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Spectroscopic Studies on Molecular Interactions. VI.[1] Mechanism of Metachromasy of 2-(4′-Hydroxyphenylazo)benzoic Acid by Serum Albumin

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Mechanism of the albumin-induced metachromasy of 2-(4′-hydroxyphenylazo)benzoic acid (HABA) has been investigated with regard to its interactions with monomeric amino acids and some dipeptides, spectral effect of polarity of the dye environment, and azo-hydrazone tautomerism of HABA. The metachromasy is concerned not with the monomeric amino acids or the primary structure of serum albumin, but with its intramolecular environment of lower polarity corresponding to that of isopropanol. HABA, once bound to serum albumin by electrostatic forces *etc.*, is buried in such an environment of lower polarity in the interior of the protein molecule, and is converted into the hydrazone form which has an absorption maximum at about 480 mμ. Thus, the metachromasy may occur. The azo-hydrazone tautomerism is supported by the infrared spectra, and is compatible with the results of quantum chemical calculations on 4-hydroxyazobenzene.

Serum albumin produces characteristic and distinctive alterations in spectral absorption of anionic dye 2-(4′-hydroxyphenylazo)benzoic acid (HABA),[3] and this metachromasy has been utilized for the quantitative determination of serum albumin[4] and for investigations of its various properties such as interactions with drugs[5] and thermal denaturations.[6] It is well known[7] that acidic compounds are non-specifically bound to serum albumin, chiefly by the electrostatic forces between acidic groups of the compounds and cationic groups of albumin. However, mechanism of the metachromasy of HABA by serum albumin has not yet been revealed in spite of the efforts of some investigators.[8] In the present investigation, the metachromasy is explained in terms of tautomerism of HABA which is concerned with the polarity of the dye environments.

Experimental and Calculation Method

Materials——HABA was of reagent grade for clinical analysis, Daiichi Kagaku Yakuhin Co. Aqueous solutions of mono- and di-sodium salts of HABA (HABA–Na and HABA–2Na, respectively) were prepared by dissolving HABA in distilled water containing calculated amounts of NaOH. Solid powder of HABA–Na, supposed to be hydrazone form, for the infrared (IR) analysis was obtained as precipitate by adding

1) Part V: I. Moriguchi, S. Fushimi, and N. Kaneniwa, *Chem. Pharm. Bull.* (Tokyo), **18**, 1553 (1970).
2) Location: *Hatanodai, Shinagawa-ku, Tokyo, 141, Japan.*
3) F. Karush, through reference 4.
4) D.D. Rutstein, E.F. Ingenito, and W.E. Reynolds, *J. Clin. Invest.*, **33**, 211 (1954).
5) I. Moriguchi, *Chem. Pharm. Bull.* (Tokyo), **16**, 597 (1968); I. Moriguchi, S. Wada, and T. Nishizawa, *ibid.*, **16**, 601 (1968); S. Wada and I. Moriguchi, *ibid.*, **16**, 1440 (1968); S. Wada, S. Tomioka, and I. Moriguchi, *ibid.*, **17**, 320 (1969); S. Wada, K. Kumaki, and S. Tomioka, *Yakuzaigaku*, **29**, 153 (1969); S. Wada, T. Nagai, H. Nogami, and S. Tomioka, *ibid.*, **30**, 26 (1970).
6) H. Terada and S. Watabe, Abstracts of Papers, 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969, p. 645.
7) I.M. Klotz, "The Proteins," Vol. I, Part B, ed. by H. Neurath and K. Bailey, Academic Press, New York, 1953, p. 727.
8) *a*) G. Markus and F. Karush, *J. Am. Chem. Soc.*, **80**, 89 (1958); *b*) J.H. Baxter, *Arch. Biochem. Biophys.*, **108**, 375 (1964).

a sufficient amount of acetone to 0.1M HABA–Na aqueous solution. *Anal.* Calcd. for $C_{13}H_9O_3N_2Na$: C, 59.10; H, 3.43; N, 10.60. Found: C, 59.09; H, 3.56; N, 10.32. Bovine serum albumin was Armour Laboratories Co. "Fraction V." For the molecular weight 69000[9] was used. Denatured albumin was prepared as follows: Bovine serum albumin (about 30 mg) was dissolved in 10 ml of 1M NaOH aqueous solution. The solution was kept at 30° for 48 hours, then adjusted to be pH 7.0 with 1M HCl, and served as a stock solution for spectral measurements. The protein denatured by such a strong alkali did not renature after neutralization.[10] All other reagents and solvents were of analytical grade, and their melting points or boiling points were found to be similar to those listed in chemical handbooks and literatures.

Measurements of Absorption Spectra——The visible and ultraviolet spectra were measured in 1-cm cells at 30° with a Shimadzu model MPS-50L multipurpose spectrophotometer. A Hitachi model 139 spectrophotometer was also used for measurements of absorbances. Infrared spectra were recorded with a Hitachi model EPI-2 IR spectrophotometer in KBr disks.

Measurement of Refractive Index——Refractive indices of aqueous isopropanol solutions were measured at 37° with an Atago model 302 Abbe-type refractometer.

Calculation Method——Energy coefficients of molecular orbitals and total energy of π-electrons for 4-hydroxyazobenzene and its hydrazone tautomer were calculated by means of the Hückel molecular orbital method with parameters[11] of $h_{\dot{N}}=0.5$, $k_{C=\dot{N}}=1$, $h_{\ddot{N}}=1.5$, $k_{C-N}=0.8$, $h_{\dot{O}}=1$, $k_{C=O}=1$, $h_{\ddot{O}}=2$, and $k_{C-O}=0.8$.

Result and Discussion

Interaction of Amino Acids with HABA

Seeing that bovine serum albumin induced a remarkable increase in the optical absorption of HABA in the vicinity of 480 mμ (Fig. 1), all of the amino acids constituting serum albumin and related dipeptides were first investigated with regard to induction of similar spectral changes of the dye. It was observed that phenylalnine, tryptophan, tyrosine, and dipeptides including any of these three amino acids caused a small increase in the absorption of HABA near 500 mμ. Accordingly, quantitative measurements were made of the interactions of the dye with L-phenylalanine, L-tryptophan, and N-glycyl-L-tyrosine: the last named was used instead of tyrosine on account of its poor solubility.

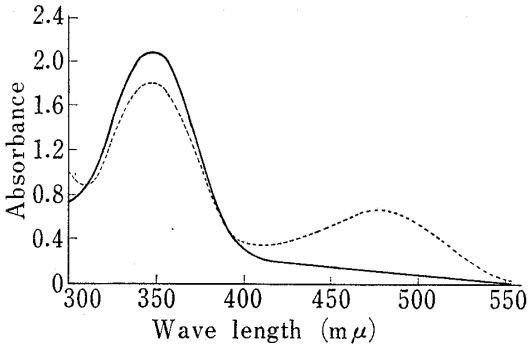


Fig. 1. Albumin-Induced Spectral Change of HABA in 0.1M Phosphate Buffer at pH 6.2

——: 10^{-4}M HABA
------: 10^{-4}M HABA and 10^{-4}M bovine serum albumin

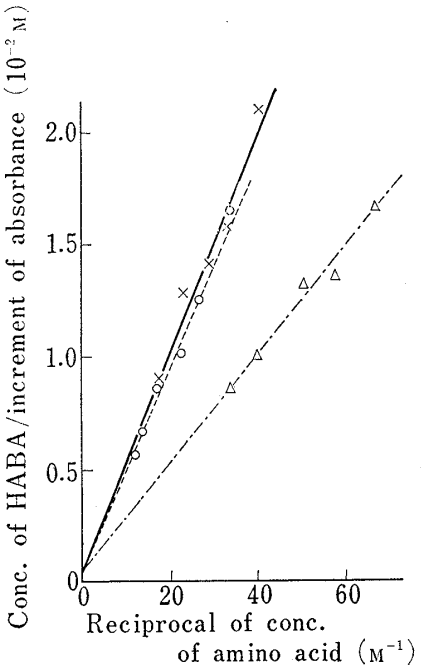


Fig. 2. Benesi–Hildebrand Plots for Complexations of Phenylalanine, Tryptophan, and N-Glycyltyrosine with HABA in 0.1M Phosphate Buffer at pH 7.0 and 30°

—×—: N-glycyl-L-tyrosine at 503 mμ
—○—: L-phenylalanine at 502 mμ
—△—: L-tryptophan at 502 mμ

9) G. Scatchard, A.C. Batchelder, and A. Brown, *J. Am. Chem. Soc.*, **68**, 2320 (1946).
10) J. Segal, K. Dornberger–Schiff, and A. Kalaidjiev, "Globular Protein Molecules," Pergamon Press, Oxford, 1960, p. 112.
11) A. Streitwieser, Jr., "Molecular Orbital Theory for Organic Chemists," John Wiley & Sons, Inc., New York, 1961, p. 135.

Table I. Constants[a] for Complexations of Phenylalanine, Tryptophan, and N-Glycyltyrosine with HABA

Compounds	λ_{max} (mμ)	$\Delta\varepsilon$	Association constant (M^{-1})
L-Phenylalanine	502	2×10^3	1.1
L-Tryptophan	502	2×10^3	2.1
N-Glycyl-L-tyrosine	503	2×10^3	1.0
Bovine serum albumin[b]	482	1.2×10^4	about 2×10^4

a) in 0.1M phosphate buffer at pH 7.0 and 30°
b) in 0.05M phosphate buffer at pH 7.4 (I. Moriguchi, S. Wada, and H. Sano, *Chem. Pharm. Bull.* (Tokyo), **16**, 592 (1968))

Fig. 2 shows the Benesi-Hildebrand plots[12] for the complexations. The difference in molar absorptivity between bound and unbound HABA at 502—503 mμ, $\Delta\varepsilon$, and the 1:1 association constant estimated from the plots are listed in Table I, indicating that the metachromasy of HABA may not be ascribable to its interactions with these monomeric amino acids because their $\Delta\varepsilon$ and association constants are very small in comparison with those for bovine serum albumin. Moreover, the spectrum of alkali-denatured bovine albumin with HABA exhibits substantially no development in the vicinity of 480 mμ (Fig. 3).

These results may indicate that the monomeric amino acids, or the primary structure of serum albumin, is not concerned with the metachromasy of HABA. The higher order structure or the intramolecular environment of the protein seems to contribute to the metachromasy.

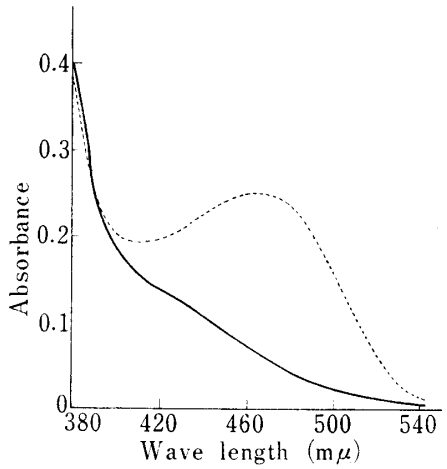


Fig. 3. Influence of Denaturation of Bovine Serum Albumin on Spectral Change of HABA at pH 7.0
——: 1.7×10^{-5}M alkali denatured albumin with 5×10^{-5}M HABA
------: 1.7×10^{-5}M native albumin with 5×10^{-5}M HABA

Influenece of Dye Environment

It has been suggested[13] that, in protein molecules, nonpolar side chains provide hydrophobic environments of lower polarity, which cause small red-shifts of the spectra of phenylalanine, tryptophan, and tyrosine when the amino acids are incorporated into native proteins. For the purpose of estimating the polarity of the intramolecular environment of bovine serum albumin, the influence of isopropanol added on the red-shift, $\Delta\lambda$, for the aqueous solution containing L-tryptophan and N-glycyl-L-tyrosine in a molar ratio of 1:15.5[14] was examined. Phenylalanine was omitted because of its small molar absorptivity,[15] although having optical absorption as well as tryptophan and tyrosine in the vicinity of 280 mμ where bovine serum albumin exhibits its spectral peak.

12) H.A. Benesi and J.H. Hildebrand, *J. Am. Chem. Soc.*, **71**, 2703 (1949); I. Moriguchi and N. Kaneniwa, *Chem. Pharm. Bull.* (Tokyo), **17**, 2173 (1969).
13) S. Yanari and F.A. Bovey, *J. Biol. Chem.*, **235**, 2818 (1960).
14) The ratio in which tryptophan and tyrosine are incorporated in bovine serum albumin (L.F. Fieser and M. Fieser, "Textbook of Organic Chemistry," D.C. Heath and Co., Cambridge, 1950, p. 374).
15) T.W. Goodwin and R.A. Morton, *Biochem. J.*, **40**, 628 (1946); R.M.C. Dawson, D.C. Elliott, W.H. Elliott, and K.M. Jones, "Data for Biochemical Research," Clarendon Press, Oxford, 1969, p. 51.

Table II includes the results as compared with peaks for native and alkali-denatured bovine serum albumin. It can be seen that the larger $\Delta\lambda$ is caused by the increased isopropanol concentration, or the decreased polarity, of the mixed amino acid solution. The data suggest that the intramolecular environment of bovine serum albumin may be of considerably low polarity, resembling with that of almost anhydrous isopropanol.

Table II. Spectral Shifts of Tryptophan–Glycyltyrosine Mixture[a] in Varied Concentrations of Isopropanol at 37°

Conc. of isopropanol (vol %)	Spectral peak position (mμ)
0	275.6
15	275.7
30	276.5
50	277.1
80	278.1
90	278.4
Bovine serum albumin in water	278.9
Alkali-denatured albumin in water	276.0

a) in the molar ratio of 1:15.5

Baxter[8b] has already found that, when HABA is dissolved in aqueous organic solvents together with a buffer of neutral pH or in the form of the sodium salt, the absorption band develops in the region near 500 mμ. Since the spectral development seemed to relate to polarity of the dye environment, the effect of isopropanol concentration on the optical absorption of HABA–Na was investigated. Fig. 4 shows the results, indicating that the spectral absorption increases with an increase in isopropanol concentration, or a decrease in polarity, of HABA–Na solution. Because the increment of the absorbance for 5×10^{-5}M HABA induced by bovine serum albumin at its saturated concentration is estimated to be about 0.6 (480 mμ) according to Fig. 5, it can be said from Fig. 4 that the polarity of the interior environment of albumin molecule may correspond to that of not less than 85% isopropanol; this is compatible with the estimation from $\Delta\lambda$ of the amino acid mixture.

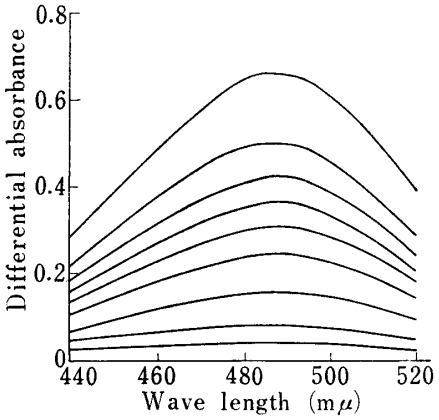


Fig. 4. Spectral Development of 5×10^{-5}M HABA–Na in Varied Concentrations of Isopropanol at 37°

reference: 5×10^{-5}M HABA–Na in distilled water
curves: upwards from the bottom, increasing isopropanol concentrations from 10 to 90%

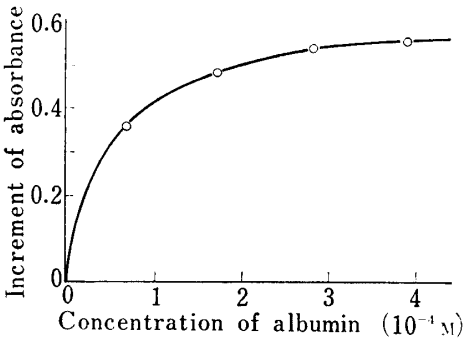


Fig. 5. Spectral Development of 5×10^{-5}M HABA–Na by Increased Concentrations of Bovine Serum Albumin in 0.095M Phosphate Buffer at pH 7.0, 37°, and 480 mμ

The hydrophobicity of the interior environment of protein molecules is expressed in terms of higher refractive index.[16] In this connection, the relations of the refractive index of isopropanol solution to ΔE (isopropanol-induced increment of the absorbance of HABA–Na at 488 mμ) and $\Delta\lambda$ of the amino acid mixture were examined. The relations are exhibited in Fig. 6, where parallel increases in ΔE and $\Delta\lambda$ with the incresased refractive index can be seen. The polarity of the intramolecular environment of bovine serum albumin may be correspond to a refractive index of about 1.37 to 1.38 according to Fig. 6, but the consideration of other factors such as dielectric constant and solvation energy[17] may be also required for the explanation of $\Delta\lambda$ and ΔE.

From these results, it may be concluded that the metachromasy is caused by the environment of lower polarity in the interior of albumin molecule where bound HABA molecule is buried. The structural change of the buried HABA molecule which provides the metachromasy is discussed in the next section with special attention to tautomerism.

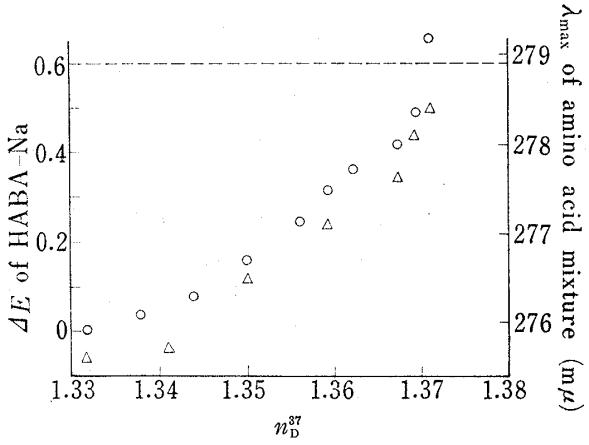
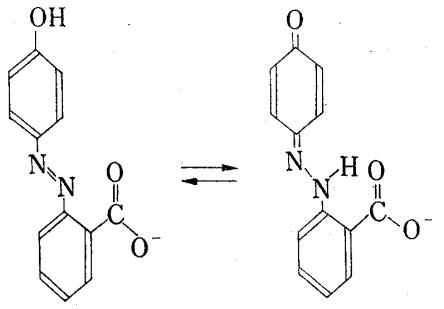


Fig. 6. Relations of Refractive Index of Aqueous Isopropanol Solution to Spectral Development of HABA–Na and Spectral Shift of Amino Acid Mixture[a]

○: spectral development of HABA–Na *vs.* n_D^{37}
△: λ_{max} of amino acid mixture *vs.* n_D^{37}
---: λ_{max} and the increment of absorbance of HABA–Na for bovine albumin in water (see Fig. 5)
a) L-tryptophan and N-glycyl-L-tyrosine in the molar ratio of 1: 15.5



azo form hydrazone form

Chart 1. Tautomerism of HABA–Na

Tautomerism of HABA Molecule

The prevention[8b] of the albumin-induced spectral change of HABA by substituting a $-OCH_3$ for the 4′-OH of the dye suggests that an azo-hydrozone tautomerism as shown in Chart 1 may be concerned with the metachromasy. The tautomerism is also expected from the fact that HABA–2Na does not exhibit any spectral development in aqueous isopropanol solutions. Therefore, the tautomerism, between azo form with an absorption maximum (λ_{max}) near 350 mμ and hydrazone form with that near 480 mμ, was investigated.

By a dding a large amount of acetone to 0.1 M HABA–Na aqueous solution, a reddish brown precipitate was obtained which exhibited a λ_{max} of about 490 mμ[18] immediately after dissolved in acetone and yielded the results of the elementary analysis compatible with HABA–Na. Its infrared spectrum, together with that of free HABA for comparison, is recorded in Fig.

16) A. Imanishi, "Atarashii Seibutsu-Butsurikagaku Kenkyūhō," Vol. 2, ed. by T. Isemura, J. Tanaka, H. Noda, and T. Miyazawa, Kagakudojin, Kyoto, 1967, p. 95.
17) T.T. Herskovitz and M. Laskowski, Jr., *J. Biol. Chem.*, **235**, PC 56 (1960).
18) The peak was disappeared about 20 minutes after dissolution.

7, indicating the presence of new absorption bands probably due to NH streching (3460 cm^{-1}) and CO (1627 cm^{-1}) and the absence of OH streching band (3220 cm^{-1}) in contrast to the spectrum of HABA. This suggests that the acetone precipitate may be the hydrazone form of HABA–Na.

For the further support of the tautomerism, the π-electronic properties of azo and hydrazone forms of 4-hydroxyazobenzene were calculated as a model of HABA–Na by means of the Hückel molecular orbital method. The spectrum of 4-hydroxyazobenzene (azo form)[19] is very similar to that of HABA–Na, both with molar absorptivity of about 2×10^4 at λ_{max} near 350 mμ. The $\pi\rightarrow\pi^*$ transition was assumed for the calculation concerning the spectra. Table III shows the results, which indicate the difference of more than 100 mμ in λ_{max} for the first $\pi\rightarrow\pi^*$ transition between azo and hydrazone forms. This may explain the large increase in λ_{max} in the metachromasy of HABA. It is also indicated that the azo form is a little stable compared with the hydrazone form; this may be compatible with the fact that an absorption development similar to that in the metachromasy occurs with aqueous solution of HABA–Na on heating. Hence some forces stabilizing the hydrazone form such as hydrogen bonding may be present and may become greater with a decrease in polarity in the dye environment, but the details remain uncertain.

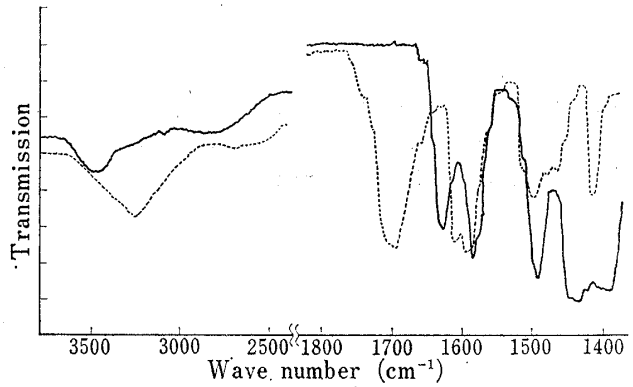


Fig. 7. Infrared Spectra of Acetone Precipitate of HABA–Na and HABA in KBr Disks

——: acetone precipitate of HABA–Na
------: HABA

Table III. π-Electronic Properties of Azo and Hydrazone Forms of 4-Hydroxyazobenzene

	Azo form	Hydrazone form
m_{lv}[a]	$-0.298\ \beta$	$-0.071\ \beta$
m_{ho}[b]	$0.601\ \beta$	$0.617\ \beta$
λ_{max} for the first $\pi\rightarrow\pi^*$ transition	(350 mμ)[c]	458 mμ[d]
Total π-electron energy	$16\alpha+24.124\ \beta$	$16\alpha+23.382\ \beta$

a) energy coefficient of the lowest vacant molecular orbital
b) energy coefficient of the highest occupied molecular orbital
c) observed value (reference 17)
d) calculated as follows: 3.54 eV (=350 mμ)$\times(-0.071-0.617)/(-0.298-0.601)$=2.71 eV (=458 m$\mu$)

The conclusions based on the results given above are that the metachromasy of HABA by serum albumin is ascribable to the azo-hydrazone tautomerism of the dye. HABA–Na, once bound to serum albumin by electrostatic forces *etc.*, is buried in the environment of lower polarity in the interior of the protein molecule, and is converted into the hydrazone form which has a spectral peak position at about 480 mμ. Thus, the metachromasy may occur.

Acknowledgement We are indebted to the members of the Analytical Center of this School for the elementary analysis and the infrared spectra.

19) A. Eucken and K.H. Hellwege, "Landolt–Börnstein Zahlenwerte und Funktionen aus Physik, Chemie, Astronomie, Geophysik, und Technik," 6. Aufl., Band I, Teil 3, Springer–Verlag, Berlin, 1951.