Interaction of Benzothiadiazides with Human Serum Albumin Studied by Dialysis and Spectroscopic Methods

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Received November 12, 1993; accepted May 17, 1994

The interaction of a series of benzothiadiazides with human serum albumin (HSA) was investigated by equilibrium dialysis (ED) and spectroscopic methods including circular dichroism (CD). The primary binding site of benzothiadiazides was designated site II, the diazepam site on the HSA molecule, as indicated by displacement experiments using different site-selective probes. Tyrosine and lysine amino acid residues were probably involved in the binding site of these compounds to HSA. Both electrostatic and hydrophobic interactions were found to play a role in the binding of these compounds to HSA. Among the compounds tested, chlorothiazide had the highest affinity ($K_1 = 5.5 \times 10^4\ M^{-1}$, $K_2 = 5.8 \times 10^3\ M^{-1}$). The primary binding affinity of the compounds for HSA was of the order: chlorothiazide > cyclopenthiazide > polythiazide > ethiazide > trichlormethiazide = methyclothiazde > hydrochlorothiazide. Binding was insensitive to the N-B transition of HSA. The binding site is proposed to consist of a cationic site on the surface of the HSA molecule with a hydrophobic crevice to accommodate the aromatic ring of the compounds. Positions 3 and 7 of the benzothiadiazide molecule is thought to affect the binding affinity to HSA.

KEY WORDS: benzothiadiazides; human serum albumin; electrostatic interaction; hydrophobic interaction; binding site.

INTRODUCTION

The plasma protein binding of thiazide compounds is well documented (1–3). Benzothiadiazides diuretics inhibit renal tubular reabsorption. In general, benzothiadiazides with relatively long duration of action show a proportionately high binding to plasma proteins. Thiazides are sometimes reported to increase the plasma concentration of other drugs such as pempidine (3), and thus can influence their pharmacokinetic properties when used simultaneously. Thiazides are reported to bind predominantly to human serum albumin (HSA). Binding of chlorothiazide to plasma proteins was shown to occur mostly at albumin (3), but the binding mode and albumin binding site were not studied. Therefore, the present work was undertaken to establish the HSA binding mode and binding site of a series of benzothiadiazide compounds. Since these compounds are structurally related to each other, their binding properties could clarify the nature of protein binding and pharmacokinetic properties of these compounds in humans.

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MATERIALS AND METHODS

Materials

HSA was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan). Chlorothiazide was purchased from Banyu Pharm. Co. (Tokyo, Japan). Cyclopenthiazide and hydrochlorothiazide were obtained from Ciba-Geigy (Japan) Ltd. Polythiazide was purchased from Yamanouchi Pharmaceutical Co., Ltd. Ethiazide, trichlormethiazide and methyclothiazide were purchased from Tokyo Tanabe Co., Ltd., Shionogi & Co., Ltd. and Dainippon Pharmaceutical Co., Ltd., respectively. The chemical structures of the benzothiadiazides used in this study are shown in Table I. The chemical purity of different benzothiadiazides was above 98.5% based on HPLC data. Dansyl-L-proline (DNSP) and dansyl-L-arginine (DNAG) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cetyltrimethylammonium bromide (CTAB), sodium lauryl sulfate (SLS) and Brij35 were obtained from Wako Pure Chemical Industries (Osaka, Japan). Polyoxyethylene lauryl ether (PLE) was purified from commercially available Brij35 according to the procedure of Ikeda et al. (4). 2-Hydroxy-5-nitrobenzyl bromide (HNBB), tetranitromethane (TNM), methylene blue (MB), and succinic acid (SA) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other reagents were of analytical grade. All the buffers used were prepared with sodium phosphate dibasic and sodium phosphate monobasic salts.

Methods

Preparation of HSA derivatives. The lone tryptophan (Trp) residue, lysine (Lys) residues, histidine (His) residues and tyrosine (Tyr) residues were modified by the methods as previously described (5). The conformation of HSA derivatives was checked by circular dichroism (CD), fluorescence measurements and SDS-PAGE. No significant structural alteration was observed as compared with the native protein.

Physicochemical Properties of Benzothiadiazides

The partition coefficient (PC) values of benzothiadiazides were determined in n-octanol/pH 5.0 phosphate buffer system according to the method of Fujita et al. (6). Experiments were carried out in triplicate.

Ionization constant (pKa) values for benzothiadiazides were determined titrametrically according to the method used by Whelpton (7). The van der Walls volumes (V_W) were calculated according to the method of Moriguchi et al. (8). Physicochemical parameters of the benzothiadiazides are illustrated in Table II.

CD experiment. CD experiments were made on a Jasco J-500 spectropolarimeter (Tokyo, Japan) using 10 mm cells at 25°C. Benzothiadiazide derivative solutions all of 20 μM were used in the CD experiments. Both native and modified HSA were also of 20 μM solution.

Equilibrium dialysis (ED). The ED method was chosen

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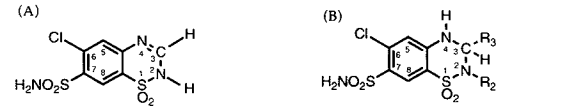
Table I. Structures of the Benzothiadiazides Used in This Study



Drug	R_2	R_3
Chlorothiazide (A)		
Hydrochlorothiazide (B)	-H	-H
Polythiazide (B)	$-CH_3$	$-CH_2SCH_2CF_3$
Trichlormethiazide (B)	-H	$-CHCl_2$
Ethiazide (B)	-H	$-C_2H_5$
Methyclothiazide (B)	$-CH_3$	$-CH_2Cl$
Cyclopenthiazide (B)	-H	$-CH_2-$

for the determination of binding parameters because of its ability to approximate physiologic conditions. ED experiments were performed using Sanko plastic dialysis cells (Fukuoka, Japan). Two cell compartments were separated by visking cellulose membranes. Aliquots of various ratios of drug (0—400 μM)-HSA (60 μM) mixture (1.2 mL) were dialyzed at 37°C for 10 hr against the same volume of buffer solution. Adsorptions of drug onto membrane or apparatus were negligible. No protein leakage was detected during the experiment. There was no volume shift during the dialysis experiment. After equilibrium was reached, the free concentration of drug was determined by UV absorption spectroscopy. For the separation of unbound drug species from both native and different modified HSA, ED method as described above, was used with little modification. The samples containing 20 μM solution (1.2 mL) of protein and drug were dialyzed against equal volume of 0.067 M phosphate buffer, pH 7.4 at 37°C. The quantitative determination of unbound drug was determined by the UV method as described above.

Fluorescence measurements were made on a Jasco FP-770 fluorometer (Tokyo, Japan). The percentage of displacement of probe was determined using the following equation according to the method of Sudlow et al. (9):

$$\frac{F_1 - F_2}{F_1} \times 100 \qquad (1)$$

where, F_1 and F_2 are the fluorescences of probe plus HSA without and with drug, respectively. The fluorescences of the probe plus HSA (1:1, 2 μM) was measured at 25°C before and after the addition of drugs (1.5–8 μM).

UV absorption spectra were recorded with a Shimadzu UV-240 spectrophotometer (Kyoto, Japan). Difference absorption spectra were measured using a pair of 10 mm split-compartment tandem cuvettes within the wavelength range 250–350 nm. The concentrations of both drugs and HSA were 10 μM, and the used concentrations for CTAB, SDS and PLE were 4×10^{-3}, 6×10^{-3}, and 1×10^{-3}M, respectively.

Table II. Physicochemical Properties of Benzothiadiazides

Compound	log PC	pKa_1	pKa_2	Vw(Å^3)
Chlorothiazide	−0.19	6.75	9.45	199
Hydrochlorothiazide	−0.30	8.20	10.30	200
Polythiazide	0.60	9.20	9.90	293
Trichlormethiazide	0.41	6.80	10.00	243
Ethiazide	−0.06	—	9.95	224
Methyclothiazide	0.60	—	9.45	254
Cyclopenthiazide	0.70	8.90	9.75	290

—, could not be determined.

Data analysis. All binding parameters were estimated by fitting the experimental values to the following equation using a non-linear squares computer program (MULTI program) (10)

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{m} \frac{N_i K_i [D_f]}{1 + K_i [D_f]} \qquad (2)$$

where r is the number of moles of bound drug per mole of protein. $[D_b]$ and $[D_f]$ are the bound and unbound drug concentrations, respectively, and $[P_t]$ is the total protein concentration. K_i and N_i are association constant and the number of binding sites for the i th class of binding sites.

The characteristics of binding constants were examined with the use of multiple regression analysis. Where necessary, statistical analyses were performed by the Student *t* test, and differences were considered significant at or below $P < 0.05$.

RESULTS

Binding of Benzothiadiazides to HSA

Scatchard analysis of the ED data showed a non-linear curve, suggesting the presence of at least two classes of sites for the binding of benzothiadiazides to HSA. The best fitting values for the binding parameters obtained by the ED method are shown in Table III.

Identification of Binding Sites

For identification of the location of binding sites of benzothiadiazides on HSA, site marker displacement experiments were carried out using site-selective fluorescent probes. Figure 1 illustrates the displacements of different site-selective probes from their binding sites by chlorothiazide. Table IV summarizes the extent of displacement of the site-selective probes by the benzothiadiazides.

Difference UV Absorption Spectra

The effect of HSA on the UV absorption spectra of benzothiadiazides was examined and compared with the spectra of drugs in the presence of detergents with different properties. The binding of benzothiadiazides to HSA caused a small shift of the absorption spectra toward longer wavelength as compared to those obtained in phosphate buffer (Figure not shown). As shown in Fig. 2, the UV difference spectrum resulting from chlorothiazide binding to HSA is characterized by one positive maximum at 266 nm and a negative maximum at 290 nm. The absorption peak at 335 nm was also characteristic. Although there was a shift of absorption peaks to a shorter wavelength, chlorothiazide showed a similar spectral pattern when bound to CTAB (Fig. 2). How-

Table III. Binding Parameters of the Benzothiadiazides to HSA as Determined by ED at 37°C

Drug	n_1	K_1 ($\times 10^4$ M^{-1})	n_2	K_2 ($\times 10^3$ M^{-1})	Binding %
Chlorothiazide	0.63	5.5	2.4	5.8	88
Hydrochlorothiazide	0.15	0.6	1.3	6.0	78
Polythiazide	0.66	2.6	2.0	7.4	82
Trichlormethiazide	0.27	1.4	2.8	3.9	71
Ethiazide	0.15	3.0	1.4	3.5	76
Methyclothiazide	0.19	2.0	1.5	3.9	56
Cyclopenthiazide	0.28	6.6	—	—	77

—, could not be determined.
For calculated binding percentage the following concentrations were used: drug, 10 μM; HSA, 60 μM.

ever, completely different spectra were produced when chlorothiazide interacted with either PLE or SLS (data not shown).

Effect of pH and Temperature on the Binding of Benzothiadiazides

The effects of pH on the CD ellipticity of the complexes of benzothiadiazides with HSA was studied. As indicated in Fig. 3, there was no noticeable change of the CD spectrum of the chlorothiazide-HSA complex upon increasing the pH from 7 to 8.3. However, there was a significant change on the CD spectrum when pH was raised from 6 to 7 (Fig. 3). Within this pH range, the CD ellipticity was found to increase with the rise of pH without changing the sign of the Cotton effect. Temperature did not have any significant effects on the CD spectrum of the chlorothiazide-HSA complex (data not shown).

Binding of Benzothiadiazides to HSA Derivatives

To investigate which amino acids were involved in the binding of benzothiadiazides to HSA, the binding of benzothiadiazides to chemically modified HSA was carried out by CD and ED methods. Results of chemical modification of HSA are shown in Table V, and the induced CD spectra and free fraction of chlorothiazide interactions with native and modified HSA are shown in Figs. 4 and 5, respectively. Free drug fraction as the result of chlorothiazide binding to Tyr-modified HSA ($p < 0.01$) and Lys modified HSA ($p < 0.05$) was increased significantly over that with native HSA.

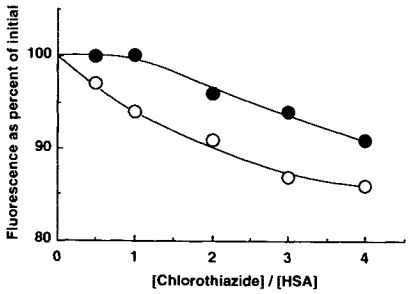


Figure 1. Effects of chlorothiazide on the fluorescence intensity of the site-selective probe-HSA systems at pH 7.4 and 25°C. (-●-), DNAG-HSA system; (-○-), DNSP-HSA system. Following concentrations were used: [HSA], 2 μM; probe, 2 μM.

To elucidate the binding mode of benzothiadiazides to HSA, quantitative relationships between binding parameters of benzothiadiazides and their physicochemical properties were investigated using multiple regression analysis. A limited correlation was obtained between PC values ($R = 0.59$) or pKa_2 values ($R = 0.65$) and binding affinities (nK) at primary binding site of the compounds. pKa_1 values failed to show any quantitative correlation with binding affinities. When PC and pKa_2 values were taken together, they correlated well with binding affinities as determined by multiple regression analysis ($R = 0.97$).

DISCUSSION

Binding of benzothiadiazides to HSA

It is generally considered that HSA binds weakly acidic drugs, while α_1-acid glycoprotein is the main serum protein for the binding of basic drugs (11–12). Serum protein binding of benzothiadiazides was almost exclusively due to their binding to HSA. Scatchard analyses from experiments with benzothiadiazides and HSA gave non-linear curves, suggesting the presence of more than one class of binding sites. As indicated in Table III, the number of primary binding sites was surprisingly low (0.15–0.66). On the other hand, the formation of a 1:1 complex by chlorothiazide with HSA was confirmed by Job's plot (13) (not shown). The reason for this discrepancy is unclear. The construction of Job's plot was

Table IV. Characterization of Benzothiadiazides Binding to Specific Sites on HSA as Determined by Site-Specific Probe Displacement Method

	Displacement of bound probe (%)	
Drug	DNAG	DNSP
Chlorothiazide	8	14
Hydrochlorothiazide	3	15
Polythiazide	11	23
Trichlormethiazide	18	30
Ethiazide	−6	12
Methyclothiazide	4	14
Cyclopenthiazide	1	10

The following concentrations were used: HSA, 2 μM; drug, 8 μM; probe, 2 μM.

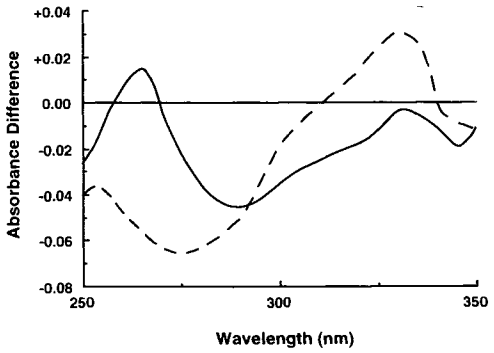


Figure 2. UV absorption difference spectra of chlorothiazide bound to HSA and CTAB detergent at pH 7.4 and 25°C. (——), drug-HSA system; (----), drug-CTAB system. Following concentrations were used: [HSA], 1×10^{-5} M; [chlorothiazide], 1×10^{-5} M; [CTAB], 4×10^{-3} M.

based on a step-wise calculation, whereas the number of binding sites shown in Table III was calculated directly from the Scatchard plot based upon the Langmuir isotherm equation. This difference might cause the difference in the number of binding sites calculated with the two methods. Further, the number of binding sites disagrees with the results reported by Breckenridge and Rosen (3). The source of this discrepancy might arise from the following facts: first, these authors used a different HSA concentration for the determination of binding parameters, and drug-protein binding parameters paradoxically depend on the HSA concentrations used (14,15). Second, the presence of any contaminants (even though in very small quantity) might also result in a different number of binding sites (16). Moreover, we found that the ratio of mercapt albumin and non-mercapt albumin in commercial albumin differs from source to source. CD results indicated that the CD ellipticity of the chlorothiazide-HSA complex was gradually increased with an isosbestic point when the fixed concentration of HSA was titrated with the increment of chlorothiazide (data not shown). This result indicates that chlorothiazide might bind to its high- and low-affinity binding sites on HSA with the same mechanism.

Characterization of Binding Sites

As all the benzothiadiazide compounds studied possess a similar benzothiadiazide ring (Table I), it was assumed that they share the same binding site. Results of fluorescence experiments indicate that the benzodiazepine site or site II is the high affinity binding site for benzothiadiazides, while, the warfarin site or site I is the low affinity binding site on HSA. Although the overall displacement of a site II probe from its binding site on HSA by benzothiadiazides was greater than the displacement of site I probe, the degree of displacement in both cases was small, because of their low HSA binding affinity compared to the probes.

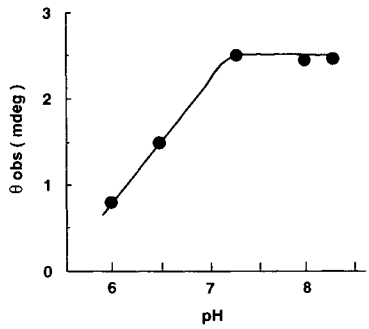


Figure 3. Effect of pH on the CD intensity of chlorothiazide-HSA system at 295 nm and 25°C. Following concentrations were used: [HSA], 20 μM; [chlorothiazide], 20 μM.

Table V. Percentage of Reacted Amino Acid Residues in the Modified HSA

	Reacted amino acid (%)			
Modified HSA	Trp (1)	Tyr (18)	Lys (59)	His (16)
HNBB-treated	93	0.0	2.3	1.5
TNM-treated	0.0	10	0.0	0.0
SA-treated	0.0	3.2	64	0.0
MB-treated	60	6.8	2.0	10

The number in parentheses represents the number of amino acids per HSA molecule.

As suggested by Fehske et al. (17,18), Tyr residues are thought to be involved in the diazepam binding site on HSA, and the lone Trp residue is part of the warfarin site, site I. Therefore, in addition to native HSA, the binding of benzothiadiazides to different chemically modified HSA was quantitatively studied by CD and ED methods to determine which amino acids were involved in the binding of these compounds to HSA. As indicated in Fig. 4, the CD intensity of chlorothiazide bound to Tyr-modified HSA was strongly reduced when compared to that obtained with native HSA. The CD intensity of the chlorothiazide-Lys-modified HSA was also reduced compared to native HSA, suggesting that Tyr and Lys residues might be involved in the binding of these compounds to HSA. The binding of benzothiadiazides to either Trp- or His-modified HSA showed no increase of free concentration of these compounds as compared to the binding to native HSA (Fig. 5). In contrast, the remarkable increase of free concentration when binding to Tyr-modified

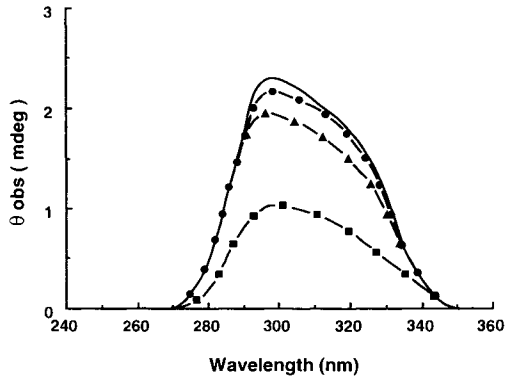


Figure 4. CD spectra of chlorothiazide bound to native and different chemically modified HSA at pH 7.4 and 25°C. (——), chlorothiazide-native HSA; (-●-), chlorothiazide-Trp modified HSA; (-▲-), chlorothiazide-Lys modified HSA; (-■-), chlorothiazide-Tyr modified HSA. Following concentrations were used: [protein], 20 μM; [drug], 20 μM.

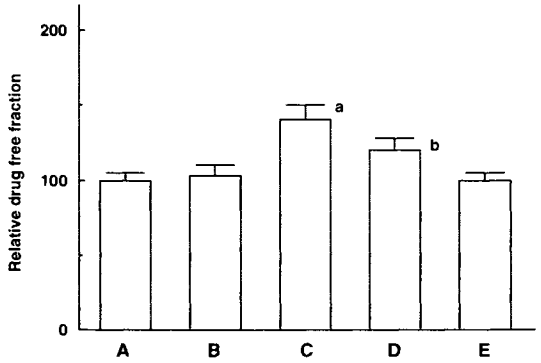


Figure 5. Relative drug free fraction of chlorothiazide to different chemically modified HSA with respect to native HSA taken as 100% at pH 7.4 and 37°C. (A), native HSA; (B), Trp modified HSA; (C), Tyr modified HSA; (D), Lys modified HSA; (E), His modified HSA. Following concentrations were used: [protein], 20 μM; [chlorothiazide], 20 μM. Each column and bar represents the mean ± S.D. of data from three experiments. a, $p < 0.01$ in Tyr modified HSA vs native HSA; b, $p < 0.05$ in Lys modified HSA vs native HSA.

HSA suggests that Tyr residues are involved. Lys residues also contributed to binding as suggested by their different to native and Lys-modified HSA.

Recently the crystallographic structure of HSA was reported by He and Carter (19), where site I and site II are located in subdomains IIA and IIIA, respectively. Based on these results, the primary binding site of benzothiadiazides is thought to be located in subdomain IIIA of the HSA molecule.

Binding Mode

The mechanism of benzothiadiazide binding was studied by different approaches.

1) pH effect. The pH-degree binding profile shows that the amount of benzothiadiazide bound remains constant over the pH range 7.4 to 8.3, which does not encompass the pKa values (6.85 and 8.45) of chlorothiazide. However, when the pH was raised from 6 to 7, the CD intensity almost trebled. The change of pH from 7 to 8 had no significant effect on the association constants of chlorothiazide to HSA, but when pH was raised from 6 to 7 both high and low affinity association constants were significantly affected, indicating that the binding of chlorothiazide to HSA was rather insensitive to N-B transition of HSA. This finding is consistent with the fact that the binding of drugs mainly bound to site II such as chlorothiazide is less sensitive to the N-B transition than that of site I drugs (5). Further, these results indicate that the formation of chlorothiazide anions had definite effects on the binding of chlorothiazide binding to HSA. Electrostatic interaction, thus, probably affect chlorothiazide to HSA. This finding, however, is in contrast to that reported by Breckenridge (3), but the source of discrepancy remains unclear.

2) Physicochemical properties. Some degree of correlation between PC values of pKa_2 values of position 7 of benzothiadiazide molecule and binding affinity (nK) at primary binding site indicate that the change in binding affinities of benzothiadiazides-HSA complexes was likely due not only to hydrophobicity of position 3 but also pKa_2 values of position 7 of these compounds. Indeed, when both pKa_2 and PC values were considered for multiple regression analysis, a significantly improved relation was noticed with 95% confidence level (log nK = 3.60 (±0.43) pKa_2 + 0.698 (±0.34) log PC + 0.020 (±.005), n = 7, R = 0.97). These results indicated that both hydrophobic and electrostatic interactions were important in the binding of the compounds to HSA. Tanford (20) proposed that the binding sites on proteins were hydrophobic patches, which suggests that binding sites have some steric limitations. When V_W was considered, the correlation did not improve, suggesting that steric effect or the size of the molecules was not important in the binding of these compounds to HSA.

3) UV difference spectra. To clarify the mechanism of binding, the UV difference spectra of chlorothiazide with different detergents of various chemical nature were taken, as detergent micelles have been reported to be used as a simple model for binding site on a protein and membrane (21,22). The UV difference spectrum of chlorothiazide with CTAB, a cationic detergent, produced a spectrum similar to that produced with HSA. However, the peak, trough and shoulder produced with CTAB were shifted slightly toward shorter wavelengths. The same measurements were performed in the presence of non-ionic and anionic detergent micelles. With all other detergents, chlorothiazide produced completely different UV difference spectra. As in the case of chlorothiazide, only CTAB among all the detergents caused an effect similar to that of HSA, the HSA binding site of chlorothiazide may consist of a hydrophobic patch to accommodate the heterocyclic ring and a cationic center at or near the chlorothiazide binding site.

In conclusion, the benzothiadiazides bind with high affinity to site II on HSA. The underlying mechanism of benzothiadiazides binding for both high and low affinity binding sites is probably the same. Both hydrophobic and electrostatic interactions are the driving forces for the binding of benzothiadiazides to HSA. It is likely that Tyr and Lys residues are involved in the binding of benzothiadiazides to HSA.

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