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Molecular Interaction Between a Reduced Riboflavin Derivative and Salicylic Acid Derivatives

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Abstract □ The interaction of reduced riboflavin-2′,3′,4′,5′-tetrabutyrate with salicylic acid, aspirin, and salicylamide has been spectroscopically investigated to determine the binding mechanism. Hydrogen-1 and carbon-13 nuclear magnetic resonance, infrared, and absorption spectra were measured in chloroform-d and chloroform. The association of the reduced riboflavin with salicylic acid derivatives is different from that oxidized one. Salicylic acid and the reduced riboflavin form a cyclic hydrogen bonded complex through the imino (3-N, 5-N) protons and the carbonyl (2-C, 4-C) oxygens of the isoalloxazine ring of the latter, and the carboxylic hydroxyl proton and carbonyl oxygen of the former. Aspirin and the reduced riboflavin form a complex by the same mode as salicylic acid. Salicylamide forms a cyclic hydrogen bonded complex with the reduced riboflavin through the imino (3-N, 5-N) protons and the carbonyl (2-C, 4-C) oxygens of the isoalloxazine ring, and the amino proton and the carbonyl oxygen of salicaylmide. It appears that both the oxidized and reduced form of riboflavin are associated with salicylic acid derivatives.

Keywords □ Reduced riboflavin, Salicylic acid derivatives, Hydrogen bonding, Nuclear magnetic resonance, Infrared and Absorption spectra.

The mechanism of the electron transfer from nicotinamide adenine dinucleotide to flavoprotein, or the charge-transfer complex formed by them, was studied by a number of authors to give a good account of the function of the respiratory chain[1-6]. Especially, Honda has described that reduced NAD-coupling enzyme complex converts spontaneously to the hypothetical intermediate as oxidized NAD-coupling enzyme, which is considered indispensable to the ATP formation in the respiratory chain[6]. Simultaneously, the electrons in $(NAD_{ox})^{-2}$ can be transfered to flavoprotein. It has been determined experimentally that higher concentrations of salicylates result in marked stimulation of respiration. It is well accepted that salicylates stimulate respiration by uncoupling oxidative phosphorylation and increasing metabolism[7-13].

Millhorn *et al.*, on the other hand, have reported recently that salicylates stimulate reapiration by mechanism other than one related to their ability to uncouple oxidative phosphorylation and increase metabolism[14,15]. However, it is generally agreed that salicylates cause the breakdown of some high-energy intermediate involved in the phosphorylation process, but the most reliable mechanism has not been found yet. It is also known that the hydrogen bonding of salicylates in biological system is relevant to their drug action[16].

In the previous papers, specific formation of hydrogen bonding between the oxidized riboflavin and salicylic acid derivatives has been reported[17,18]. The isoalloxazine ring of riboflavin takes hydrogenated and dehydrogenated forms and hence it can be an electron carrier in respiratory chain. Therefore, it seems worthwhile to examine the effect of hydrogenation on the association of a riboflavin derivative with salicylic acid derivatives.

In this paper, molecular interaction between a fully reduced riboflavin tetrabutyrate and salicylic acid derivatives in chloroform-d and chloroform was examined by the spectroscopic methods (through a detailed analysis of the IR, NMR, and absorption spectra of the complex). The results of this study may provide a basis for understanding the redox reactions of flavoenzyme and interpreting the mode of action of salicylate.

EXPERIMENTAL METHODS

Materials

Riboflavin-2', 3', 4', 5'—tetrabutyrate(RFTB) was obtained from Dae Woong Pharm. Co., Ltd., Korea. It was recrystallized from chloroform and its purity was checked by TLC. Salicylic acid (SA) and salcylamide (SM) were obtained from Pacific Pharm. Co., Ltd., Korea and were used after recrystallization from chloroform. Aspirin (AS) was purchased from E. Merk, Darmstat, Germany, which was used without further purification. chloroform-d was purchased from E. Merk. Chloroform was treated with one-half its volume of water several times, dried with calcium chloride and distilled fractionally from phosphorous pentoxide through a 120cm column packed with glass helices. The distillate was refluxed and redistilled fractionally.

Methods

The absorption spectra were measured in a Unicam SP 1750 Ultraviolet Spectrophotometer connected to a Unicam AR 25 Linear Record, using a 10 mm quartz cells. Infrared spectra were recorded on a Beckman IR 20 A Infrared spectrophotometer. Fused quartz cells (5mm were used in the 3 μ region. Hydrogen-1 an carbon-13 NMR spectra were recorded on Varian 80 MHz FT-NMR Spectrometer equippe with a temperature-control unit. Chemical shift were read relative to the resonance of intern tetramethylsilane in both cases.

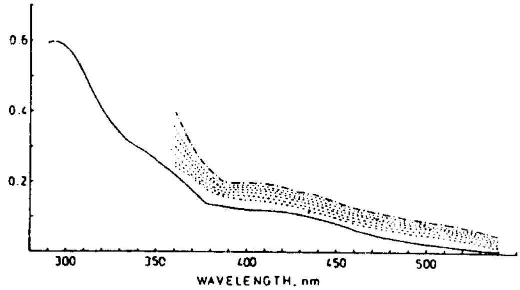
To prepare the reduced RFTB (RH) sampl a $CDCl_3$ solution of RFTB (R) in a samp tube (or cell) was treated with an aquous solu tion of sodium dithionite in an amount of suffi cient to reduce the riboflavin. After shakin the tube (or cell) was sealed anaerobicall Since even a slight amount of paramagnet flavin radicals causes brodening of the signal the water layer was held over the $CDCl_3$ sol tion to keep the flavin in its fully reduc state[19,20]. The reduced riboflavin could reversibly oxidized by bubbling oxygen in the solution.

RESULTS

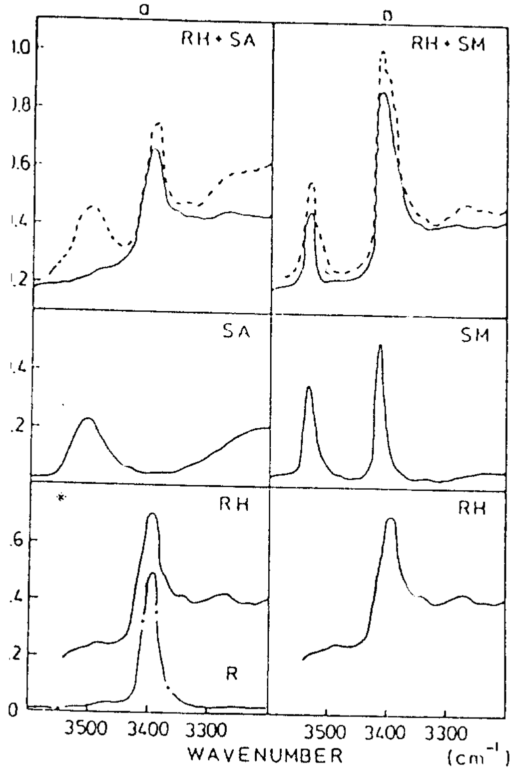
Absorption spectra

The absorption spectrum of RFTB treated the above-mentioned reduction was compar with that of the oxidized RFTB in CHCl Disappearance of absorptions at 450 and 350 n and appearance of a strong band at 300 n were observed. This may be a evidence th the reduction of riboflavin was completed[21-2

As shown in Fig. 1, a marked spectral chan was produced upon adding SA to RH $CHCl_3$. The red and hyperchromism w observed. It may be assumed that these spect changes are due to solely to the formation association between RH and SA. Frequen shifts due to association such as hydrogen bo were determined from the spectra of the f and hydrogen bonding species. These phenom



g. 1: Effects of SA on the absorptron spectrum of 5×10^{-5}M RH in $CHCl_3$. SA is added from 0 to 5×10^{-2}. Key (——) free molecule, (……) spectra in the present of SA in order to increasing concentration of SA, (- - -) spectra in the presence of 5×10^{-2}M SA.



r. 2: Infrared spectra of (※) oxidized RFTB(R) and RH, and 1 : 1 mixture of (a) RH and SA, (b) RH and SM. These spectra show the observed (——) and the calculated sum of the lower two spectra (……). The concentrations are 4 mM in $CHCl_3$ for the measurments in 3 μ region.

were also observed with AS or SM in $CHCl_3$.

Infrared spectra

The IR spectrum of 4 mM RH showed very broad strong band around 3400 cm^{-1} contradicting with the sharp 3-NH stretching band at 3380 cm^{-1} of the oxidized one in 3 μ region (Fig. 2). According to Yu, the broad band comes from the hydrogenated NH groups at the 1-N and 5-N positions of the reduced isoalloxazine ring and indicates the strong self-association of RH[21]. In the spectrum of 4mM SA in $CHCl_3$, a medium band due to the nonbonded carboxylic hydroxyl stretching vibration was observed at 3510 cm^{-1} and a quitely broad band due to the bonded carboxylic hydroxyl vibration was also observed below 3350 cm^{-1}.

When eqimolar solution of RH and SA were mixed together, the nonbonded bands of the imino groups of RH and the carboxylic hydroxyl group of SA decreased drastically in intensity (Fig. 2). These spectral changes are apparently caused by hydrogen bonding. From these facts, it may be suggested that the hydrogen atoms of imino groups of RH and the carboxlic hydroxyl group of SA be used in the association. Similar phenomena were observed with RH upon the addition of AS.

The spectrum of 4 mM SM in 3 μ region showed two sharp bands with medium intensity at 3415 and 3535cm^{-1}, which are respectively due to symmetric and antisymmetric stretching vibration of the nonbonded amino group. The IR spectra of the 1 : 1 mixture of RH and SM also showed us binding of RH with SM (Fig. 2). The nonbonded bands of amino group of SM became weak. Therefore, it may be considered that amino group of SM participates in hydrogen bonding.

Hydrogen-1 NMR spectra

^{1}H-NMR spectrum of the reduced state of flavin in comparison with the oxidized one has been reported[19,20]. In the spectrum of RH in $CDCl_3$, most of proton nuclei of RH resonated in higher fields than those of R. This may be due to the increase of the total electron densities by reduction as discussed previously[20]. Nevertheless, the 3-N proton signal of RH was observed at a lower field than than of R, which may be due to the stronger self-association of RH. And 5-N proton signal was positioned at about 4.8 ppm, but 1-N proton was not evident at the present condition (not shown).

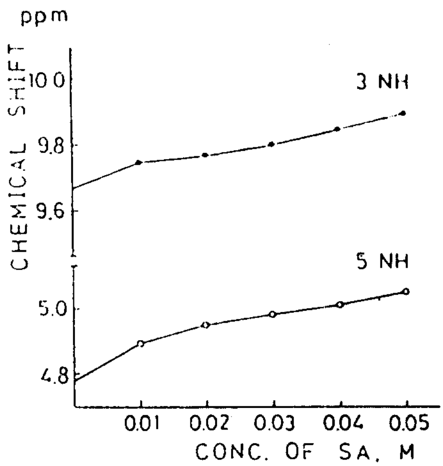
In the spectrum of SA solution in $CDCl_3$, the chemical shift of the carboxylic hydroxyl proton was observed below 10 ppm, the phenol proton did not appear at low concentrations but was weakly observed at high concentrations below 9 ppm and the benzene ring protons were observed in 6.9—8.1 ppm. The carboxyl proton of AS was also observed weakly at high concentration (below 9 ppm). In the spectrum of SM, absorption of amino proton was observed at about 6 ppm and that of hydroxyl proton below 12 ppm.

The imino protons of RH moved slightly downfield by the increase of the concentration (not shown). It may be considered that this evi dence indicates the strong self-association of RH through imino protons. If some proton takes part in hydrogen bonding, it becomes less shielded and its resonance shifts downfield. Therefore, the exact positions of the hydroxyl, amino and imino resonances depend on the degree of association and hydrogen bond formation: they vary with concentration and temperature.

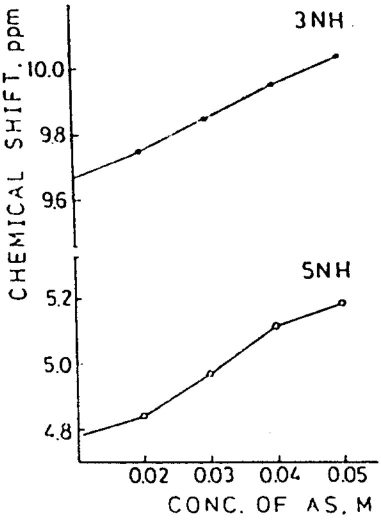
To confirm the formation of hydrogen bonds between RH and salicylic acid derivatives in $CDCl_3$, the shifts of the imino proton resonances of RH were measured on addition of salicylic acid derivatives. The chemical shifts of 3-N and 5-N protons of RH were plotted against concentration of SA at 37°C, keeping the c centration of RH constant at 0.08 M (Fig.3- The imino signals shifted slightly downfi particularly 3-N proton signal appeared to l aden, as the concentration of SA increased: slopes of 3-N and 5-N curves were found be slightly greater than those of the chem shifts due to the self-association of RH. U the addition of AS to RH, the chemical sh of the imino protons of RH were similar those of SA (Fig. 3-b). From the results can be inferred that the association betw RH and SA or AS is stronger than the association of each compound and imino (3 5-N) protons of RH also participate in hydrogen bonding. The carboxylic hydr proton signals of SA and AS disappeare addition of SA or AS to RH. This may be to the rapid chemical exchange between carboxylic hydroxyl proton of SA or AS hydroxyl proton of water molecule. The ph proton signal of SA decreased in intensity then disappeared with shielding effect as concentration of RH was increased.

In the case of SM, the imino protons of moved downfield as the concentration of increased and the slope of 3-N curve observed to be a little greater than th 5-N curve (Fig. 3-c). Fig. 3-d shows chemical shift of the amino proton of plotted against the concentration of RH, keeping the concentration of SM consta 0.08 M. As the concentration of RH incre the amino proton signal of SM shifted d field and appeared to sharpen but the phe hydroxyl proton signal did not move and was located at about 12 ppm constantly.

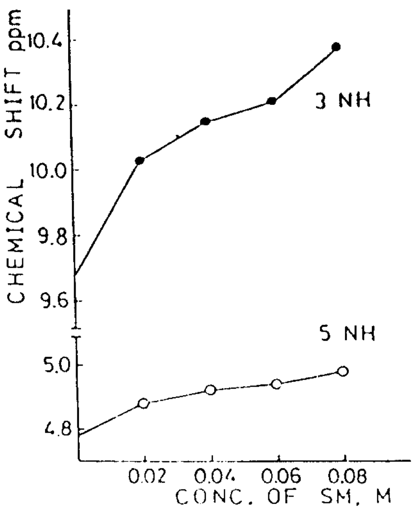
From above results, it may be suggested RH and SM form a hydrogen bonded con



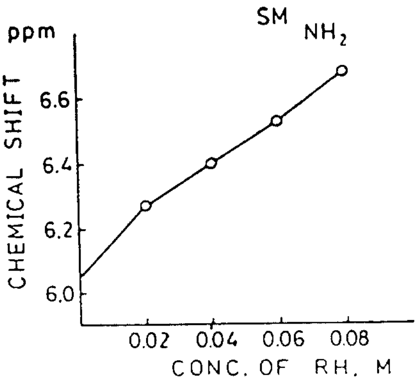
(a)



(b)



(c)



(d)

Fig. 3-a: Effects of the concentration of SA on the chemical shifts of RH 3-N(•) and 5-N(o) protons in $CDCl_3$, keeping the concentration RH constant at 0.08 M.

3-b: Effects of the concentration of AS on the chemical shifts of RH 3-N(•) and 5-N(o) protons in $CDCl_3$, keeping the concentration of RH constant at 0.08 M.

3-c: Effects of the concentration of SM on the chemical shifts of RH 3-N(•) and 5-N(o) protons in $CDCl_3$, keeping the concentration of RH constant at 0.08 M.

3-d: Effects of the concentration of RH on the chemical shifts of SM amino proton in $CDCl_3$, keeping the concentration of SM contant at 0.08 M.

through the imino (3-N, 5-N) protons of RH and the amino proton of SM.

Carbon-13 NMR spectra

^{13}C-NMR spectra of the fully reduced form of flavin has been reported with riboflavin tetrabutyrate[19,20]. Proton decoupled ^{13}C-resonance spectra of the oxidized and reduced riboflavin ^{13}C at natural abundance level were observed (not shown). As the results of ^{1}H-NMR spectra, most of carbon nuclei of RH gave signals at higher field than those of the R, which is well explained by the increase in total electron densities.

To obtain information about characteristics of carbon atoms following the complex formation in $CDCl_3$, the shifts of all carbon resonances of RH were measured on addition of SA (Table I). The resonance signals of 2- and 4-carbonyl carbons of RH moved to upfield and those of other carbons of the isoalloxazine ring moved slightly.

Table. I. ^{13}C Chemical-Shift Values of the Isoalloxazine Ring-Carbons of Reduced RFTB (RH) upon Addition of Salicylic Acid*

carbon	chemical shift (ppm)	
	RH	RH+SA
C(4a)	112.7	103.9
6-CH	117.4	115.8
9-CH	119.0	117.0
C(9a)	127.6	127.6
C(8)	129.5	128.5
C(7)	130.8	133.2
C(5a)	135.7	135.1
C(10a)	139.7	139.2
2-CO	147.8	150.0
4-CO	150.4	156.4

* Measured from proton-decoupled ^{13}C NMR spetra with ^{13}C at natural abundance.
Reduced PETBRH): 98.6mg/0.5ml$CDCl_3$ (0.3M)
Salicylic acid(SA): 41.6mg/0.5ml$CDCl_3$ (0.15M)
** Measured from internal standard, TMS.

It is known that variations in local-electron densities primarily govern ^{13}C shielding in aromatic rings[24]. If some carbonyl oxygen participates in hydrogen bonding, the carbonyl carbon becomes the more shielded and its resonance shifts upfield. This phenomenon can be similar to the solvent effect[25,26].

From above results, therefore, it may be assumed that 2- and 4-carbonyl oxygens of RH are used in hydrogen bonding. And probably, the pertubations of other carbon signals may be also due to the formation of hydrogen bonding that changes the local-electron densities of each carbon. Similar phenomena were also observed upon addition of AS or SM to RH (not shown).

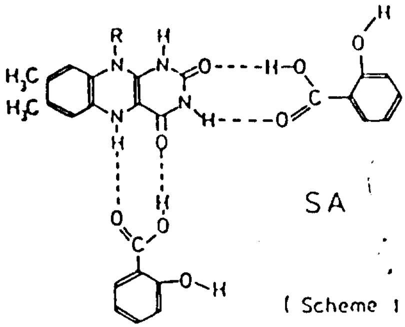
DISCUSSION

The selective formation of hydrogen bonding between the oxidized form of riboflavin and salicylic acid derivatives has been elucidated through our previous investigations[17,13].

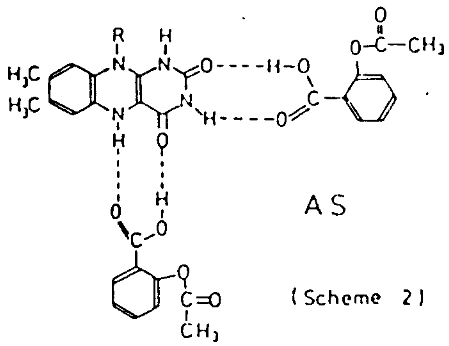
Infrared and nuclear magnetic resonance techniques provide a direct observation of the hydrogen bonded association in solution. As shown in the ^{1}H-NMR spectra, the 3- and 5-N imino protons of RH and the amino proton of SM seem to participate in the hydrogen bonding. However, participation of the 1-N imino proton of RH in the bonding is not clear because of no experimental evidence in the present condition. The ^{1}H-NMR method utilized here measures the chemical shifts of the donor proton in the hydrogen bond formation. In the observation of ^{13}C-NMR spectra, which can give a direct information on the identity of the acceptor atom. Hence it can be inferred that not only 2-C but also 4-C carbonyl group of RH takes part in the hydrogen bonding. This suggestion can be illustrated by the fact the charge density of the 2-C carbonyl oxygen atom is similar to that of the 4-C one in RH, while the charge density of the 2-C carbonyl oxygen atom is greater than that of the 4-C one in R[6,27].

Participation of the carboxyl groups of SA and AS in the association is also assumed by the results of IR spectral observation. And the changes of absorption spectra support the hydrogen bond formation at the afore-mentioned binding sites.

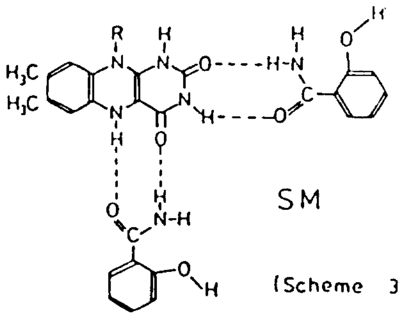
Based on these above points, thus, the most probable hydrogen binding modes are represented in the following manner. A likely association between RH and SA is a hydrogen bonded complex through the imino (3-N, 5-N) protons and the carbonyl (2-C, 4-C) oxygens of RH, and the carboxylic hydroxyl proton and the cabonyl oxygen of SA (Scheme 1). In the case of AS, the similar mode of the association with SA can be considered (Scheme 2). SM forms a hydrogen bonded complex with RH through the imino (3-N, 5-N) protons and the carbonyl (2-C, 4-C) oxygens of the isoalloxazine ring of RH, and the amino proton and carbonyl oxygen of SM (Scheme 3).


SA

(Scheme 1)


AS

(Scheme 2)


SM

(Scheme 3)

Because the association constants of RH with salicylic acid dervatives in the present condition can not be obtained unfortunately, the intensity of the hydrogen bonding between salicylic acid derivatives is not able to compared. However, it seems to be distinct that both the oxidized and reduced form of riboflavin associate with salicylic acid derivatives.

The formation of various types of hydrogen bonding affects the frontier orbital density of 5-N of the isoalloxazine ring[28]. It is still not clear that the hydrogen bonding of RH affects the electron densities of 4a-C and 5-N. Considering that salicylates interact with the oxidized and reduced riboflavin, the electron affinity of the isoalloxazine ring may be increased, which accelerates the electron flow from the substrate to the coenzyme.[27-31]

The NMR studies have been restricted to the oxidized form of free and protein-bound flavin, except for several studies of N-alkylated flavins, because the line-broadening was caused by small amounts of semiquinone radicals provoked by the trace of oxygen and the strong self-association of the reduced flavin[19,20]. Then, it is well known that riboflavin tetrabutyrate is a useful compound to circumvent these difficulties[19].

The use of nonpolar solvents, such as $CDCl_3$ and $CHCl_3$, is resonable, because this environment may mimic in someway the inside of the enzyme-substrate complex. This expression is related to the suggestion that FAD in flavoenzyme is surrounded by the hydrophobic environment[32-34].

ACKNOWLEDGMENTS

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