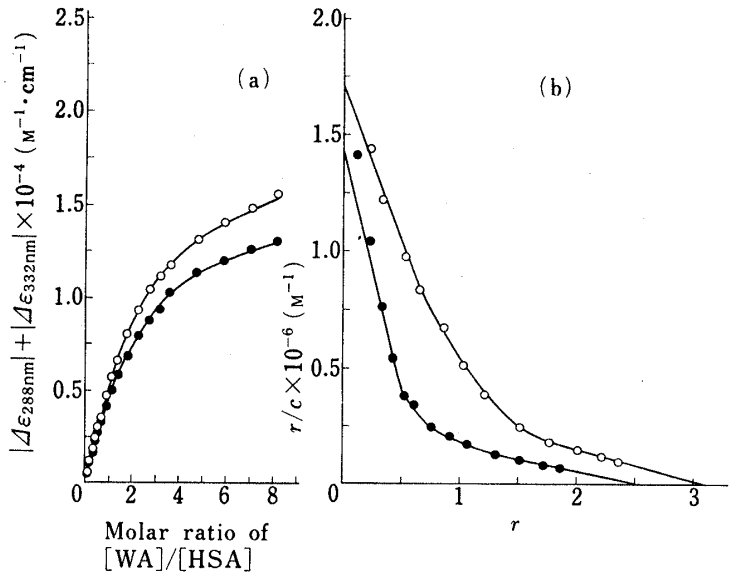

Fig. 9a. The Effect of Increasing Amounts of WA on the Difference UV Absorption of HSA and NBS-oxidized HSA

The difference spectra were expressed as $|\Delta\varepsilon_{288nm}|+|\Delta\varepsilon_{332nm}|$. Protein concentrations were 20 μM.

Fig. 9b. Scatchard Plots of the Binding of WA to HSA and NBS-oxidized HSA (determined from the Difference UV Spectra Shown in Fig. 9a)

—○—, native HSA; —●—, NBS-oxidized HSA.

overlap with the indole and benzodiazepine binding site.

(c) Binding of NBS-oxidized HSA with WA. The binding of WA to HSA induced a difference UV spectrum having positive bands at 247 and 332 nm and negative bands at 278, 288 and 300 nm. Fig. 9a showed the titration of HSA and NBS-oxidized HSA with various concentrations of WA. Scatchard plots were drawn based on the data shown in Fig. 9a (Fig. 9b). The K and n values of both high and low affinity sites of WA–HSA and WA–NBS-oxidized HSA calculated from Fig. 9b are shown in Table II. The results indicate that the affinity of the primary binding site was not affected by oxidation of the tryptophan residue, while the low affinity binding sites were affected markedly. The situation was very similar to that of the PB binding site.

Discussion

In contrast to the chemical modification studies reported by Fehske *et al.*[7-9] the results presented here suggest that the tryptophan residue of HSA is located in or near the indole–benzodiazepine binding site and not in the primary binding site for WA and PB (warfarin–azapropazone binding site). The fluorescence intensity due to the tryptophan residue in HSA is known to be partially quenched by increasing addition of WA and PB.[22,23] The results suggest that the tryptophan residue is located near the binding site of WA and PB. Similar experiments performed with CDO gave similar results (not shown in this paper). Therefore, it is most probable that (1) the tryptophan residue is located near the indole–benzodiazepine and warfarin–azapropazone binding sites, (2) oxidation of the tryptophan residue by NBS is most unfavorable for the binding of CDO (indole–benzodiazepine site) and less so for the WA and PB sites, indicating a close relation of the tryptophan residue with the CDO binding site, (3) on chemical modification of the tryptophan residue by 2-hydroxy-5-nitrobenzyl bromide and *o*-nitrophenylsulfenyl chloride, the bulky substituents project towards the WA binding

NII-Electronic Library Service

site causing the marked decrease in the affinity for WA, but not for PB. The results of further binding studies of various drugs with NBS-oxidized HSA will be published elsewhere.

References

1) K.J. Fehske, W. Müller and U. Wollert, *Biochem. Pharmacol.*, **30**, 687 (1981).
2) P.Q. Behrens, A.M. Spiekerman and J.R. Brown, *Fed. Proc.*, **34**, 591 (1975).
3) B. Meloun, L. Moravek and V. Kostka, *FEBS Lett.*, **58**, 134 (1975).
4) J.E. Walker, *FEBS Lett.*, **66**, 173 (1976).
5) D. Hawkins, R.N. Pinckard and R.S. Farr, *Science*, **160**, 780 (1968).
6) C.F. Chignell and D.K. Starkweather, *Mol. Pharmacol.*, **7**, 229 (1971).
7) K.J. Fehske, W.E. Müller and U. Wollert, *Hoppe-Seyler's Z. Physiol. Chem.*, **359**, 709 (1978).
8) K.J. Fehske, W.E. Müller and U. Wollert, *Mol. Pharmacol.*, **16**, 778 (1979).
9) K.J. Fehske, W.E. Müller and U. Wollert, *Mol. Pharmacol.*, **21**, 387 (1982).
10) C. Jacobsen, *Eur. J. Biochem.*, **27**, 513 (1972).
11) K.J. Fehske, W.E. Müller and U. Wollert, *Biochim. Biophys. Acta*, **577**, 346 (1979).
12) N. Roosdorp, B. Wänn and Z. Sjöholm, *J. Biol. Chem.*, **252**, 3876 (1977).
13) T.F. Spande and B. Witkop, "Methods in Enzymology," ed. by C.H.W. Hirs, XI, Academic Press, N.Y., 1967, p. 506.
14) R.F. Chen, *J. Biol. Chem.*, **242**, 173 (1967).
15) H.E. Schultz, N. Heimburger and G. Frank, *Biochem. Z.*, **336**, 388 (1962).
16) D.H. Spackman, W.H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
17) A. Dalby and C-Y. Tsai, *Anal. Biochem.*, **63**, 283 (1975).
18) Y.H. Chen, J.T. Yang and H.M. Martinez, *Biochemistry*, **11**, 4120 (1972).
19) A.L. Shapiro, E. Vineula and J.V. Maizel, *Biochem. Biophys. Res. Comm.*, **28**, 815 (1967).
20) A. Rosen, *Biochem. Pharmacol.*, **19**, 2075 (1970).
21) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51**, 660 (1949).
22) C.F. Chignell, *Mol. Pharmacol.*, **6**, 1 (1970).
23) V. Maes, J. Hoebeke, A. Vercruysse and L. Kanarek, *Mol. Pharmacol.*, **16**, 147 (1979).

NII-Electronic Library Service