Journal of the Pharmaceutical Society of Korea
20, 130-137 (1976)

A Spectroscopic Study of Hydrogen Bonding between Riboflavin and Salicylic Acid Derivatives

Jae Wook Huh* and Byung Sul Yu

College of Pharmacy, Seoul National University, Seoul 151, Korea

(Received June 16, 1976)

Abstract—Specific association phenomena of riboflavin-2′, 3′, 4′, 5′, - tetraacetate and salicylic acid derivatives, such as salicylic acid, aspirin and salicylamide have been measured by infrared and fluorescence spectroscopy. Salicylic acid and riboflavin tetraacetate form the 1 : 1 cyclic hydrogen bonded dimer through the imino group and the 2-C carbonyl group of isoalloxazine ring of the latter, and the carbonyl group and carboxylic hydroxyl group of the former. Asprin and riboflavin tetraacetate form the 1 : 1 cyclic hydrogen bonded dimer by the same mode. Salicylamide forms the 1 : 1 cyclic hydrogen bonded dimer with riboflavin tetraacetate by using its amide group and carbonyl group. Salicylic acid derivatives are effective quenchers of the fluorescence of riboflavin tetraacetate. It is appeared that salicylamide is the strongest quencher among them. The quenching effect is attributed to the formation of association dimer.

It is commonly known that the hydogen bonding of salicylates in biological system is related to their drug actions[1].

The role of hydrogen bonding in biological system has been the topic of numerous experimental studies and much speculation. Kyogoku *et al.*[2] have shown that the strong hydrogen bonding between barbiturates and adenine derivatives may concern their physiological activities. And Yu[3] has suggested that barbiturates inhibit the interaction between adenine and riboflavin so that they may affect the oxidative phosphorylation.

It has been shown experimentally that large doses of salicylates results in marked stimulation of respiration[4], and salicylates uncouple oxidative phosphorylation[5].

It is generally agreed uncoupling agents act by causing the breakdown of some high-energy intermediate involved in the synthesis of ATP, but the most reliable mechanism has not yet been found.

However, it seems true that salicylates may affect the respiratory chain mechanism. So it seemed worthwhile to examine the hydrogen-bonded interaction between salicylates

* This work is presented in partial fulfilment of the requirements for the M.Sc. degree.

and riboflavin, that concerns the respiratory chain metabolism.

EXPERIMENTAL

Materials—Riboflavin-2′, 3′, 4′, 5′, -tetraacetate was prepared by the method described by Yagi[6]. A yellow flaky crystal was obtained (3 mg, mp 246°, Found: C, 53.95; H, 5.22; N, 10.41%). Commercial aspirin and salicylamide were recrystallized from chloroform. Salicylic acid was obtained from E. Merck, Darmstadt, Germany, it was used without further purification. Chloroform and chloroform-d were used as solvents. The chloroform (reagent grade, Tedia Company, Inc.,Canada) was distilled after being dried with phosphorus pentoxide overnight and then passed through an alumina gel column 25 cm long. Chloroform-d was purchased from E. Merck, Darmstadt, Germany. It seemed to contain a slight amount of exchangeable deuterium, which caused exchange at the amino group of the solutes in dilute solutions. The chloroform-d was therefore purified by passing it through an alumina gel columin 5 cm in length.

Spectral determination—Infrared spectra were observed with a Beckmann Model 20 A double beam infrared spectrophotometer. Fused quartz cell ranging 5mm was used for measurement in the 3800~3200 cm^{-1} region, and potassium bromide cell ranging 1 mm was used for 1800~1500 cm^{-1} region. The infrared spectra shown in the figures of this paper were given in the absorbance scale, which was calculated from the absorbed transmission with the aid of the solvent curves as base lines.

The fluorescence measurements were carried out with a Hitachi 204 fluorescence spectrophotometer equipped with a mercury lamp and a grating monochromator. The activating wave length was set at 330 nm and the fluorescence was read in the range 380~640 nm. A fused quartz cell of 1 cm width and 1 cm length with a cap was used.

To avoid self quenching at higher concentration, the solution of riboflavin derivatives should have a concentration less than 10^{-4}M.

RESULTS AND DISCUSSION

Infrared spectra—In the spectrum of 4×10^{-3}M riboflavin-2′, 3′, 4′, 5′, -tetraacetate in chloroform solution a sharp band is observed at 3380cm^{-1} in the region of 3600~3200 cm^{-1}, and a strong band is observed at 1745 cm^{-1} and two bands with medium intensity at 1710 cm^{-1} and 1690cm^{-1} in the region of 1800~1500 cm^{-1} (Fig. 1). The 3380cm^{-1} band is assigned to the nonbonded N-H stretching vibration, and the 1745cm^{-1} band which appears at such high frequency region is assigned to the carbonyl stretching band of the acetyl groups according to Yagi[6]. The 1710cm^{-1} band is considered to arise mainly from the 4-C carbonyl group and the 1685 cm^{-1} band from the 2-C carbonyl stretching vibrations coupled with the N-H bending mode since the 2-C carbonyl bond, being conjugated with the C-N bonds, should have lower frequency vibration than the 4-C

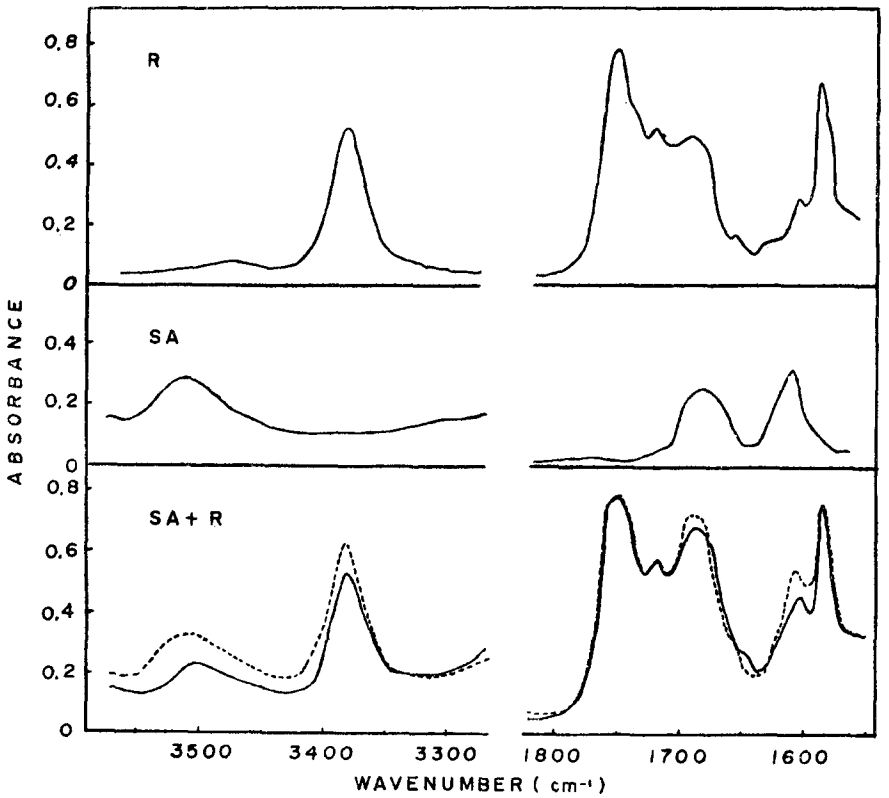


Fig. 1—Infrared spectra of riboflavin tetraacetate, salicylic acid and 1 : 1 mixed solution.
3 μ region; 4×10^{-3}M in $CDCl_3$ 5 mm quartz cell.
6 μ region; 4×10^{-3}M in $CHCl_3$ 1 mm KBr cell.
Solid line; observed spectra.
Dotted line; calculated sum of the upper two spectra.

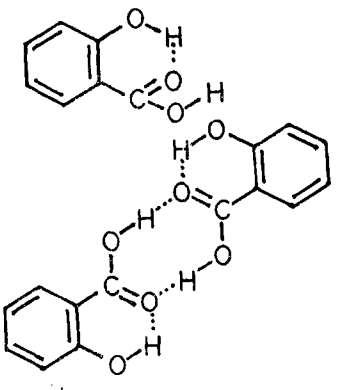


Fig. 2—Intramolecular and intermolecular H bonding in salicylic acid.

carbonyl bond.

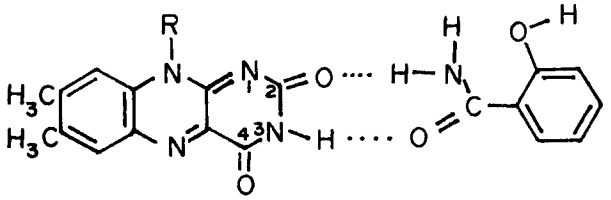
In the spectrum of 4×10^{-3}M solution of salicylic acid in chloroform a broad band is observed at 3510cm^{-1} in the region of 3600~3200 cm^{-1} and two medium bands at 1680 and 1610 cm^{-1} in the region of 1800 ~1500 cm^{-1} (Fig. 1).

It is known that salicylic acid forms the intramolecular as well as intermolecular hydrogen bond (Fig. 2). We should determine whether those bands are due to intramolecular bonded or intermolecular bonded or nonbonded free form. According to

Pimentel *et al.*[7] the most direct and readily obtainable test to distinguish intramolecular H bonds from intermolecular H bond is the observation of changes in the infrared spectrum at low concentration. Intermolecular H bonding (and its spectral characteristics) disappear at low concentration in an inert solvent, whereas intramolecular H bonds do not. It is, therefore, obvious that the association does not occur at such low concentration about 4×10^{-3}M. It has been shown[10] that intramolecular H bonding reduces the frequency of the carbonyl stretching absorption to a greater degree than does Intermolecular H bonding. In intramolecular H bonding the hydroxyl group of salicylic acid absorbs at 3200 cm^{-1} and the carbonyl group at 1665 cm^{-1}, but the carbonyl group of para-hydroxy benzoic acid that cannot form intramolecular H bonding absorbs at 1680 cm^{-1}. As shown by Fig. 1, the carbonyl stretching occurs at rather 1680 cm^{-1} than 1665 cm^{-1}, and the hydroxyl stretching at rather 3510 cm^{-1} than 3200 cm^{-1}. Therefore it may be considered that those bands are mainly due to the free salicylic acid, and by analogy, the other salicylic acid derivatives do not form either intra or intermolecular H bonding in very dilute chloroform solution. The 1610 cm^{-1} band is assigned to the phenyl nucleus resonance vibration (skeletal vibration involving C to C stretching within the ring).

When equimolar solutions of riboflavin tetraacetate and salicylic acid are mixed together, the nonbonded bands decrease in intensity but new bands are not found out in 3μ region, and the band ranging 1690~1680 cm^{-1} and the 1610 cm^{-1} band become weak, and the shoulderlike band ranging 1675~1630 cm^{-1} is now appeared (Fig. 1). The 1745 cm^{-1} band and 1710cm^{-1} band are not changed. From the above result, we can recognize that the hydogen atom of the imino group and the oxygen atom of 2-C carbonyl function of the isoalloxazine ring of riboflavin tetraacetate are used in the association, but the oxygen atom of the 4-C carbonyl function of the isoalloxazine ring seems to be scarcely used since the intensity of the 1710 cm^{-1} band is not changed.

In salicylic acid the hydrogen atom of the carboxylic hydroxyl group and the oxygen atom of the carbonyl group are used in the association. Therefore it may be considered that 1 : 1 cyclic H bonded dimer through the imino group and the 2-C carbonyl group of isoalloxazine ring of riboflavin tetaercetate and the carbonyl group and carboxylic hydroxyl group of salicylic acid is the most probable.



In the spectrum of asprin the broad band at 3510 cm^{-1} is assigned to the nonbonded hydroxyl stretching vibration of the carboxyl group by analogy to salicylic acid. In 6μ region the broad band near 1730 cm^{-1} is assigned to the overlapped band of the nonbonded

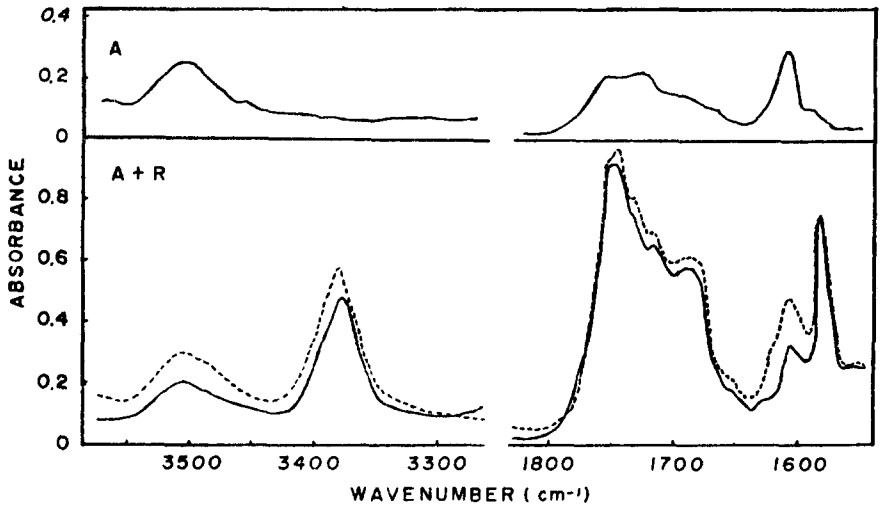
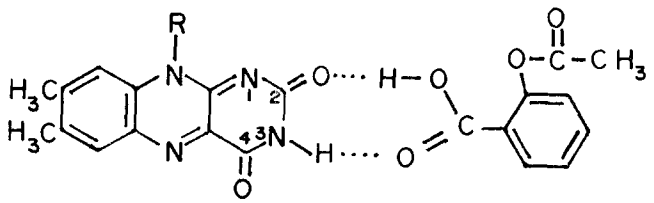


Fig. 3—Infrared spectra of aspirin and 1 : 1 mixed solution with riboflavin tetraacetate.
3 μ region; 4×10^{-3}M in $CDCl_3$ 5 mm quartz cell.
6 μ region; 4×10^{-3}M in $CHCl_3$ 1 mm KBr cell.
Solid line; ocserved spectra.
Dotted line; calculated sum of the upper spectrum and the spectrum of riboflavin tetraacetate.

carbonyl stretching vibration of the carboxyl group and the carbonyl stretching vibration of the aromatic ester, and the medium band at 1605 cm^{-1} is assigned to the phenyl nucleus resonance vibration (Fig. 3).

When equimolar solutions of aspirin and riboflavin tetraacetate are mixed together, there is no new band and all bands except 1580 cm^{-1} decrease in intensity. Though the mode of interaction cannot be deduced only with given spectrum, it is expected that the similar mode of association to salicylic acid may occur because of its structural anology with salicylic acid.



The spectrum of salicylamide in 3μ region shows two sharp bands with medium intensity at 3535 and 3415cm^{-1} which are respectively due to antisymmetric and symmetric stretching vibration of the nonbonded amino group (Fig. 4). In 6μ region a sharp band is observed at 1660cm^{-1} and two medium bands are found at 1625, 1605 cm^{-1}. The 1660 cm^{-1} band is assigned to the nenbonded carbonyl stretching vibration of the carbonyl amide group, and the 1625 cm^{-1} band is due to the bending vibration of the nonbonded free amide group. The 1605 cm^{-1} band is considered to arise mainly from the amino bending vibration

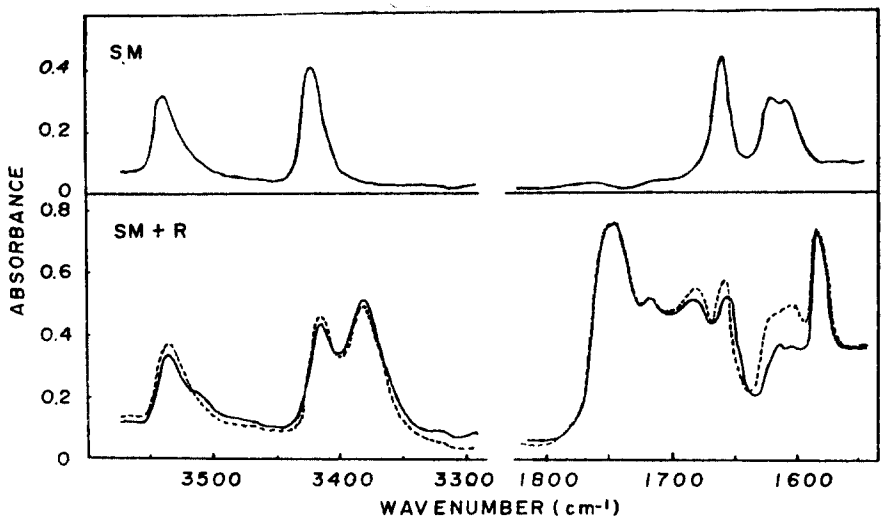


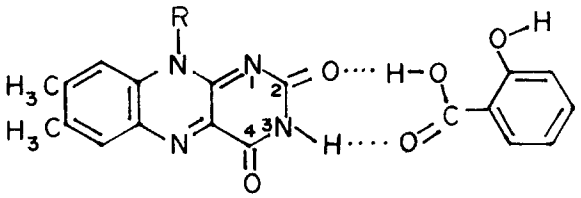
Fig. 4—Infrared spectra of salicylamide and 1 : 1 mixed solution with riboflavin tetraacetate.
3 μ region; 4×10^{-3}M in $CDCl_3$ 5 mm quartz cell.
6 μ region; 4×10^{-3}M in $CHCl_3$ 1 mm KBr cell.
Solid line; observed spectra.
Dotted line; calculated sum of the upper spectrum and the spectrum of riboflavin tetraacetate.

coupled with phenyl nucleus vibration mode.

When equimolar solutions of riboflavin tetraacetate and salicylamide are mixed together, the nonbonded bands of salicylamide become weak and association bands appear at 3480 cm^{-1} and 3355~3200 cm^{-1} in 3μ region. The former looks like a shoulder of the 3535cm^{-1} band and the latter is very broad. The 3380 cm^{-1} band becomes stronger than riboflavin tetraacetate alone, but in fact, it is probably the overlapped sum of nonbonded N-H of riboflavin tetraacetate and bonded N-H of salicylamide, and it may be considered that the nonbonded N-H stretching band of riboflavin tetraacetate decreases in intensity. In 6μ region, the 1690 cm^{-1} band and the broad band ranging 1625—1605 cm^{-1} become weak, and the 1660cm^{-1} band decreases in intensity and tends to shift to lower frequency. The 1745 cm^{-1} band and 1710 cm^{-1} band remain unchanged.

From the result of the spectrum of mixed solution, it is expected that the association occurs by using the hydrogan atom of N-H and the oxygen atom of carbonygroup of salicylamide and the hydrogen atom of N-H and the oxygen atom of 2-C carbonyl group of the isoalloxazine ring of riboflavin tetraacetate. Therefore it may be considered that 1 : 1 cyclic H bonded dimer through the amide group and carbonyl group of salicylamide and the imino group and the 2-C carbonyl group of isoalloxzine ring of riboflavin tetraacetate is the most probable.

The association between salicylamide and riboflavin tetraacetate is expected to be the strongest among three cases of association between salicylates and riboflavin tetraacetate,



because its spectrum shows apparent new association bands than the other cases, and the association with salicylic acid is more stronger than with aspirin from the inspection of the existence of the association band in 6 μ region.

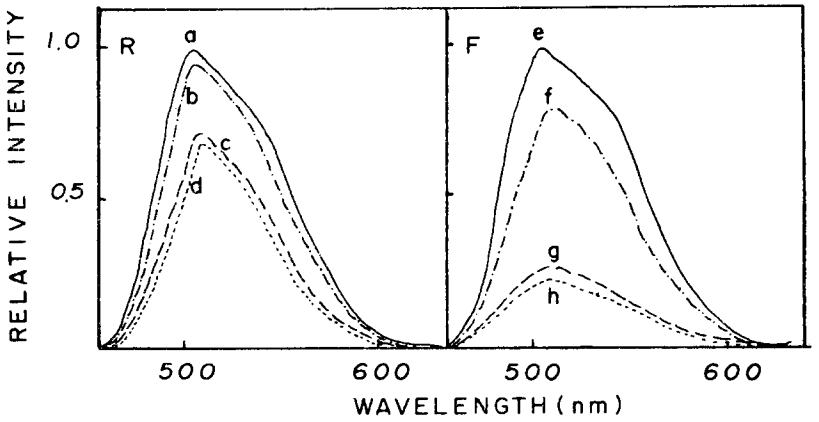


Fig. 5—Fluorescence spectra of riboflavin tetraacetate (R) in $CHCl_3$ solution and flavin mononucleotide (F) in aqueous solution.
a) R(2×10^{-5}M); b) R(2×10^{-5}M) and aspirin (2×10^{-2}M); c) R(2×10^{-5}M) and salicylic acid (2×10^{-2}M); d) R(2×10^{-5}M) and salicylamide (2×10^{-2}M); e) F(2×10^{-5}M); f) F(2×10^{-5}M) and aspirin (2×10^{-2}M); g) F(2×10^{-5}M) and salicylic acid (2×10^{-2}M); h) F(2×10^{-5}M) and salicylamide (2×10^{-2}M)

Fluorescence spectra—Fluorescence spectra of 2×10^{-5} M riboflavin derivatives are shown in Fig. 5.

When the same amount of salicylic acid derivatives was added to the solution respectively, there was no change in the fluorescence spectra of riboflavin tetraacetate and flavin mononucleotide (FMN). In this experiment we used dried commericial flavin mononucleotide. As the concentration of salicylic acid derivatives was increased obvious quenching was detected. Those quenchings are included in Table I. The 2×10^{-2}M solution of salicylic acid derivatives in Table I is due to the consideration of solubility of them on chloroform and water. We observed that quenching was released from 39 per cent to 36 per cent as temperature was increased from 20° to 40° in the case of riboflavin tetraacetate plus salicylamide solution. Therefore the quenching appears to be related to the formation of complex with salicylic acid derivatives through hydrogen bond as discussed by Kyogoku and Yu[8].

Table I—Quenching of fluorescence of riboflavin tetraacetate and FMN by salicylates

Quencher 2×10^{-2}M	Riboflavin tetraacetate 2×10^{-5}M (in $CHCl_3$)	FMN 2×10^{-5}M (in H_2O)
None	100	100
Aspirin	93	75
Salicylic acid	69	28
Salicylamide	61	22

The figures in the Table are values relative to the intensity of the fluorescence of pure solution at 20°. The error is estimated less than 2%.

The magnitude of quenching is increased in order of aspirin, salicylic acid and salicylamide. This is almost compatible with the anticipation by inspectron of infrared spectra.

However, according to common textbook of pharmacology[9], aspirin is more potent than salicylic acid as an analgesic and antipyretic, and salicylamide is much less effective than either. This is just reverse of our experimental result. Therefore it seems that the analgesic and antipyretic action of salicylates is not related to the effect of salicylates on the oxidative phosphorylation metabolism. But our view point is at the effect of salicylates on respiration. Salicylamide is, therefore, expected to be much potent in stimulant effect on respiration, insofar as this depends on hydrogen bonding with riboflavin.

Acknowledgment—The authors express their thanks to Miss Soo Young Choo for her assistance at the beginning of this work.

REFERENCES

1. Wilson, Gisvold, Doerge (Editors), *Textbook of Organic Medicinal and Pharmaceutical Chemisty*, Philadelphia, J.B. Lippincott Co., **1971**, p-49.
2. Y. Kyogoku, R.C. Lord, and A. Rich, *Nature*, **218**, 69 (1968).
3. B.S. Yu, *Korean Biochem. J.*, **3**, 61 (1970).
4. J.D.P. Graham, and W.A. Parker, *Quart. J. Med.*, **17**, 153 (1948).
5. T.M. Brody, *J. Pharmacol. Exp. Ther.*, **117**, 39 (1956).
6. K. Yagi, Y. Yamamoto and M. Kobayashi, *J. Vitaminol.*(Kyoto), **14**, 271 (1968).
7. G.C. Pimentel, A.L. McCellan, *The hydrogen bond*, San Francisco, W.H. Freeman & Co., **1960**, p-169.
8. Y. Kyogoku and B.S. Yu, *Bull. Chem. Soc. of Japan*, **42**, 1387 (1969).
9. A. Goth *Medical Pharmacology*, St. Louis, The C.V. Mosby Co., **1974**, p-328.
10. H. Horikuchi, *Sekigai Kyukotosetsu soran*, Tokyo, Sankyo Publ. Co., **1973**, p-114.