Characterization of Microenvironments of 2-(4′-Hydroxyphenylazo)benzoic Acid Bound to Bovine Serum Albumin by Studying the Solvent Effects

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Abstract □ The binding and thermodynamic parameters of the two binding forms of 2-(4′-hydroxyphenylazo)benzoic acid to bovine serum albumin were estimated. The number of the binding sites of the azo form was 3.2 and that of the hydrazone form was 0.7. The binding of the hydrazone form was affected only by enthalpy changes, in contrast to that of the azo form which was affected by both enthalpy and entropy changes. To estimate the complex microenvironments of 2-(4′-hydroxyphenylazo)benzoic acid molecules on bovine serum albumin, the solvent effects were studied. The 2-carboxyl group of 2-(4′-hydroxyphenylazo)benzoic acid participates in the azo–hydrazone tautomerism. The 2-carboxylate ion forms an ion pair with triethylamine in ethylene dichloride, chloroform, and benzene, resulting in the appearance of the hydrazone form. The hydrazone formation in the system of 2-(4′-hydroxyphenylazo)benzoic acid–triethylamine (1:1) in chloroform was affected only by enthalpy changes, in the same manner as in the system of 2-(4′-hydroxyphenylazo)benzoic acid–bovine serum albumin. We speculate the presence of the two kinds of ion pairs on the basis of the changes of the azo–hydrazone tautomerism in chloroform, and that the azo form takes the contact ion pair and the hydrazone form takes the solvent-separated ion pair. A new possible model for the interaction of the azo and hydrazone forms and bovine serum albumin is proposed.

Keyphrases □ Microenvironments—2-(4′-hydroxyphenylazo)benzoic acid bound to bovine serum albumin, solvent effects, characterization □ 2-(4′-Hydroxyphenylazo)benzoic acid—binding to bovine serum albumin, solvent effects, characterization of microenvironments □ Protein binding—microenvironments of 2-(4′-hydroxyphenylazo)benzoic acid bound to bovine serum albumin, solvent effects

In a previous paper (1), it was shown that 2-(4′-hydroxyphenylazo)benzoic acid binds to two different classes of binding sites on bovine serum albumin. This binding can be distinguished spectrally since it involves the preferential binding of either the azo or hydrazone forms of this molecule to these sites. 2-(4′-Hydroxyphenylazo)benzoic acid has therefore become a useful probe when studying drug affinities and competitive binding of drugs at the two classes of binding sites (2).

To investigate the circumstances of the two classes of binding sites, the thermodynamic parameters of the azo and the hydrazone forms bound to bovine serum albumin in pH 7.40 phosphate buffer were sought. It was found that the binding of the hydrazone form was affected by the contribution of enthalpy changes only ($\Delta H°$), in contrast with that of the azo form which was affected by both enthalpy ($\Delta H°$) and entropy ($\Delta S°$) changes.

We attempted to simulate the situation leading to the hydrazone stabilization in the binding sites by measuring the changes of the azo–hydrazone tautomerism in various solvents with different dielectric constants. Baxter (3) observed the spectral change of 2-(4′-hydroxyphenylazo)benzoic acid in some organic solvents containing small amounts of the aqueous solution or in the cationic detergent. His observation has been taken to suggest the importance of the hydrophobic environments in development of the hydrazone form. However, this interpretation obscures the explanation of the thermodynamic parameters mentioned above. This paper reveals the participation of the 2-carboxyl group of 2-(4′-hydroxyphenylazo)-benzoic acid, rather than of the dielectric constant of the solvent, to the azo–hydrazone tautomerism and discusses a new possible model for the interaction of the azo and hydrazone forms in two classes of binding sites on bovine serum albumin.

EXPERIMENTAL

Materials—Bovine serum albumin (fraction V[1]) was used without further purification. 2-(4′-Hydroxyphenylazo)benzoic acid was recrystallized. Methanol, ethanol, and isopropyl alcohol were dried with molecular sieves and distilled. *tert*-Butyl alcohol, benzene, chloroform, and ethylene dichloride were refluxed over calcium hydride and distilled. Dimethyl sulfoxide and dimethylformamide were dried with molecular sieves, followed by distillation under nitrogen and reduced pressure. *n*-Butyl alcohol and isobutyl alcohol were redistilled. Triethylamine dried with calcium hydride was distilled under nitrogen and reduced pressure.

Apparatus—The visible[2], UV[2], and IR spectra[3] were obtained by spectrophotometers. Conductivity measurements[4] were made at 25°C (cell constant, 1 mho/cm).

Binding Constants of the Azo and Hydrazone Forms—Five milliliters of the solution containing 2-(4′-hydroxyphenylazo)benzoic acid (0.2×10^{-4} M–4.0×10^{-4} M) and 0.3% bovine serum albumin was dialyzed against 15 mL of the buffer solution, (0.067 M phosphate buffer, pH 7.40) for 20 h at 20°C or 37°C. After equilibration, the concentration of the total bound 2-(4′-hydroxyphenylazo)benzoic acid was determined by analyzing the free concentration in 15 mL of buffer solution. The analysis of the concentration of the azo and hydrazone forms has been described previously (1). The binding of the azo form, the hydrazone form, and total 2-(4′-hydroxyphenylazo)-benzoic acid was drawn in Scatchard plots; the binding constants were obtained from the slopes of these plots.

Equilibrium Constants of Azo–Hydrazone Tautomerism—2-(4′-Hydroxyphenylazo)benzoic acid and triethylamine were mixed at the same concentration (0.5×10^{-4} M) in the solvent, and the absorption spectra were measured at 0°C, 10°C, 20°C, and 30°C. The equilibrium constant (K) of the azo–hydrazone tautomerism is obtained by the concentration ratio (4):

$$K = \frac{(\text{hydrazone})}{(\text{azo})} = \frac{A_h/E_h}{A_a/E_a} = \frac{A_h}{A_a} \cdot \frac{E_a}{E_h} \qquad \text{(Eq. 1)}$$

where A_h, A_a, E_h, and E_a are the absorbances of the hydrazone form at 480 nm, that of the azo form at 350 nm, the molar extinction coefficient of the hydrazone form, and that of the azo form, respectively. The decrease of the concentration of the azo form, $\Delta A_a/E_a$, equals the increase of that of the hydrazone form, $\Delta A_h/E_h$. Therefore:

$$\Delta A_a/E_a = \Delta A_h/E_h \qquad \text{(Eq. 2)}$$

$$E_a/E_h = \Delta A_a/\Delta A_h \qquad \text{(Eq. 3)}$$

As E_a/E_h was 0.5 by Eq. 3 in this experiment, K was obtained by substituting this value and A_h/A_a at each temperature into Eq. 1.

pK_a of the 2-Carboxyl Group by Conductivity Measurements—The conductivity water was obtained by passing water through ion-exchange resin, followed by distillation (relative conductivity $< 1 \times 10^{-6}$ mho/cm). As the first step, 1×10^{-4} M 2-(4′-hydroxyphenylazo)benzoic acid solution was prepared under nitrogen, and the equivalent conductances ($\Lambda_{I(C)}$) at the acid

[1] Armour Co.
[2] Hitachi 557 spectrophotometer.
[3] JASCO IRA-1 spectrophotometer.
[4] Model CA-2A; Toa Denpa Kogyo Co. Ltd.

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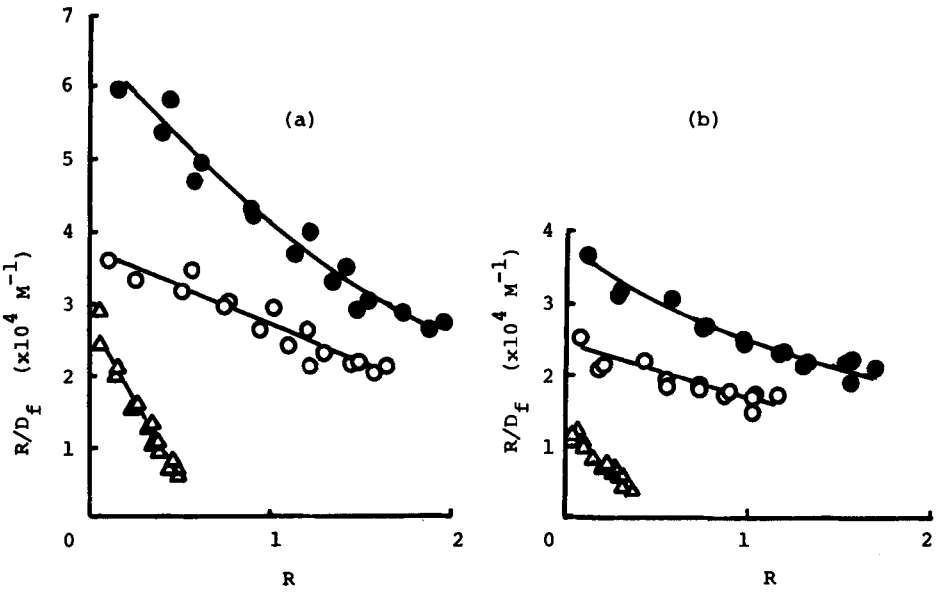


Figure 1—*Scatchard plots of the binding of the azo form (○), hydrazone form (△), and total (●) 2-(4′-hydroxyphenylazo)benzoic acid to 0.3% bovine serum albumin at 20°C (a) and 37°C (b). R represents the molar ratio of the bound form to bovine serum albumin; D_f is the concentration of the free form.*

concentrations (C) of 0.9×10^{-4}, 1.05×10^{-4}, 1.2×10^{-4}, 1.35×10^{-4}, and 1.5×10^{-4} M were measured twice. As the second step, the limiting conductance of 2-(4′-hydroxyphenylazo)benzoic acid ($\mathring{\Lambda}_I$) was obtained as follows. A solution of the sodium salt of 2-(4′-hydroxyphenylazo)benzoic acid (5×10^{-3} M) was prepared by dissolving the acid in 0.005 M NaOH ($f = 0.98$), and the equivalent conductances of the sodium salt of 2-(4′-hydroxyphenylazo)benzoic acid ($\Lambda_{I\text{-}Na}$) were measured twice for each concentration using the diluted solution (0.2×10^{-3}–5×10^{-3} M). The limiting conductance of the sodium salt of the acid ($\mathring{\Lambda}_{I\text{-}NA}$) was graphically estimated by plotting $\Lambda_{I\text{-}Na}$ against the square root of the concentration of the sodium salt of 2-(4′-hydroxyphenylazo)benzoic acid and was found to be 70.3. $\mathring{\Lambda}_I$ was then calculated as 370.3, taking the limiting conductance of Na^+ and that of H^+ as 50 and 350, respectively (5).

The degree of ionization (α) is equal to $(\Lambda_{I(C)})/(\mathring{\Lambda}_I)$, and the ionization constant (K_a) can be obtained by the following Ostwald's equation, taking C as the concentration of 2-(4′-hydroxyphenylazo)benzoic acid:

$$K_a = \frac{\alpha^2 \cdot C}{1 - \alpha}$$

The pK_a value of the 2-carboxyl group of 2-(4′-hydroxyphenylazo)benzoic acid was estimated to be 3.66 at 25°C by averaging the values obtained from the aforementioned five concentrations.

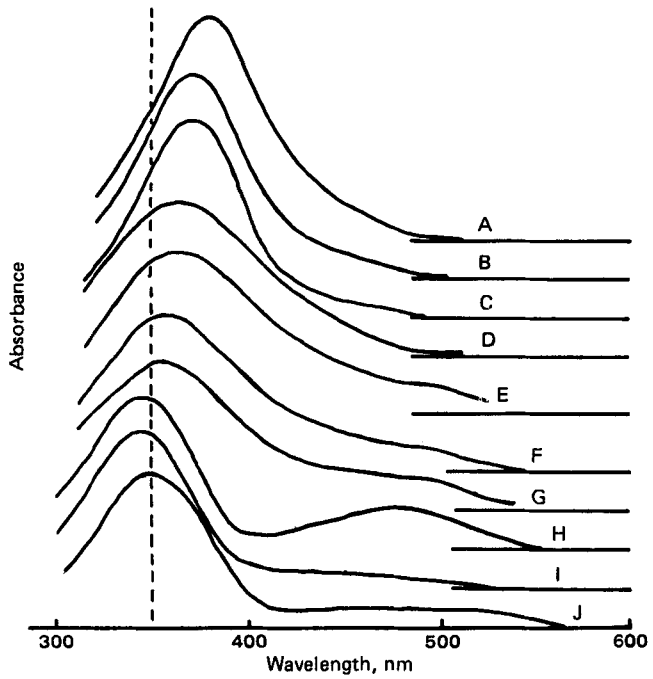


Figure 2—*Absorption spectra of 2-(4′-hydroxyphenylazo)benzoic acid (0.5×10^{-4} M) in various solvents. The ordinate is arbitrary; the broken line represents 350 nm; the solid lines to the right of the diagram are the baselines for each solvent. Key for solvents (dielectric constant): (A) chloroform (4.8 at 20°C); (B) ethylene dichloride (9.1 at 20°C); (C) benzene (2.3 at 20°C); (D) tert-butyl alcohol (10.9 at 30°C); (E) n-butyl alcohol (17.1 at 25°C); (F) isopropyl alcohol (18.3 at 25°C); (G) isobutyl alcohol (17.7 at 25°C); (H) ethanol (24.3 at 25°C); (I) methanol (32.6 at 25°C); (J) dimethyl sulfoxide (48.9 at 20°C).*

RESULTS AND DISCUSSION

Thermodynamics of the Azo and Hydrazone Forms—Figure 1 shows the Scatchard plots of the binding of the azo form, the hydrazone form, and total 2-(4′-hydroxyphenylazo)benzoic acid with bovine serum albumin at 20°C and 37°C. In this concentration range, the plots of both the azo and hydrazone forms showed straight lines and were extrapolated to 3.2 and 0.7 on the x-axis, respectively, which corresponds to the number of the binding sites. On the other hand, the binding of total 2-(4′-hydroxyphenylazo)benzoic acid showed a curvature; this binding curve satisfactorily corresponded to the line obtained by the graphical summation of the plots of the azo and hydrazone forms according to the method described by Rosenthal (6).

The thermodynamic parameters are shown in Table I. The binding of both forms shows an exothermic reaction, as has been reported for many protein bindings. However, the negative value of free energy changes ($\Delta G°$) of the hydrazone form was derived from the contribution of $\Delta H°$ only, in contrast with the concentration of both $\Delta H°$ and $\Delta S°$ in that of the azo form. Therefore, the binding of the hydrazone form is entropically unstable, but energetically stable. On the other hand, the binding of the azo form is stable both entropically and energetically. These facts show that two different microenvironments of 2-(4′-hydroxyphenylazo)benzoic acid molecules produced two binding forms, the azo and the hydrazone.

Solvent Effects of the Azo–Hydrazone Tautomerism—To consider the situation leading to the hydrazone stabilization, the solvent effects of the azo–hydrazone tautomerism were investigated. Figure 2 shows the absorption spectra of 2-(4′-hydroxyphenylazo)benzoic acid in various solvents. In

Table I—Thermodynamic Parameters for Azo and Hydrazone Forms Bound to Bovine Serum Albumin at pH 7.40

Form	Temp.	log K^a	$\Delta G°$, kcal/M	$\Delta H°$, kcal/M	$\Delta S°$, eu
Azo	20°C	4.01	−5.37	−3.02	7.88
	37°C	3.87	−5.49	−3.02	7.96
Hydrazone	20°C	4.47	−6.30	−7.99	−5.97
	37°C	4.11	−5.87	−7.99	−6.97

[a] Binding constant.

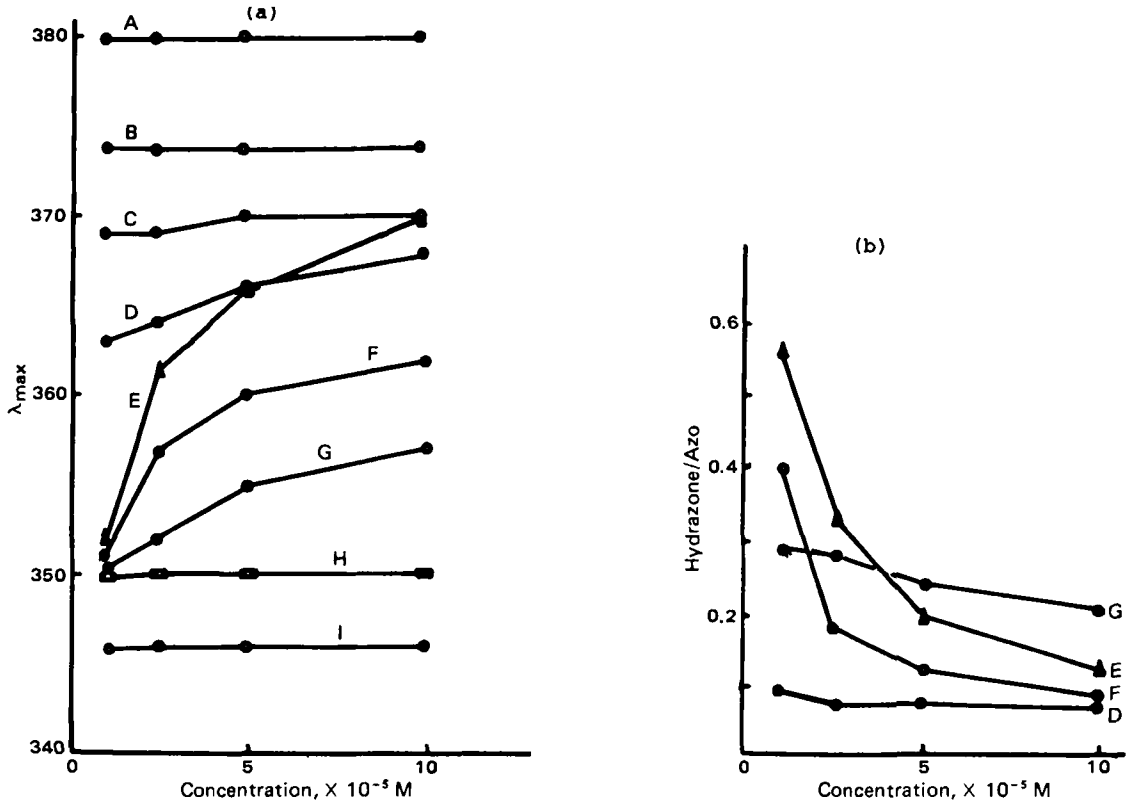


Figure 3—*Concentration dependence of the absorption spectra of 2-(4′-hydroxyphenylazo)benzoic acid utilizing λ_{max} (a) and the expedient concentration ratio of hydrazone to azo forms (b). Key: (A) chloroform; (B) ethylene dichloride; (C) benzene; (D)* tert*-butyl alcohol; (E)* n*-butyl alcohol; (F) isopropyl alcohol; (G) isobutyl alcohol; (H) dimethyl sulfoxide; (I) methanol and ethanol.*

methanol, ethanol, isopropyl alcohol, *n*-butyl alcohol, and isobutyl alcohol, λ_{max} was observed at ~350 nm with the shoulder or broad peak at ~500 nm; in *tert*-butyl alcohol λ_{max} was at 365 nm without a shoulder.

According to the calculation by means of Hückel molecular orbital by Moriguchi (7), $\pi \rightarrow \pi^*$ transition of the azo and the hydrazone forms of 4-hydroxyazobenzene shows λ_{max} at 350 and 458 nm, respectively. Terada *et al.* (8) reported that the absorption spectra with λ_{max} at ~350 nm and at ~480 nm in the aqueous solutions of 2-(4′-hydroxyphenylazo)benzoic acid under the different conditions are the azo and hydrazone forms on the basis of the resonance Raman spectra. We considered the λ_{max} values at ~350 nm and the broad peak at ~500 nm observed in the solvents as the azo and hydrazone forms, respectively.

In the solvents with low dielectric constants (ethylene dichloride, chloroform, and benzene), λ_{max} was observed at 374, 380, and 370 nm, shifting to a longer wavelength than in alcoholic solvents with higher dielectric constants. As a general rule, the absorption spectrum due to the $\pi \rightarrow \pi^*$ transition shifts to the shorter wavelength in the solvent with the lower polarity. To determine which of the absorption spectra in the solvents with low dielectric constants are assigned to the azo and hydrazone forms respectively, the IR spectra were measured. In the IR spectra of 2-(4′-hydroxyphenylazo)benzoic acid in eth-

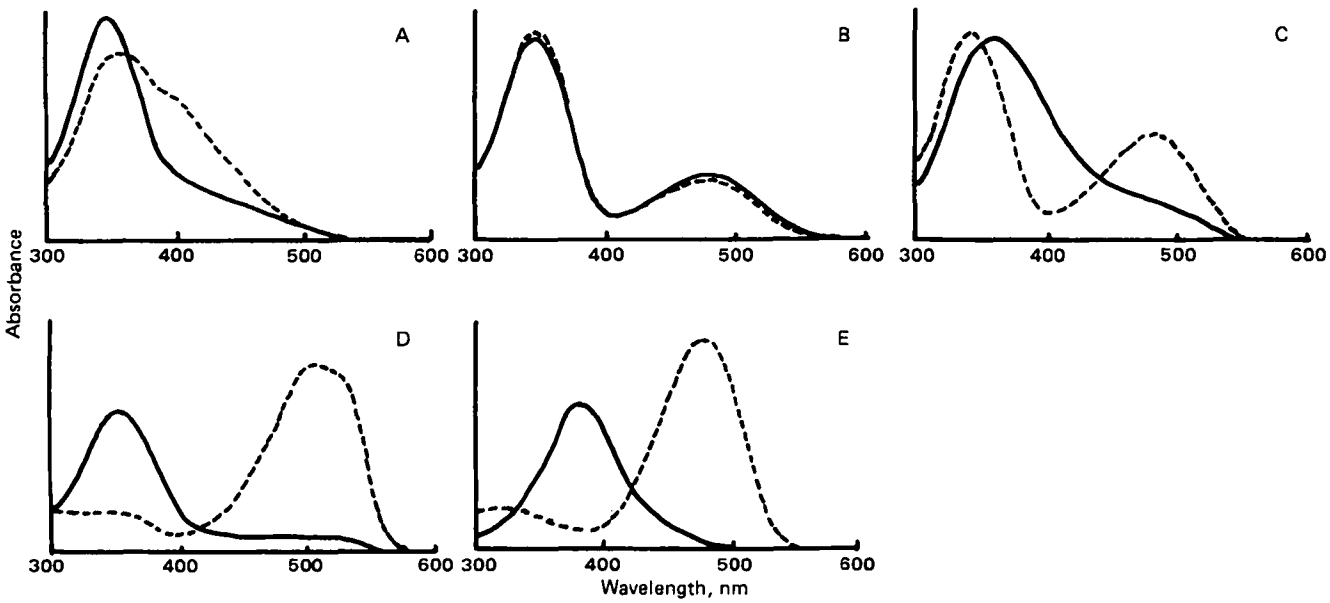


Figure 4—*Absorption spectra of 2-(4′-hydroxyphenylazo)benzoic acid in solvents with (----) or without (—) 0.1 M triethylamine. Key: (A) methanol; (B) ethanol; (C) isopropyl alcohol; (similar change observed with* n*-butyl and isobutyl alcohols); (D) dimethylformamide (similar change observed with dimethyl sulfoxide); (E) chloroform (similar change observed with ethylene dichloride and benzene).*

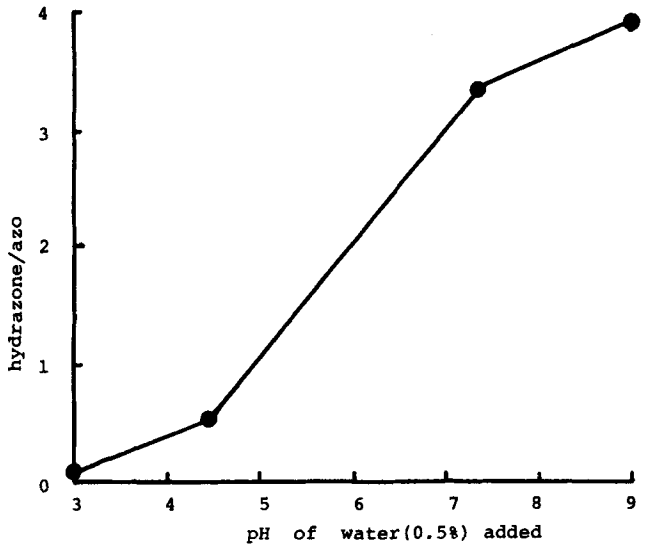
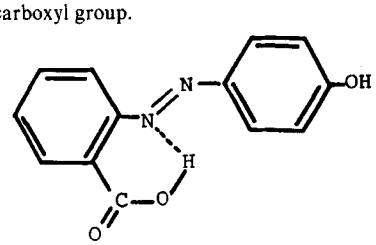


Figure 5—*Effects of the pH of water added to dimethylformamide on hydrazone formation. The ordinate was obtained as the ratio of the absorbance at 510 nm to that at 350 nm.*

ylene dichloride and chloroform, there was an absorption maximum at 3670 cm^{-1} due to ν_{OH} of the 4′-hydroxy group monomer, but no appearance of a peak at ~1660 cm^{-1} due to $\nu_{C=O}$ of the conjugated ketone group. Therefore, 2-(4′-hydroxyphenylazo)benzoic acid takes the azo form in ethylene dichloride and chloroform. As the solvent effects of the absorption spectra of 4-hydroxyazobenzene obeyed the ordinary rule in this experiment, it seems that the 2-carboxyl group in the 2-(4′-hydroxyphenylazo)benzoic acid molecule takes part in the unusual spectral shift to the longer wavelength in the solvents with lower dielectric constants.

So, to investigate the intra- or intermolecular interaction, the effects of the concentration of 2-(4′-hydroxyphenylazo)benzoic acid on the absorption spectra were examined. As shown in Fig. 3a λ_{max} shifted to the longer wavelength with the increase of the concentrations of 2-(4′-hydroxyphenylazo)-benzoic acid in isobutyl, isopropyl, *n*-butyl, and *tert*-butyl alcohols, indicating the presence of the intermolecular interaction (9), while no shift was observed in chloroform, ethylene dichloride, benzene, dimethyl sulfoxide, ethanol, and methanol.

It seems that dimethyl sulfoxide, ethanol, and methanol with relatively high dielectric constants can solvate the hydroxy moiety of the 2-carboxyl group easily. On the other hand, chloroform, ethylene dichloride, and benzene could not solvate the hydroxy moiety of the 2-carboxyl group easily. Therefore, it is suggested that in these solvents the intramolecular interaction stabilizes the azo form (I). Figure 3b shows the concentration dependence of the ratio of the maximum absorbance at ~500 nm to that at ~350 nm, which expediently corresponds to the concentration ratio of the hydrazone form to the azo form. The increase of the intermolecular interaction in isobutyl, isopropyl, *n*-butyl, and *tert*-butyl alcohols with the increase of the concentration of 2-(4′-hydroxyphenylazo)benzoic acid resulted in the decrease in the hydrazone form. This may indicate the stabilization of the azo form due to the dimerization of the 2-carboxyl group.



I

Solvent Effects on the 2-Carboxylate Ion—As mentioned above, the formation of the hydrazone form in various solvents cannot be estimated only from the dielectric constants of the solvents, but depends on their solvation to 2-(4′-hydroxyphenylazo)benzoic acid molecules and the intra- or intermolecular interaction of the 2-carboxyl group.

As the 2-carboxyl group dissociates in the pH 7.40 phosphate buffer used in the study of the binding of 2-(4′-hydroxyphenylazo)benzoic acid with bovine serum albumin, the participation of the 2-carboxylate ion in the azo–hydrazone tautomerism in various solvents was investigated. Figure 4 shows the absorption spectra at the addition of 0.1 M triethylamine to 2-(4′-hydroxyphenylazo)benzoic acid in some solvents. In the IR spectra, the addition of triethylamine to 2-(4′-hydroxyphenylazo)benzoic acid in all the solvents caused the disappearance of the peak at ~1700 cm^{-1} due to the carboxyl group and the appearance of the peak at ~1600 cm^{-1} due to the carboxylate ion, indicating the dissociation of the 2-carboxyl group. The absorption spectra in methanol showed the spectrum similar to that in the alkaline aqueous solution assigned to the azo form (10), and in ethanol the spectral change was not observed. In dimethylformamide, with a high dielectric constant, the hydrazone form predominated. On the other hand, in chloroform, with a low dielectric constant, the same change was observed as in dimethylformamide. However, the addition of 0.1 M triethylamine to 4-hydroxyazobenzene did not cause the spectral change in any solvent.

Table II—Direct Conductivity Measurements of 2-(4′-Hydroxyphenylazo)-benzoic Acid–Triethylamine (1×10^{-3}M:0.1 M) at 25°C

Solvent	0.1 M Triethylamine, μmho/cm[a]	2-(4′-Hydroxyphenylazo)benzoic Acid–Triethylamine System, μmho/cm
Dimethyl sulfoxide	1.2[b]	17.6
Methanol	49.7[b]	123.5
Ethanol	8.0[b]	24.7
Isopropyl alcohol	<1	2.3
tert-Butyl alcohol	<1	<1
Ethylene dichloride	<1	<1
Chloroform	<1	<1
Benzene	<1	<1

[a] Triethylamine only was added to the solvent. [b] This indicates the ionization of triethylamine.

Moreover, to estimate the participation of the 2-carboxylate ion in the formation of the hydrazone form, aqueous solutions at four pH values were all added to dimethylformamide at a concentration of 0.5% (Fig. 5). According to the principle of the specific sorting of solvents, a component with a stronger solvating power in the mixtures of solvents selectively solvates the solute. So, in the dimethylformamide–water mixtures, water solvates the 2-carboxyl group. Therefore, the pH of a small quantity of water may significantly affect the ionization of the 2-carboxyl group. With pH >3.66 (the pK_a value obtained by conductivity measurements), the ratio of the hydrazone form increased. These facts show that the ionization of the 2-carboxyl group resulted in the accelerated formation of the hydrazone form.

Next, conductivity measurements were carried out on the system of 1×10^{-3} M 2-(4′-hydroxyphenylazo)benzoic acid–0.1 M triethylamine in some solvents (Table II). In the 2-(4′-hydroxyphenylazo)benzoic acid–triethylamine system, the conductivity was not observed in *tert*-butyl alcohol, ethylene dichloride, chloroform, and benzene. This observation indicates that 2-carboxylate ion exists as an ion pair with a triethylammonium ion exclusively in these solvents. In the other solvents, the free ions and ion pair are in equilibrium. Therefore, the predominant appearance of the hydrazone form in such solvents as ethylene dichloride, chloroform, and benzene after the addition of triethylamine (Fig. 4) is attributed to the formation of ion pairs.

Table III shows the thermodynamic parameters for hydrazone formation in the system of 2-(4′-hydroxyphenylazo)benzoic acid–amines (1:1) in chloroform. When the temperature was lowered, the hydrazone form increased. Only $\Delta H°$ contributed to the hydrazone formation (−4130 cal·M^{-1} in triethylamine and −5880 cal·M^{-1} in diethylamine), in the same manner as in the system of 2-(4′-hydroxyphenylazo)benzoic acid–bovine serum albumin (Table I). The same result was obtained in ethylene dichloride and benzene. It seems that this change of the azo–hydrazone tautomerism depending on temperature reflects the presence of two species of ion pairs. One ion pair takes the azo form and the other the hydrazone form. Hogen-Esch and Smid (11) presented evidence for two kinds of ion pairs for 9-fluorenyl-alkali metal, *viz.*, the contact ion pair and the solvent-separated ion pair, which were distin-

Table III—Thermodynamic Parameters for Hydrazone Formation in Chloroform

Amine	Temp., °C	K^a	$\Delta G°$, cal/M	$\Delta H°$, cal/M	$\Delta S°$, eu
Triethylamine	0	0.356	560	−4130	−17.2
	10	0.270	737	−4130	−17.2
	20	0.232	852	−4130	−17.0
	30	0.164	1087	−4130	−17.2
Diethylamine	0	0.197	883	−5880	−24.8
	10	0.135	1123	−5880	−24.8
	20	0.102	1326	−5880	−24.6
	30	0.078	1530	−5880	−24.5

[a] Equilibrium constants of azo–hydrazone tautomerism.

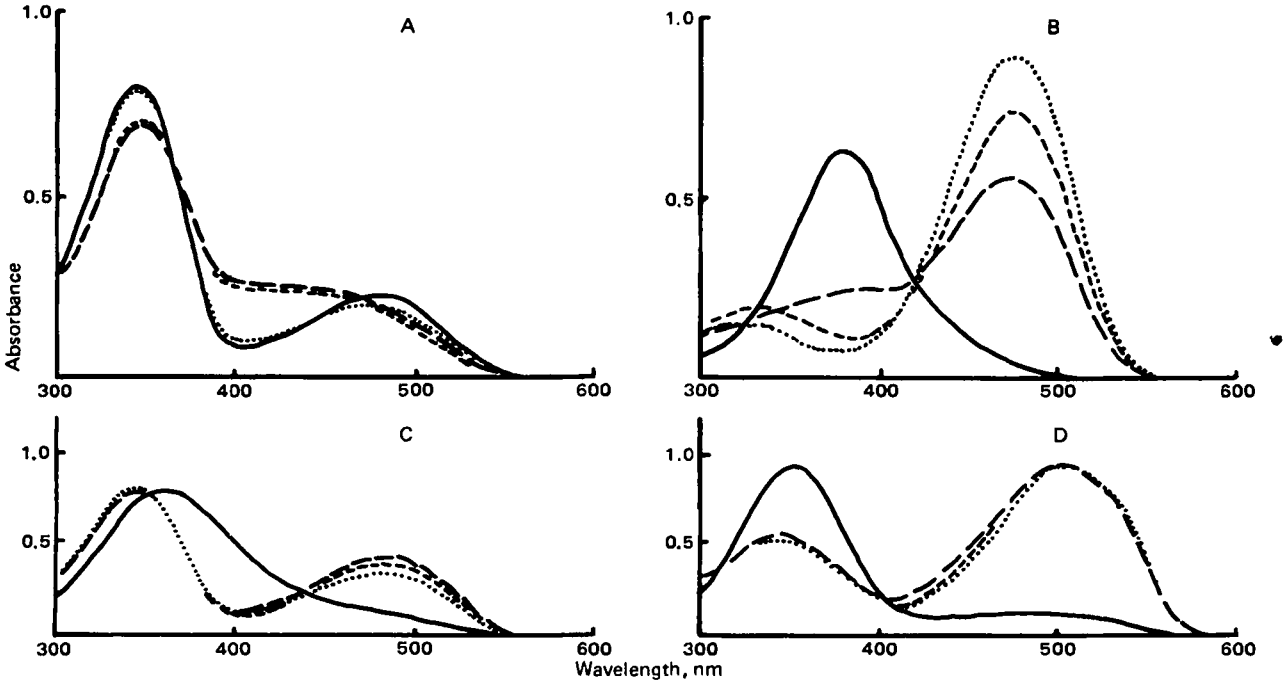


Figure 6—*Effects of* n*-butylamine (—), diethylamine (---), piperidine (---), and triethylamine (·····) on the absorption spectra of 2-(4′-hydroxyphenylazo)benzoic acid (0.5 × 10⁻⁴ M). Piperidine induced the same change as diethylamine. The solid line represents no addition of amines. Key: (A) ethanol; (B) chloroform; (C) isopropyl alcohol; (D) dimethyl sulfoxide.*

guishable by the absorption spectra. The relative amounts of these two kinds of ion pairs were found to be the sensitive functions for the solvating power, temperature of the medium, and for the types of anion and cation. In the same solvent, an ion pair of any ion with a counterion with a larger molecular volume is likely to take the form of the solvent-separated ion pair (12). According to Grunwald's description (13), the distance between a cation and an anion is ~3 Å for a contact ion pair and ~6 Å for a solvent-separated ion pair.

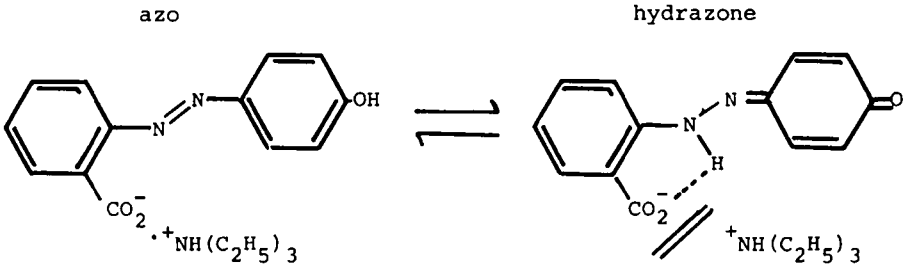
Investigation was carried out on the effects of some amines, which dissociated the 2-carboxyl group of 2-(4′-hydroxyphenylazo)benzoic acid in methanol, ethanol, isopropyl alcohol, dimethyl sulfoxide, benzene, ethylene dichloride, and chloroform, but are considered to have different steric hindrances in forming ion pairs. Figure 6 shows the absorption spectra of 2-(4′-hydroxyphenylazo)benzoic acid with four amines. Among the solvents investigated, triethylamine, with a larger molecular volume, induced the hydrazone formation more strongly than the other amines only in ethylene dichloride, chloroform, and benzene.

Solvent-separated ion pairs become stable when the temperature is lowered due to the large gain of solvation enthalpy (11). Therefore, the hydrazone formation in chloroform (Table III) is attributed to the solvent-separated ion pairs. At the same temperature, as diethylamine more easily introduces the contact ion pairs than triethylamine because of a small steric hindrance, the hydrazone formation decreases (Table III). From these considerations, the origin of the hydrazone formation is speculated to be the formation of the solvent-separated ion pairs, in which the 2-carboxylate ion may take an energetically stable form due to the intramolecular hydrogen bond (Scheme I).

It seems that the accelerated formation of the hydrazone form was not observed (Fig. 4) in methanol and ethanol, because of the easy solvation of 2-carboxylate ion. These facts imply that 2-(4′-hydroxyphenylazo)benzoic acid molecules do not take the hydrazone form in the solvents in which the contact ion pairs can be formed or the free ions can solvate easily.

On the other hand, that the hydrazone form predominated in dimethyl sulfoxide or dimethylformamide (Fig. 4) in spite of the high dielectric constants of these solvents can be interpreted by the solvating power of these solvents. Because these solvents are powerful solvating agents to a cation, but not to an anion (14), no contact ion pairs may be formed. The fact that no difference was observed among the four amines in their effects in dimethyl sulfoxide (Fig. 6) may prove the predominant presence of the solvent-separated ion pairs.

Thomas and Merlin (15) inferred from their resonance Raman spectroscopic study that hydrazone formation on protein binding is stabilized by the intermolecular hydrogen bond between the —NH— group of the hydrazone form and the carboxylate of a suitable amino acid residue in the binding sites. Unlike that of Thomas and Merlin, another possible model can be proposed for the 2-(4′-hydroxyphenylazo)benzoic acid–bovine serum albumin interaction. The 2-carboxyl group of the 2-(4′-hydroxyphenylazo)benzoic acid dissociated at pH 7.40 may be attracted by a long-range electrostatic force due to the arginine or lysine residues in the proposed binding sites (16) of Brown's model (17). At the binding sites of the azo form, 2-(4′-hydroxyphenylazo)benzoic acid may be able to form contact ion pairs with the basic amino acid residues. On the other hand, at the binding sites of the hydrazone form, the microenvironment of 2-(4′-hydroxyphenylazo)benzoic acid molecules may structurally restrict the proximity of the 2-carboxylate ion to the amino acid residues, resulting in no formation of the contact ion pairs. Consequently, the distance between the 2-carboxylate ion and the basic amino acid residue may be similar to that between the two counterions of the solvent-separated ion pair, *viz.*, 6 Å. This speculation may be corroborated by the fact that the binding capacity of bovine serum albumin to the hydrazone



Scheme I

form is smaller than that to the azo form (Fig. 1); *i.e.*, the number of the binding sites of the former is 0.7, although that of the latter is 3.2. Moreover, as was shown previously (18), the circular dichroism spectra of the azo form induced by bovine serum albumin was larger than that of the hydrazone form. Taking into consideration this result and the theory of Takenaka *et al.* (19) that the contact ion pair formation between unchiral benzoic acid derivatives and chiral amines introduces the larger circular dichroism, the azo form may be present as the contact ion pairs in the 2-(4′-hydroxyphenylazo)benzoic acid–bovine serum albumin interaction.

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Polymorphism of Butyrophenones Related to Haloperidol

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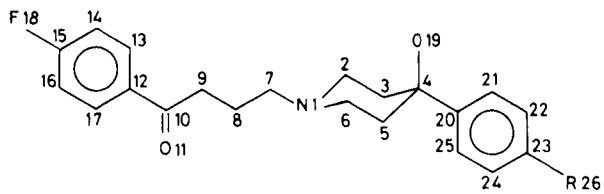
Abstract □ A comparison of X-ray powder diffraction patterns, IR spectra, and crystal structures of structurally related compounds belonging to the butyrophenone family has been undertaken to obtain information about the elements of chemical structure which predispose a substance to exhibit polymorphism. Five butyrophenones, differing by the nature of only one substituent, were selected. After crystallization from 15 solvents, it appears that two compounds of the group exhibit more than one crystalline form. An explanation of the absence of polymorphism in the other compounds of the group is proposed and discussed.

Keyphrases □ Polymorphism—disubstituted butyrophenones, comparison of IR spectra, X-ray diffraction patterns, and crystal structures □ Haloperidol—polymorphism, comparison of IR spectra, X-ray diffraction patterns, and crystal structures □ Moperone—polymorphism, comparison of IR spectra, X-ray diffraction patterns, and crystal structures □ Bromperidol—polymorphism, comparison of IR spectra, X-ray diffraction patterns, and crystal structures

Many papers have been published in the past 10 years about drug polymorphism, generally describing the way of obtaining the polymorphs of a given substance (1–3); their IR spectra (2, 4, 5), thermal behaviors (6–8), and X-ray diffraction patterns (3, 8, 9); their dissolution and solubility profiles (2, 8, 10, 11); and more rarely their *in vivo* rates of release (12–15). Studies of polymorphism are thus often empirical. Few studies attempted to determine what structural characteristics predispose an organic compound to exhibit polymorphism; in a paper concerning the polymorphism of sulfamides, Yang and Guillory (16) investigated that problem.

This work was undertaken to study the polymorphism of closely related compounds belonging to the butyrophenone family; to compare their IR spectra, X-ray diffraction patterns, and crystal structures; and to correlate, if possible, the frequency of polymorphism occurrence and chemical structure. The substances were chosen because of their therapeutic importance, structural simplicity, and crystalline nature (17–21).



HALOPERIDOL: R = Cl
BROMPERIDOL: R = Br
I: R = F
II: R = H
MOPERONE: R = CH_3

EXPERIMENTAL

Materials—Five butyrophenones were selected for study: haloperidol[1], bromperidol[2], 4-[4-hydroxy-4-(4-fluorophenyl)-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone[3] (I), moperone[4], and 4-(hydroxy-4-phenyl-1-piperidinyl)-1-(4-fluorophenyl)-1-butanone[5] (II). The difference between these substances lies only in the nature of the substituent at the 26 position.

[1] Haloperidol: 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone.

[2] Bromperidol: 4-[4-(4-bromophenyl)-4-hydroxy-1-piperidinyl]-1-(-4-fluorophenyl)-1-butanone.

[3] I: 4-[4-(4-fluorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone (no generic name).

[4] Moperone: 4′-fluoro-4(4-hydroxy-4-*p*-tolylpiperidino)butyrophenone.

[5] II: 4-(4-hydroxy-4-phenyl-1-piperidinyl)-1-(4-fluorophenyl)-1-butanone (no generic name).

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