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**ACKNOWLEDGMENTS**

Support was obtained in part from the professorship provided to A. Martin by Coulter R. Sublett.

The authors thank Alan Burbower, Energy Center, University of California at San Diego for advice and assistance.

# Molecular Interaction Between Riboflavin and Salicylic Acid Derivatives in Nonpolar Solvents

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Received November 23, 1981, from the *Department of Physical Pharmacy, College of Pharmacy, Seoul National University, San 56-1, Shinrim-Dong, Kwanak-Ku, Seoul 151, Korea.* Accepted for publication June 2, 1982.

**Abstract** □ The interaction of riboflavin-2′,3′,4′,5′-tetrabutyrate (I) with salicylic acid (II), aspirin (acetylsalicylic acid, III), and salicylamide (IV) has been spectroscopically investigated to determine the binding mechanism. NMR and absorption spectra were measured in nonpolar solvents. The association constant $K$ of the formation of complex was calculated from the absorption spectra. Compounds I and II form a 1:1 cyclic hydrogen-bonded dimer through the N-3 proton and the C-2 carbonyl oxygen of the isoalloxazine ring, and the carboxylic hydroxyl proton and carbonyl oxygen of II. Compounds I and III form a 1:1 cyclic hydrogen-bonded dimer by the same mode. Compound IV forms a 1:1 cyclic hydrogen-bonded dimer with I through the N-3 proton and the C-2 carbonyl oxygen of the isoalloxazine ring, and the amino proton and the carbonyl oxygen of IV. Salicylates produce marked changes in the absorption spectra of I. These spectral changes are attributed to the formation of the hydrogen-bonded dimer. It appeared that the strongest complex was formed with salicylic acid, a weaker one with aspirin, and an even weaker one with salicylamide.

**Keyphrases** □ Riboflavin—molecular interactions with salicylic acid derivatives, NMR and absorption spectroscopy in nonpolar solvents, association constant determinations for hydrogen-bonded dimers □ Salicylates—molecular interactions with riboflavin, NMR and absorption spectroscopy in nonpolar solvents, association constant determinations for hydrogen-bonded dimers

Salicylate, one of the oldest synthetic drugs, remains the most widely used analgesic and antipyretic agent. It is known that the hydrogen bonding of salicylates in biological systems is related to their drug action (1). It has been determined experimentally that higher concentrations of salicylates result in marked stimulation of respiration while low concentrations depress this function (2–7). Salicylic acid derivatives act as uncoupling agents on the isolated mitochondrial respiration and lower the respiration rate of dinitrophenol-uncoupled mitochondria; inhibition of state 3 mitochondrial respiration by salicylic acid derivatives is accompanied by an increase in the oxidized state of all electron transport systems (8).

The electron-transfer from nicotinamide adenine dinucleotide (NADH) to flavoprotein, or the charge-transfer complex thus formed, was studied by a number of authors to give an account of the function of the respiratory chain (9–13). It has been determined that reduced NAD-coupling enzyme[1] complex converts spontaneously to the hypothetical intermediate as oxidized NAD-coupling enzyme[2], which is considered indispensable to the formation of adenosine triphosphate (ATP) in the respiratory chain (14). Simultaneously, the electrons in $(NADox)^{-2}$ can be transferred to flavoprotein. It is generally agreed that salicylates, which act as uncoupling agents, cause the breakdown of some high-energy intermediate involved in the phosphorylation process. However, the mechanism of such a breakdown has not previously been determined. The mechanism of action of salicylate could be described as: (*a*) salicylate associates with the adenine moiety of flavin adenine dinucleotide (FAD) or NAD, (*b*) salicylate inhibits the interaction of the flavin moiety with the adenine moiety of FAD or NAD, (*c*) salicylate affects the electronic environment due to an association with flavin, or (*d*) salicylate inhibits the interactions of FAD and the flavin mononucleotide (FMN) with apoprotein. Therefore, it seems worthwhile to examine the molecular interaction between salicylate and riboflavin. In this paper a detailed analysis of the NMR and absorption spectra of the complex will be presented and a structure of the complex will

[1] Enzyme-coupled reduced nicotinamide adenine dinucleotide.
[2] Enzyme-coupled oxidized nicotinamide adenine dinucleotide.



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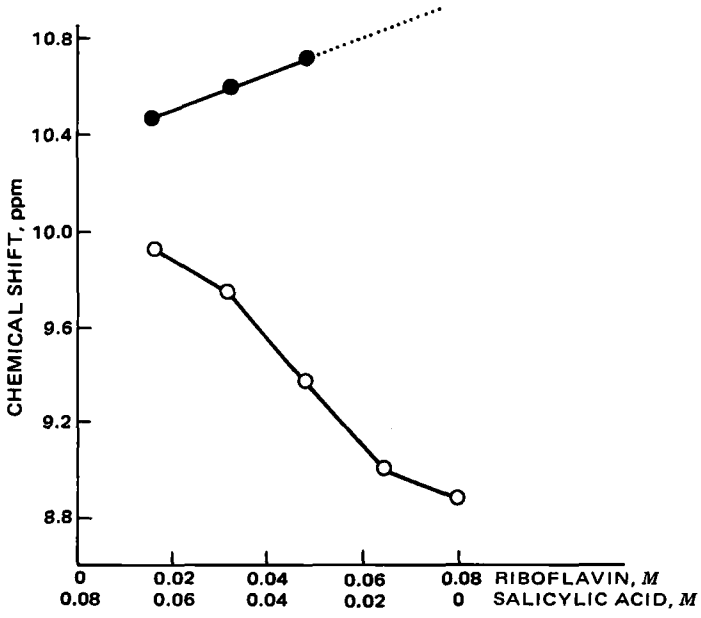



**Figure 1**—*Stoichiometric pairing of I and II: dependence of the chemical shifts of the riboflavin N-3 (○) and salicylic acid carboxyl (●) protons in a mixing experiment of I and II. Total concentration was constant at 0.08* M.

be proposed. These results may provide a basis for interpreting the mode of action of salicylic acid derivatives.

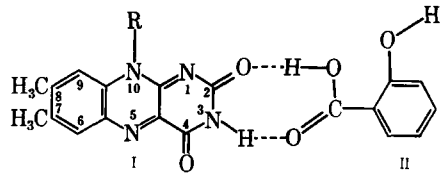
## EXPERIMENTAL

**Materials**—Riboflavin-2′,3′,4′,5′-tetrabutyrate[3] (I) was obtained commercially. It was recrystallized from chloroform and the purity was confirmed by TLC. Salicylic acid[4] (II), aspirin[5] (III), and salicylamide[5] (IV) were used after recrystallization from chloroform. Deuterochloroform was purified by filtration through an alumina gel column, 5 cm in length. Carbon tetrachloride was treated with methanolic potassium hydroxide solution, washed with water several times, dried over calcium chloride overnight, and fractionally distilled from phosphorus pentoxide through a 120-cm column packed with glass helices. The distillate was refluxed and again fractionally distilled. Benzene was treated with concentrated sulfuric acid several times. After repeated washing with potassium hydroxide solution, it was dried over calcium chloride overnight, fractionally distilled from phosphorus pentoxide through a 120-cm column packed with glass helices, and crystallized twice. It was refluxed and again fractionally distilled.

**Methods**—$^1$H-NMR spectra were recorded on a 90-MHz spectrometer equipped with a temperature-control unit[6]. For the measurement of NMR spectra, the samples of I and salicylate at various molar ratios were dissolved in deuterochloroform. Sample volumes of 5 ml in 5-mm diameter tubes with polytef caps were used. Tetramethylsilane (0.3%) was added to the deuterochloroform to provide the field lock signal; chemical shifts were measured from the signals. The probe temperature was 35°. Absorption spectra were measured in a UV visible spectrophotometer[7] connected to a linear recorder[8]. Fused quartz cells of 10-mm light path were used, each of which was fitted with a polytef stopper. The slit width was chosen so that the effective band width of the exit beam was sufficiently narrow compared with the width of absorption bands.

To determine the association constant of complex of the absorption spectra as precisely as possible, ternary solutions which contained small amounts of I and II (or III) in nonpolar solvents, such as carbon tetrachloride and benzene, were used. For each set of I and II (or III), several solutions containing equal quantities of I and different amounts of II (or III) were prepared. The actual concentration of I was of the order of $10^{-5}$ $M$ ($3 \times 10^{-5}$, $5 \times 10^{-5}$, and $8 \times 10^{-5}$ $M$), while that of II (or III) ranged from 0 to $10^{-2}$ $M$ (or $2 \times 10^{-2}$ $M$). The concentration of I was small enough to allow the effect of self-association of I molecules to be ignored. The absorbance changes of I were measured at 436 and 446 nm, the association constants were calculated, and the experimental data were treated statistically.

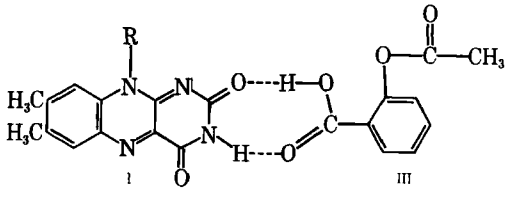
[3] Dae Woong Pharm. Co. Ltd., Seoul, Korea.
[4] E. Merck, Darmstadt, West Germany.
[5] Il Sung Corp., Seoul, Korea.
[6] Perkin-Elmer R 32 NMR Spectrometer.
[7] Unicam SP 1750 Ultraviolet Spectrophotometer.
[8] Unicam AR 25 Linear Recorder.



V

## RESULTS

**NMR Spectra**—The $^1$H-HMR spectra of riboflavin and salicylic acid derivatives in deuterochloroform are relatively simple in the downfield region. In the spectrum of II, the absorption of the carboxyl proton is observed below 10 ppm, the phenol proton is not evident at low concentrations but is weakly observed at high concentrations, and the aromatic protons appear at 6.9–8.1-ppm. The carboxyl proton of III is not evident. In the spectrum of IV, absorption of the amino proton was observed at ~6 ppm and that of the hydroxyl proton below 12 ppm. As the concentration of IV increased, the signal for the hydroxyl proton moved only slightly, but the signal for the amino proton shifted downfield.

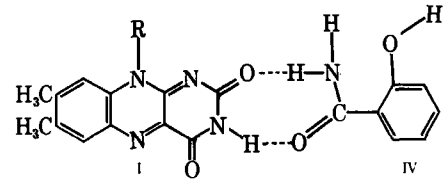


VI

The N-3 proton of I is the most deshielded proton in the molecule and occurs farther downfield than all the other resonances. The C-6 and C-9 protons of I are both downfield from the residual nondeuterated chloroform peak. These two protons are concentration independent. When these protons take part in hydrogen bonding to form cyclic self-association dimers or I–salicylate pairs or triplets, they become less shielded and their resonances shift downfield. The exact positions of the hydroxyl, amino, and imino resonances depend on the degree of association and hydrogen-bond formation; therefore, both vary with temperature and concentration.

To obtain information on the characteristics of complex in deuterochloroform, experiments were performed at constant total concentration. Unlike the results obtained from optical methods, these curves do not show a maximum (or minimum) at the relative concentrations indicative of the stoichiometry of the reaction (15). Figure 1 shows the dependence of the chemical shifts for the N-3 proton of I and the carboxyl proton of II when I was mixed with II. The N-3 proton signal of I shifted downfield as the relative concentration of II increased. The carboxyl proton signal of II also moved downfield as the concentration of I increased. From these observations it can be inferred that the association between I and II is stronger than the self-association of either compound. When I was mixed with III, the dependence of the chemical shifts on the N-3 proton of I was similar to that of II (Fig. 2). In the case of IV, the N-3 proton signal of I changed slightly and the amino proton signal of IV moved downfield slightly as the concentration of I increased and that of IV decreased (Fig. 3). This implies that the association between I and IV is stronger than the self-association of IV and approximately the same as the self-association of I. It may be assumed by the shape of the slope of all three curves that 1:1 complexes are formed in each case (15, 16).

The chemical shifts of the N-3 proton of I were plotted against the



VII

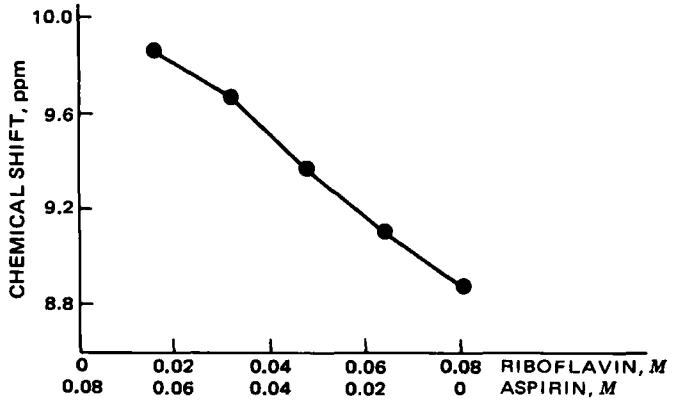



**Figure 2**—*Stoichiometric pairing of I and III: dependence of the chemical shifts of the riboflavin N-3 proton in a mixing experiment of I and III. Total concentration was constant at 0.08* M.

concentration of the three salicylic acid derivatives at 35°, keeping the concentration of I constant at 0.04 *M* (Fig. 4). The slopes of the N-3 proton curves of I for II and III are much greater than that of the chemical shift due to the self-association of I, while that for IV is slightly greater. These data can be interpreted to indicate that I associates somewhat more strongly with II or III than with IV, which is consistent with the other data obtained. The carboxyl proton signal of II was more upfield as the concentration of II was increased while keeping the concentration of I constant (not shown). From this fact alone, it can be inferred that the association between I and II is stronger than the self-association of II and the mole ratio of complex is the 1:1 type.

Figure 5 shows the chemical shift of the carboxyl and phenol protons of II plotted against the concentration of I, while keeping the concentration of II constant at 0.1 *M*. The curve of the carboxyl proton of II is convex upward, while the curve of the phenol proton is concave upward. As the concentration of I increased, the carboxyl proton signal appeared to broaden whereas the phenol proton signal sharpened. The downfield shift and broadening effect of the carboxyl proton are presumably because this proton takes part in the hydrogen bonding to form a I–II complex. The upfield shift and sharpening effect of the phenol proton indicate the possibility of a stacked configuration or two components of the complex not being in one plane. The N-3 proton signal of I was more upfield as the concentration of I was increased while keeping the concentration of II constant (not shown). From this fact, it can be suggested that the association between I and II is stronger than the self-association of I and the mole ratio of the complex is the 1:1 type. A similar phenomenon was observed with III on addition of I while keeping the concentration of III constant at 0.06 *M* (not shown). In the case of IV, the curve of the amino proton is straight upward (Fig. 6). The phenol proton signal decreased in intensity and then disappeared without a shielding effect as the concentration of I was increased (not shown). This implies that only the amino proton takes part in hydrogen bonding to form a I–IV complex.

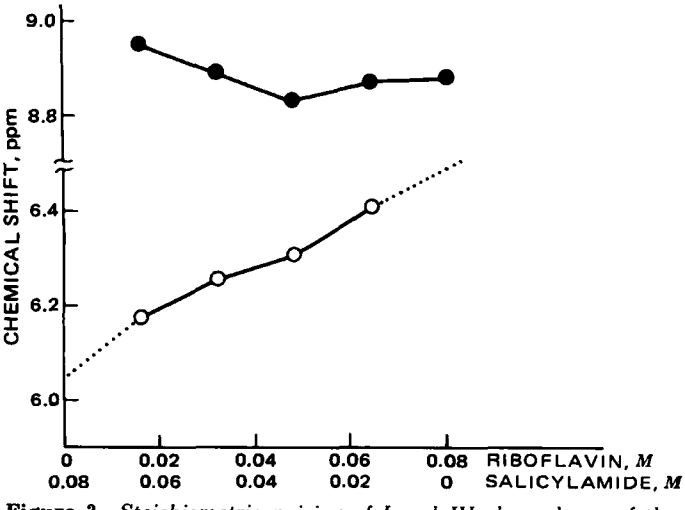



**Figure 3**—*Stoichiometric pairing of I and IV: dependence of the chemical shifts of the riboflavin N-3 (●) and salicylamide amino (○) protons in a mixing experiment of I and IV. Total concentration was constant at 0.08* M.

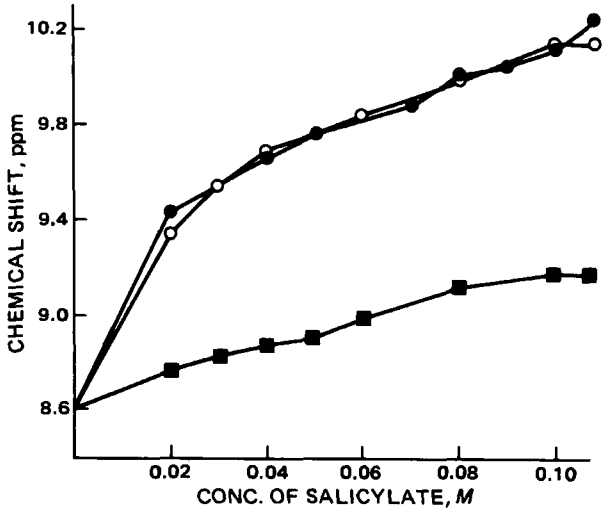



**Figure 4**—*Effects of the concentrations of salicylates on the chemical shifts of the riboflavin N-3 proton in deuterochloroform, keeping the concentration of I constant at 0.04* M. *Key: (●) salicylic acid, (○) aspirin, and (■) salicylamide.*

Besides the observation of complex formation in the downfield shift of the protons directly involved, a secondary effect also may be noted which is due to a change in the electron density at sites where several bonds are altered in the donor or acceptor atoms during hydrogen-bond formation. Small changes may be produced in the ring currents and the electron densities in the acceptor molecules, resulting in shifts in the resonances of the other protons. It is also possible to produce small changes in the ring resonance positions due to neighboring unsaturated ring systems. The resonances of the C-6 and C-9 protons of I are not shielded. But small upfield shifts were observed in all of the ring protons of II and III. In the case of IV, some of the ring protons moved downfield slightly and others shifted upfield.

**Absorption Spectra**—As shown in Fig. 7, a marked spectral change was produced on adding II to I in a nonpolar solvent, carbon tetrachloride. It may be assumed that this spectral change is due solely to the formation of a hydrogen bond between I and II. This view is further substantiated by the existence of several isosbestic points in each set of spectra. The shorter wavelength band shifted to the red, the longer band shifted to the blue, and hyperchromism of the longer band was observed. Frequency shifts due to hydrogen bonding were determined from the spectra of the

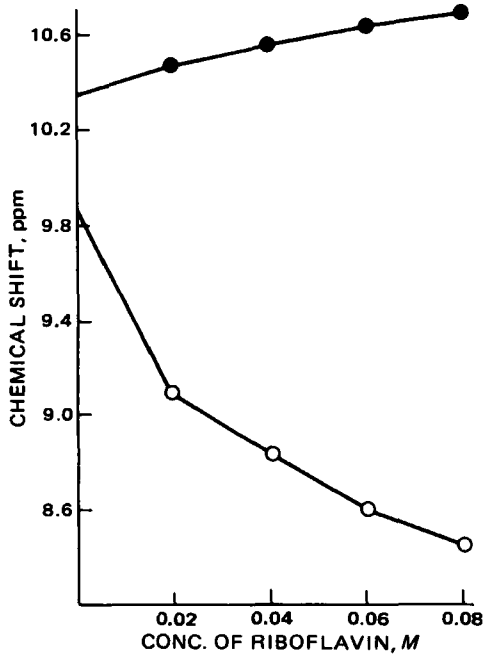



**Figure 5**—*Effects of the concentration of I on the chemical shifts of the carboxyl (●) and the phenol (○) protons of II in deuterochloroform, keeping the concentration of II constant at 0.1* M.

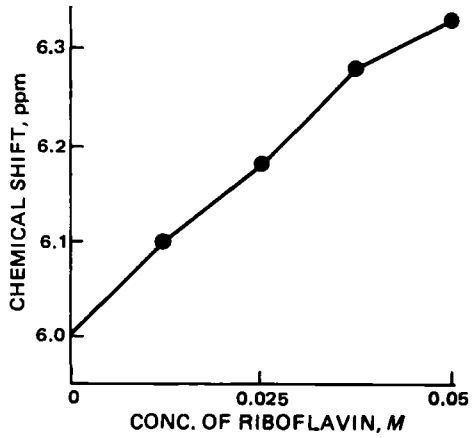



**Figure 6**—*Effects of the concentration of I on the chemical shifts of the salicylamide amino protons in deuterochloroform, keeping the concentration of IV constant at 0.05* M.

free and hydrogen-bonded species (resulting from the difference between the energy of the hydrogen bond in the excited and ground states). These phenomena were observed with III or IV in carbon tetrachloride. In the other nonpolar solvent, benzene, similar phenomena also were observed.

Determination of the association constant of the complex is possible from spectral data if the complex shows a significantly different spectrum from that of its components. The association constant of the hydrogen-bonding complex was obtained using the following:

$$\frac{1}{\Delta\epsilon_{obs}} = \frac{1}{K(\epsilon_c - \epsilon_a)}\frac{1}{C_b} + \frac{1}{\epsilon_c - \epsilon_a}$$

where $\Delta\epsilon_{obs} = \epsilon_{obs} - \epsilon_a$, $\epsilon_a$ and $\epsilon_c$ are the molar absorptivities at a given frequency of I in uncomplexed form and in pure complex, respectively, $\epsilon_{obs}$ is the observed absorptivity at a given frequency of I in complexed media, $C_b$ is the concentration of salicylates (on some scale defined in the *Experimental* section), and $K$ is the association constant of the complex (17). This can be easily derived with the assumption that the concentration of I is negligibly small compared with that of salicylate; such is actually the case in the present study. From this equation, a plot of $I/\Delta\epsilon_{obs}$ *versus* $1/C_b$ is expected to give a straight line. When both the concentration of I and the cell length are kept constant throughout a set of spectra, the absorptivities ($\epsilon$) in the equation may be replaced by the corresponding absorbances ($A$). Thus $1/\Delta A_{obs}$ was plotted against $1/C_b$ for various frequencies. These plots gave straight lines, and the extrapolation of the lines for each of the salicylates led to $K$ values which were

**Table I—Measured and Calculated Properties of I and Salicylates on Absorption Spectra**

| Salicylate | Range of Salicylate Concentration, $M$ | Solvent | Association constant ($K$), $M^{-1}$ |
|---|---|---|---|
| II | $1 \times 10^{-3}$–$1 \times 10^{-2}$ | Benzene | 450 |
| III | $1 \times 10^{-3}$–$1 \times 10^{-2}$ | Benzene | 370 |
| II | $1 \times 10^{-3}$–$1 \times 10^{-2}$ | Carbon tetrachloride | 435 |
| III | $2 \times 10^{-4}$–$1 \times 10^{-3}$ | Carbon tetrachloride | 350 |

equal to one another within experimental error. One example of such plots is shown in Fig. 8. The $K$ values were calculated from the plots and are summarized in Table I. These association constants were obtained by averaging the values from three different concentrations of I ($3 \times 10^{-5}$, $5 \times 10^{-5}$, and $8 \times 10^{-5}$ $M$).

## DISCUSSION

High-resolution $^1$H-NMR techniques provide a direct observation of the hydrogen bonding in solution. As shown in the NMR spectra, the N-3 proton of I, the carboxyl protons of II and III, and the amino protons of IV seem to participate in the bonding. The NMR method utilized here measures the chemical shifts of the donor proton in hydrogen-bond formation. The acceptor atoms do not have protons and therefore do not give rise to resonances. Hence, no direct information can be gained on the identity of these acceptors. Using IR spectra, it was determined which carbonyl group of riboflavin was involved (18). In the 6-$\mu$m region of the spectra, the association through the C-2 carbonyl group of the isoalloxazine ring predominates. This is consistent with the suggestion that, in the hydrogen bonding on the isoalloxazine ring, the C-2 carbonyl group is preferred to the one at C-4 (19). This can be illustrated by the fact that the proton affinities of the heteroatoms in the isoalloxazine ring are actually different from one another (14, 19–21). As determined previously (14), the charge density of the C-2 carbonyl oxygen atom in the isoalloxazine ring is greater than that of the C-4 carbonyl oxygen atom; the N-3 proton is extremely labile and a good electron acceptor since the charge density of N-3 nitrogen atom is higher than any of the other heteroatoms. The change of the absorption spectra supports hydrogen-bond formation at the aforementioned binding sites (22).

Thus, the most probable hydrogen bonding is represented in the following manner. Compounds I and II form the 1:1 cyclic hydrogen-bonded dimer (V) through the N-3 proton and the C-2 carbonyl oxygen of the isoalloxazine ring, and the carboxylic hydroxyl proton and carbonyl oxygen of II. Compounds I and III form the 1:1 cyclic hydrogen-bonded dimer (VI) in the same manner. Compound IV forms the 1:1 cyclic hydrogen-bonded dimer (VII) with I through the N-3 proton and the C-2

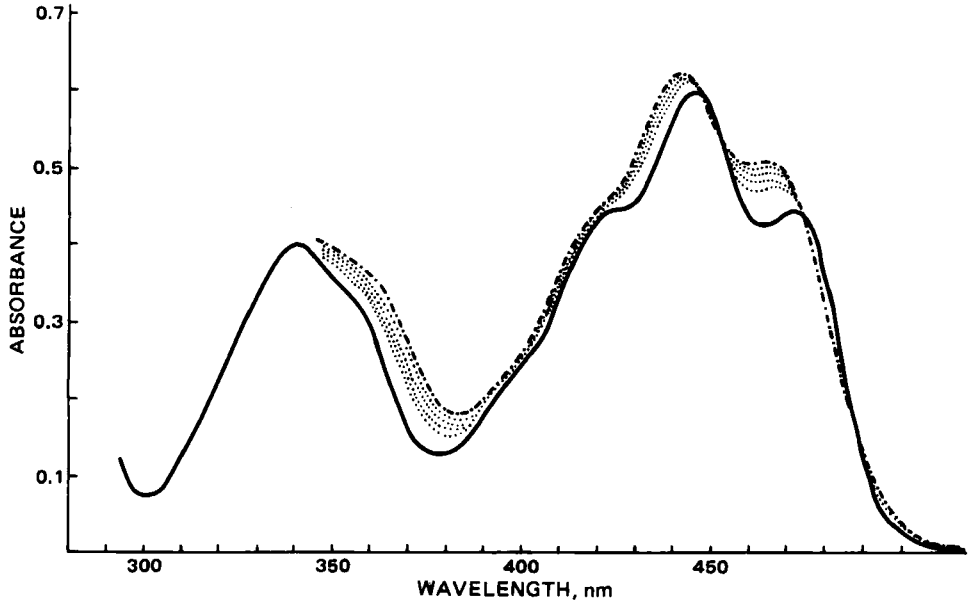



**Figure 7**—*Effects of II on the absorbance spectrum of I ($5 \times 10^{-5}$ M) in carbon tetrachloride. Salicylic acid was added from 0 to $1 \times 10^{-2}$ M. Key: (—) free molecule, (· · ·) spectra in the presence of II in order of increasing concentration of II, (· - · - ·) spectra in the presence of $1 \times 10^{-2}$ M II.*

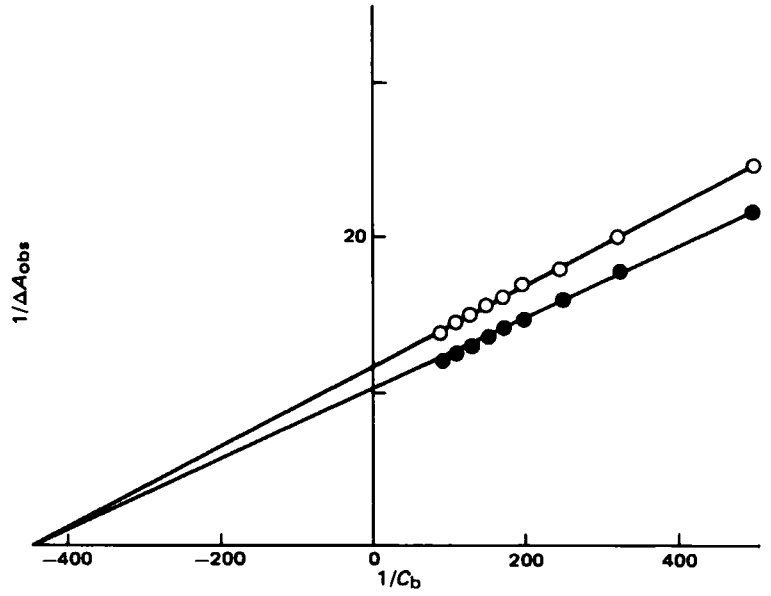



**Figure 8**—*Relationship between $1/A_{obs}$ and $1/C_b$ values of the I–II system in benzene obtained according to the association constant equation. Key: (○) 436 nm, (●) 446 nm.*

carbonyl oxygen of the isoalloxazine ring, and the amino proton and carbonyl oxygen of IV. The frontier orbital density of N-5 is the greatest of all the atoms in the isoalloxazine ring (20). Therefore, N-5 seems to be the entrance of electrons. The formation of various types of hydrogen bonding affects the frontier orbital density of N-5 of the isoalloxazine ring (22). Considering that salicylates interact with riboflavin at the aforementioned sites, the electron affinity of the isoalloxazine ring and the frontier electron density of N-5 increase, which accelerates the electron flow from the substrate to the coenzyme (19–26). The hydrogen bonding involved in flavoproteins may be considered to be significant with respect to their catalytic activity.

In nonpolar solvents (carbon tetrachloride and benzene) marked absorption spectral changes due to hydrogen bonding were produced when salicylates were added to I. Frequency shifts were observed. It is known that the energy of the hydrogen bond in the excited state is differentiated from that in the ground state. The association constants of I with II or III in nonpolar solvents are large, however, with IV it is too small to be calculated from the absorption spectral data. The NMR data produced similar results. On the other hand, in chloroform solution the absorption spectral change due to hydrogen bonding was weak (not shown). This could be attributed to the solute–solvent interaction.

Although II, III, and IV are very similar structurally, their effects on the body may be quite diverse. Sodium salicylate and III effectively depress oxidative phosphorylation while IV is a weak or ineffective uncoupling agent. Compound III is more potent than sodium salicylate as an analgesic and antipyretic, and IV is much less effective than either (27, 28). These facts are consistent with the experimental results obtained in this study. The use of deuterochloroform, carbon tetrachloride, and benzene as solvents in these experiments enabled observation of these interactions free of strong solute–solvent association effects. This environment may mimic in some ways the inside of the enzyme–substrate complex from which water may be excluded. It has been suggested that FAD in the enzyme is surrounded by a hydrophobic environment even in the aqueous phase (29, 30). If one considers that the oxidation–reduction process in these systems occurs in a lipophilic rather than an aqueous environment, it may be worthwhile to research the direct interaction between salicylates and the riboflavin moiety in a nonpolar system. Further quantitative analysis of these data should lead to a more precise fundamental understanding of the riboflavin–salicylate complex formation.

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## ACKNOWLEDGMENTS

This research was supported by the 1981 grant from the Korea Science and Engineering Foundation.