Journal of the Pharmaceutical Society of Korea
18, 209-216 (1974)

Specific Association of Riboflavin and Penicillin Derivatives in Chloroform Solution

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(Received September 10, 1974)

Abstract—From the measurements of infrared and fluorescence spectra riboflavin-2′, 3′, 4′, 5′-tetraacetate has been found to associate with penicillin-V more than strongly with themselves. They form the 1 : 1 cyclic hydrogen bonded dimer through the imino and the 2-C carbonyl groups of the isoalloxazine ring and the imino group of the penicillin-V ressidue. Penicillin-V is an effective quencher of the fluorescence of riboflavin tetraacetate. The quenching appears mainly due to the coplanar interction through hydrogen bonds partly due to the collision interaction with the penicillin ring.

It has been known that flavin deficiency from the use of antibiotics, in particular penicillin, oxytetracycline and chlortetracycline [1-6].

Several investigator have previous reported that the mechanism of appearance of riboflavin deficiency caused by antibiotics [2-5].

They suggested the inhibition of flavin enzyme is due to interaction between chlortetracycline and riboflavin [7].

The use of infrared spectroscopy has become customary for detecting the specific interaction of biologically important molecules in chloroform solutions. The method has been extended to the survey of the interaction of other kinds of molecules like drugs [8-10].

This paper will report that some of antibiotics such as penicillins, can inhibit the enzyme due to interaction with riboflavin which might be present in flavoenzyme. The results might help to interpret the nature of previously reported theory [6].

EXPERIMENTAL

Materials — Riboflavin-2′, 3′, 4′, 5′-tetraacetate was prepared by the method described

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previously [7]. A yellow flaky crystal was obtained (3 mg) (mp 246°C Found ; C, 53.95 ; H, 5.22 ; N, 10.41%).

Crude 6-phenoxyacetamido penicillinic acid (penicillin-V) was obtained by the addition of hydrochloric acid to commercial sodium salt. Resultant precipitate was recrystallized from hot water.

Procedures — For the measurement of infrared spectra the samples were dissolved in chloroform-d (E. Merck Co.) which was purified by passing through alumina gel column.

Infrared spectra were measured with a Perkin Elmer Model 421 (3μ region) and Beckmann Model 20 A infrared spectrophotometer. Fused quartz cell ranging 5 mm was used for measurement in the 3700-3000 cm^{-1} region and potassium bromide cell ranging 0.2mm 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 which the aid of the solvent curves as base lines. None deuterated chloroform was used as the solvent for the measurements of visible-ultraviolet and fluorescence spectra. The solvent was purified by distillation and by succesive passage through alumina gel column.

Visible-ultraviolet spectra were recorded with Hitachi 124 spectrophotometer. The fluorescence measurements were carried out with a Hitachi 204 fluorescence spectrophotometer equipped with a mercury lamp and a grating monochrometer. The activating wavelength was set at 360 mμ and the fluorescence was read in the range 380-640 mμ. 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 — Infrared spectra of the 0.004 M deuterochloroform solution of riboflavin tetraacetate, penicillin-V and the same mole mixture of them are given in figure 1.

The spectrum of riboflavin tetraacetate a strong sharp band is observed at 3380 cm^{-1} and a broad band at 3200 cm^{-1}. Since both of the bands disappear on the deuterium and methyl substitution at the 3-N position, they are associated with the N-H stretching vibrations. The apparent extingtion coefficient of the 3380 cm^{-1} band increases and that of the 3200 cm^{-1} band increases and that of the 3200 cm^{-1} band decreases with dilution of the solution. Therefore the former band is assignable to the nonbonded N-H stretching

vibration and the latter is considered to be related to the bonded N-H stretching vibration as discussed previously [7].

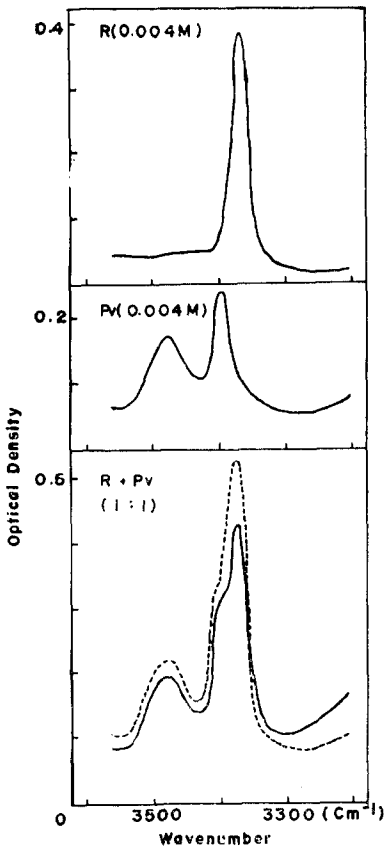


Fig. 1 — Infrared spectra of the deuterochloroform solutions in a 5 mm quartz cell.

Solid line ; observed spectra.
Dotted line ; calculated sum of the upper two spectra.

The spectrum of penicillin-V shows two strong bands at 3475 and 3400 cm^{-1} which are respectively due to O-H stretching vibration of the nonbonded carboxylic acid group and nonbonded N-H stretching vibration of imino group.

When both the solutions are mixed together the nonbonded bands decrease in intensity and new association bands appear at 3300-3200 cm^{-1} region strong and broad. It is difficult to assign each association band of mixture to one or both components.

In order to get further information on the structure of R-PV complex in solution the spectra in the carbonyl stretching region were studied. In the spectrum of the dilute solu-

tion of riboflavin tetraacetate a strong band is observed at 1745 cm^{-1} and two bands with medium intensity at 1710 cm^{-1} and 1960 cm^{-1} (Figure 2). On the deuteration of the 3-N position the strongest band remains unchanged whereas the 1710 cm^{-1} band moves to 1713 cm^{-1} and the 1690 cm^{-1} band shifts to 1675 cm^{-1}. The 1745 cm^{-1} band which appears at such high frequency region must be the carbonyl stretching band of the acetyl groups as discussed previously [7]. The 1710 cm^{-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 carbonyl bond.

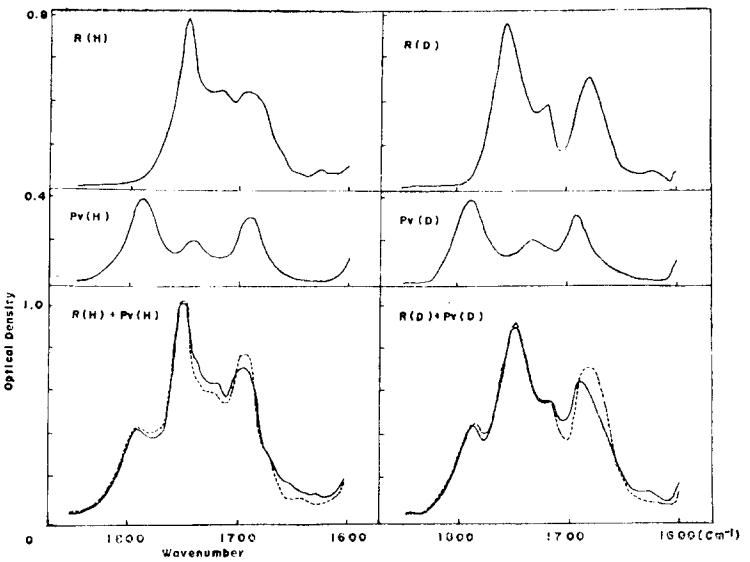


Fig. 2 — Infrared spectra of the carbonyl stretching region of riboflavin tetraacetate (R) and penicillin-V (Pv). The concentration of each solute is 0.004 M and path length is 0.2 mm.

H ; nondeuterated compound, D ; N-deuterated compound.
Solid line ; observed spectra. Dotted line : calculated sum of the upper two spectra.

The spectrum of penicillin-V shows three medium bands at 1800, 1740 and 1710 cm^{-1} which were assigned to the carbonyl stretching vibration band and N-H bending. The 1800 cm^{-1} band which appears at such high frequency region must be the carbonyl stretching band of 7-C in penicillin-V. The 1745 cm^{-1} band is considered to arise from non-bonded N-H vibration because on the deuteration of the imino group the band moves to 1735 cm^{-1} and the 1690 cm^{-1} band is assignable to the carbonyl stretching band of free carboxylic acid of 2′-C.

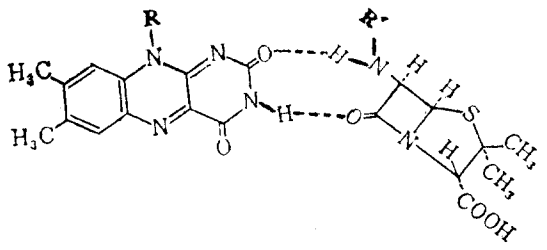
When both the solutions are mixed together, new association bands appear at 1730 and 1650 cm^{-1} and the band at 1690 cm^{-1} become weak. The 1745 cm^{-1} band remains unchanged. The 1710 and 1690 cm^{-1} bands of the coupled vibration of the carbonyl stretching and the N-H bending motions of riboflavin tetraacetate shift to 1700 and 1665 cm^{-1} respectively by the formation of hydrogen bonds.

The spectrum of penicillin-V in the 1800-1600 cm^{-1} region shows the carbonyl stretching band at 1800 cm^{-1}, which becomes weak and shifts to the lower frequency when riboflavin is added to the solution (Figure 2).

It is clear that a hydrogen bond is formed at the carbonyl group of 7-C. On the other hand in the N-deuterated compounds the bands at 1745 and 1713 cm^{-1} of the mixture are uneffected. Only the 1675 and 1800 cm^{-1} bands of carbonyl groups decrease in intensity and new band appears 1710 cm^{-1}. Since only the 1675 cm^{-1} band of N-deuterated compound shifts to lower frequency by the addition of penicillin-V, the 2-C carbonyl group of riboflavin tetraacetate seems to be used for the formation of hydrogen bond which penicillin-V as previous condition [8].

Besides this, it is doubtless that the imino group of the riboflavin tetraacetate and the imino group of penicillin-V are employed for the complex formation. This fact indicates the importance of the cyclic dimer formation for the association of riboflavin tetraacetate and penicillin-V.

From the present results, however, the following structure can be proposed for the association.



It is generally believed that there is some kind of interaction between isoalloxazine ring and adenine moieties of the FAD molecule [11-15] as a co-enzyme of flavoenzyme.

Antibiotics, especially penicillins or chlortetracycline, are known to be inhibitors in the flavoenzyme where FAD serves as a co-enzyme [16,17]

It is suggested, therefore, that penicillin-V inhibit the flavoenzyme by disrupting the structure of FAD ; it takes the flavin moiety from the intramolecular adenine and isoalloxazine complex.

Fluorescence Spectra — Fluorescence spectra of 1×10^{-1}M chloroform solution of riboflavin tetraacetate are given in figure 3.

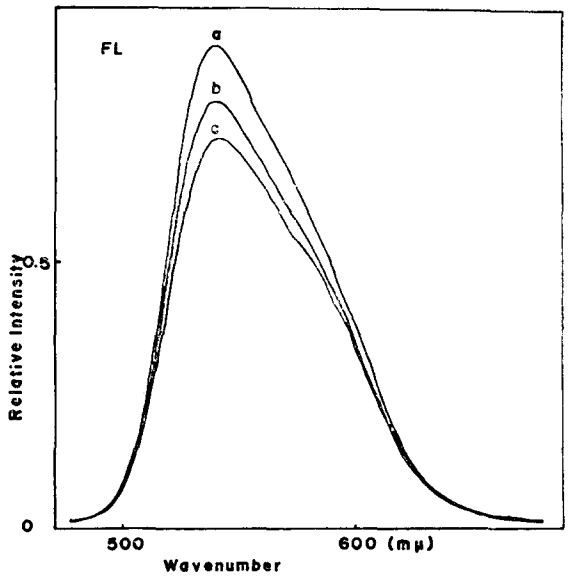


Fig. 3 — Fluorescence spectra of riboflavin tetraacetate in chloroform solution.
a ; R (1.2×10^{-4}M) b ; R (1.2×10^{-4}M) and Pv (1.2×10^{-3}M)
c ; R (1.2×10^{-4}M) and Pv (1.2×10^{-2}M)

When the same amount of penicillin-V was added to the solution, there was no change in the fluorescence spectrum of riboflavin tetraacetate. As the concentration of penicillin-V was increased, however, decisive quenching was detected. 15 percent quenching was observed for the solution containing 1×10^{-4}M riboflavin tetraacetate and 1×10^{-2}M penicillin-V.

The quenching appears to be related to the formation of the complex with penicillin-V through hydrogen bonds as discussed previously [8].

In this paper the number of the complex molecules at a given concentration did not considered.

Ultraviolet and Visible Spectrum — The spectrum of riboflavin tetraacetate and mixture of penicillin-V in chloroform are given in figure 4.

Spectrum in the same region were recorded for all of the mixture solutions that were

examined by the fluorescence measurements.

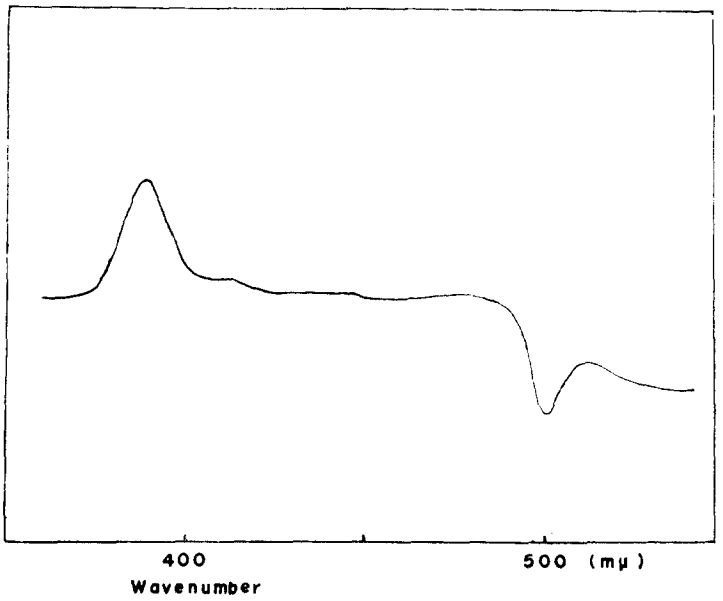


Fig. 4 — Differential UV spectrum of riboflavin tetraacetate (1.2×10^{-4}M) and mixture with penicillin-V (1.2×10^{-3}M) in chloroform solution.

The absorption at 320 mμ of the penicillin-V solution at 1×10^{-2}M is so strong that the spectrum of the mixture solution could not be recorded below 320 mμ. The shift and hypochromicity were appeared to be related to the formation of the complex with penicillin-V through hydrogen bonds, because the spectrum of riboflavin tetraacetate show red shift in 490 mμ region and blue shift in 380 mμ region when it is mixed together with penicillin-V.

The same phenomena were recognized from differential UV spectrum of mixture of 5×10^{-2}M penicillin-V.

Acknowledgment — The author expresses his sincere thank to Miss. E.J. Park for her experimental assistance. Thank is also due to Mr. S.U. Kim for the permisson to use fluorescence spectrometer.

This work was partly supported by a grant from the Ministry of Education, Republic of Korea (1974).

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