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Specific Association of Riboflavin and Adenine Derivatives in Chloroform Solution and the Effect of Barbiturates on the Association

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Riboflavin-2′,3′,4′,5′-tetraacetate and tetrabutyrate have been found to associate with 9-ethyladenine. They form stronger complexes with each other than with themselves and with other nucleic acid purine and pyrimidine derivatives. The association constants of the formation of the complexes were determined from the infrared spectra to be 130 and 95 M^{-1} respectively. The complex is a 1 : 1 cyclic hydrogen bonded dimer through the imino and the 2-C carbonyl groups of the isoalloxazine ring and the amino group of the adenine residue. 9-Ethyladenine is an effective quencher of the fluorescence of riboflavin tetraacetate. The quenching appears mainly due to the coplanar interaction through hydrogen bonds and partly due to the collision interaction with the purine rings. Some barbiturates were found to prevent the formation of the complex of the adenine and riboflavin derivatives by the formation of more stable complexes with ethyladenine.

The use of infrared spectroscopy has become customary for detecting the specific interaction of biologically important molecules in chloroform solutions. The method was first used for the detection of the specific association of the nucleic acid purine and pyrimidine derivatives.[1-7] It has been extended to the survey of the interaction of other kinds of molecules like drugs and coenzyme derivatives.[8-10] In a previous communication it was reported that riboflavin derivatives show fairly strong association only with an adenine compound and not with other nucleic acid base derivatives.[10] In this paper a detailed analysis of the infrared spectra of the complex will be presented and the structure of the complex will be proposed.

Previous investigators have reported that purine derivatives work as the quenchers of the fluorescence of riboflavin derivatives in aqueous solutions.[11-15] A similar phenomenon has been found in chloroform solutions. The mechanism of the quenching will be discussed in relation to the structure of flavin adenine dinucleotide (FAD).

Barbiturates were found to interact strongly with adenine derivatives.[8] In this paper it will be reported that some of barbiturates can disturb the interaction between riboflavin and adenine derivatives which might be present in the FAD molecule. Barbiturates can inhibit the respiration in mitochondria, which is thought to be related to their sedative activity in the central nervous system.[16,17] The present results might provide a basis for interpreting their mode of action.

Experimental

Materials. Riboflavin-2′,3′,4′,5′-tetraacetate (R) was prepared by the reaction of riboflavin with acetic

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anhydride in pyridine. The mixture of anhydrous pyridine (35 ml) and acetic anhydride (4 ml) suspended with dried riboflavin (3 g) was stirred for fifty hours at room temperature. The solution became clear and its color changed to deep orange with green fluorescence. Pyridine was removed by the addition of ethanol and successive evaporation. The sample was recrystallized from the ethanol and chloroform mixture. A yellow flaky crystal was obtained (5 g) (mp 246°C, Found: C, 53.95; H, 5.22; N, 10.41%). 3-*N*-Methylriboflavin-2′,3′,4′,5′-tetraacetate (MeR) was obtained from 3-methylriboflavin by applying the same procedure described above (mp 185°C, Found: C, 55.62; H, 5.49; N, 10.50%). 3-*N*-Methylriboflavin and ribofiavin-2′, 3′,4′,5′-tetrabutyrate (RB) were kindly provided by Professor K. Yagi of Nagoya University and by Professor J. Okuda of Meijo University.

9-Ethyladenine (A), 1-cyclohexyluracil (U), 1-cyclohexyl-5-bromouracil (BrU), 2′,3′-benzylidene-5′-trityl-guanosine (G) and 2′,3′-benzylidene-5′-trityl-cytidine (C) were purchased from Cyclo Chemical Co., Los Angeles, U.S.A. Commercial 5-ethyl-5-phenylbarbituric acid (phenobarbital) (PB) was used after recrystallization from hot water. Crude 1-methyl-5-cyclohexenyl-5-methylbarbituric acid (CB) was obtained by the addition of hydrochloric acid to the commerical sodium salt. Resultant precipitate was recrystallized from hot water.

Procedures. For the measurement of infrared spectra the samples were dissolved in chloroform-*d* (Showa Denko Co., Kawasaki) which was purified by passing through alumina gel column. Infrared spectra were measured with a Perkin Elmer Model 621 infrared spectrophotometer. Fused quartz cells ranging from 1 to 25 mm were used for the measurement in the 3700—3000 cm^{-1} region and potassium bromide cells (including a variable thickness cell) ranging from 0.1 to 5 mm were used for the 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 observed transmission with the aid of the solvent curves as base lines.

From the concentration dependence of the intensity of the monomer bands, association constants were calculated on the theory described previously.[5,6] Six to ten spectra were recorded at different concentrations between 0.1 and 0.001M for the determination of each association constant. It is estimated that the experimental errors of measurement yield relative association constants which are accurate to ±15 per cent.

Non-deuterated chloroform was used as the solvent for the measurements of visible-ultraviolet and fluorescence spectra. The solvent was purified by distillation and by successive passage through alumina gel column. Visible-ultraviolet spectra were recorded with a Hitachi 124 spectrophotometer. The fluorescence measurements were carried out with a Hitachi MPF-2A fluorescence spectrophotometer equipped with a xenon lamp and a grating monochromator. The activating wavelength was set at 360 mμ with a slit width of 10 mμ and the fluorescence was read in the range 380—700 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

Specific Association of Riboflavin Tetraacetate with Ethyladenine. *Infrared Spectra.* In the spectrum of the 0.02M solution of riboflavin tetraacetate a strong sharp band is observed at 3380 cm^{-1} and a broad band at 3200 cm^{-1} (Fig. 1). Since both of the bands disappear on the deuterium and methyl substitution at the 3-N position, they are associated with the NH stretching vibrations. The apparent extinction coeffecient of the 3380 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 NH stretching vibration and the latter is considered to be related to the bonded NH stretching vibration.

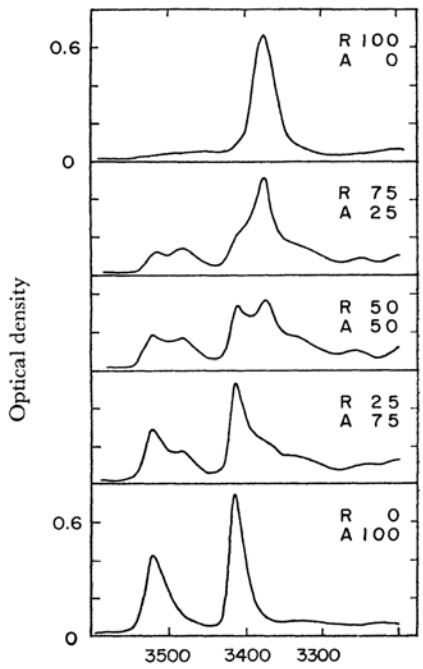


Fig. 1. Infrared spectra of various mixtures of riboflavin tetraacetate (R) and ethyladenine (A). The total concentration of the material is 0.02M and the path length is 2 mm.

The spectrum of 9-ethyladenine shows two strong bands at 3525 and 3414 cm^{-1} which are respectively due to the antisymmetric and symmetric NH stretching vibrations of the nonbonded amino group as discussed previously[5] (Fig. 1). The self-association of the ethyladenine molecules at the 0.02M solution must be negligibly small because the self-association bands at 3485, 3310, 3250 and 3200 cm^{-1} are very weak.

When both the solutions are mixed together, the nonbonded bands decrease in intensity and new

association bands appear at 3485, 3330, 3260, and 3200 cm^{-1}. The positions of some of these bands are different from those of the self-association bands of ethyladenine and riboflavin tetraacetate. It is difficult to assign each association band of the mixture to one or both components. The spectra of various mixtures of riboflavin tetraacetate and ethyladenine are given in Fig. 1. The intensity of the association bands at 3485 and 3330 cm^{-1} changes as a function of the mole ratio and reaches its maximum in the 1 : 1 region (Fig. 2). Similar

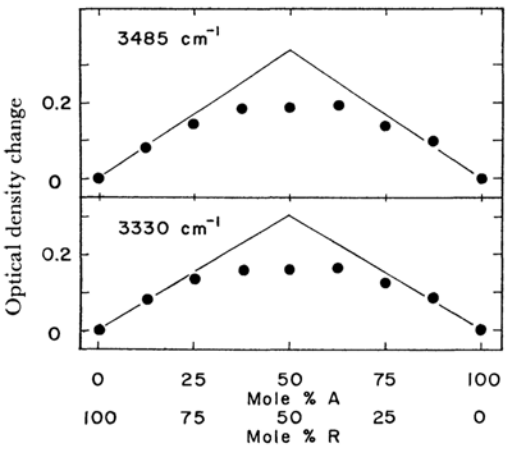


Fig. 2. Change in the optical density of the association bands found at 3485 and 3330 cm^{-1} as a function of the mole ratio of riboflavin tetraacetate and ethyladenine. The optical density of the pure solution is adjusted to zero.

phenomenon was observed for the riboflavin tetrabutyrate and ethyladenine mixture. But the spectrum of ethyladenine does not change to any observable extent by the addition of *N*-methylriboflavin tetraacetate. There is no interaction between them at this concentration.

The association constants for the formation of the R-A complex and the RB-A complex at 25°C were determined to be 130 and 95 M^{-1} respectively. The self-association constant of riboflavin tetraacetate is 4.5 and that of riboflavin tetrabutyrate is 2.3M^{-1}. The monomer band of the riboflavin derivatives at 3380 cm^{-1} and that of ethyladenine at 3525 cm^{-1} were used for the determination.

Although riboflavin tetraacetate and tetrabutyrate associate with ethyladenine fairly strongly, their interaction with other nucleic acid purine and pyrimidine derivatives are negligibly small. Figure 3 shows the infrared spectra of the equimolar mixtures of R-A, R-U, R-C and R-G at 0.008M. Except for the R-A mixture the observed spectra (solid line) are virtually identical to the sum (dotted line) of the component spectra.

In order to get further information on the structure of the R-A complex in solution the spectra in the carbonyl stretching region were studied. In the spectrum of the dilute solution of riboflavin tetraacetate a strong band is observed at 1745 cm^{-1} and two bands with medium intensity at 1710 and 1690 cm^{-1} (Fig. 4). 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 infrared spectrum of 3-*N*-methylriboflavin tetraacetate shows three bands at 1745, 1705 and 1660 cm^{-1}). The 1745 cm^{-1} band which appears at such high frequency region must be the carbonyl stretching band of the acetyl groups. The 1710 cm^{-1} band is considered to arise mainly from the

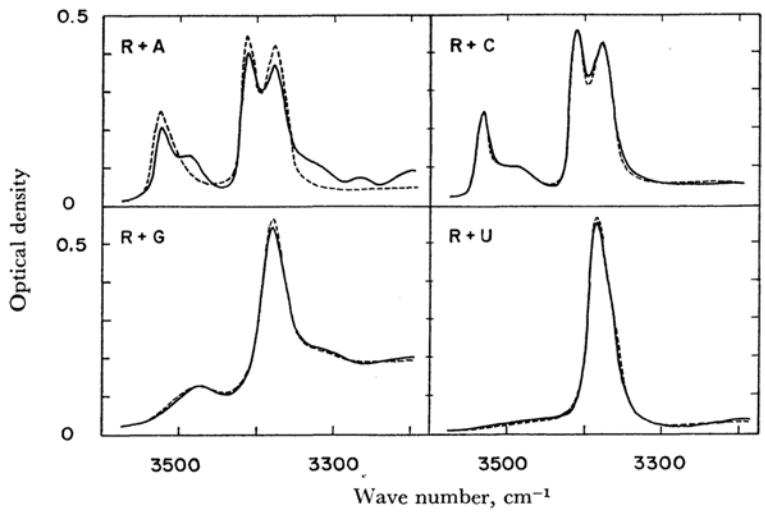


Fig. 3. Infrared spectra of the equimolar mixture solutions of riboflavin tetraacetate and one of the A, U, G and C compounds in chloroform. The total concentration is 0.008M and the path length is 5 mm. See text.

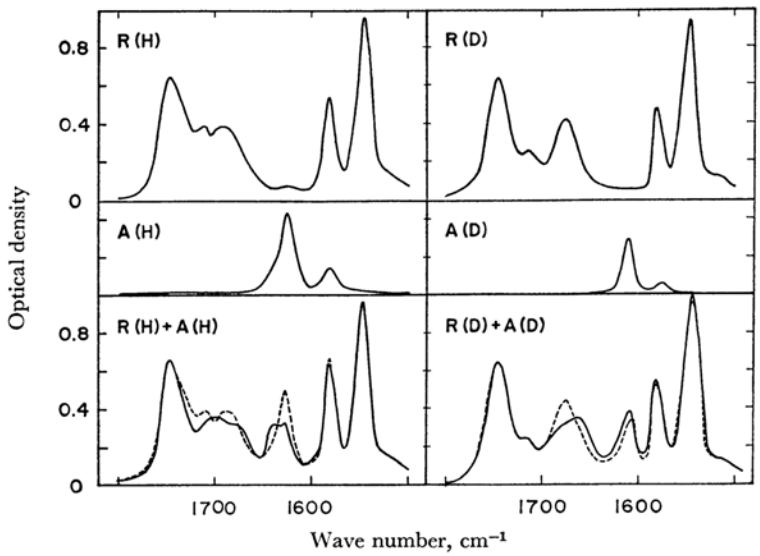


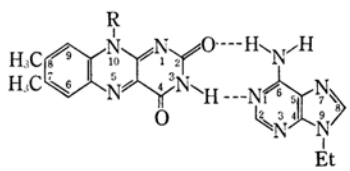
Fig. 4. Infrared spectra of the carbonyl stretching region of riboflavin tetraacetate (R) and ethyladenine (A). The concentration of each solute is 0.05M and the path length is 0.1 mm. H, nondeuterated compound; D, *N*-deuterated compound. Solid line, observed spectra; dotted line, calculated sum of the upper two spectra.

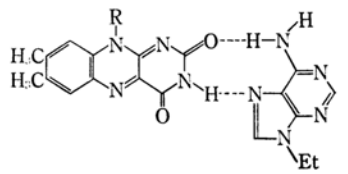
4-C carbonyl group and the 1685 cm^{-1} band from the 2-C carbonyl stretching vibrations coupled with the NH bending mode, since the 2-C carbonyl bond, being conjugated with the C=N bonds, should have lower frequency vibration than the 4-C carbonyl bond. Therefore the bands at 1713 and 1675 cm^{-1} of the *N*-deuterated compound can be assigned to the pure stretching modes of the 4-C and 2-C carbonyl groups respectively.

The spectrum of ethyladenine shows a medium band at 1625 cm^{-1} which was assigned to the coupled vibration of the NH_2 scissors vibration and the ring stretching motions.[5] On the deuteration of the amino group the band moves to 1612 cm^{-1}.

When both the solutions are mixed together, new association bands appear at 1700, 1668 and 1640 cm^{-1} and the bands at 1710, 1685 and 1625 cm^{-1} become weak. The 1745 cm^{-1} band remains unchanged. The 1710 and 1690 cm^{-1} bands of the coupled vibrations of the carbonyl stretching and the NH bending motions of riboflavin tetraacetate shift to 1700 and 1665 cm^{-1} respectively by the formation of hydrogen bonds. The NH_2 scissors vibration at 1625 cm^{-1} of ethyladenine must be moved to 1640 cm^{-1}. On the other hand in the spectrum of the mixture solution of the *N*-deuterated compounds the bands at 1745 and 1713 cm^{-1} of riboflavin tetraacetate are unaffected. Only the 1675 cm^{-1} band of the 2-C carbonyl group decreases in intensity and a new band appears at 1660 cm^{-1}. The 1612 cm^{-1} band of *N*-deuterated ethyladenine shifts to 1615 cm^{-1}.

Since only the 1675 cm^{-1} band of the *N*-deuterated compound shifts to lower frequency by the addition of ethyladenine, the 2-C carbonyl group of riboflavin tetraacetate seems to be used for the formation of the hydrogen bond with ethyladenine. Besides this it is doubtless that the imino group of the riboflavin tetraacetate and the amino group of ethyladenine are employed for the complex formation. Ethyladenine does not interact with *N*-methylriboflavin tetraacetate which is still capable of forming linear hydrogen bond with ethyladenine. This fact indicates the importance of the cyclic dimer formation for the association of riboflavin tetraacetate and ethyladenine. From the present results, however, it can not be concluded which nitrogen atom at the 1 or 7 position of ethyladenine is used for the complex formation.





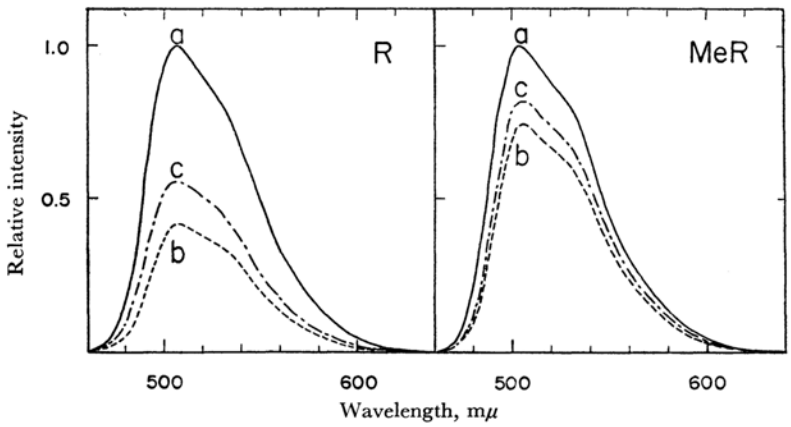


Fig. 5. Fluorescence spectra of riboflavin tetraacetate (R) and *N*-methylriboflavin tetraacetate (MeR) in chloroform solution.
a) R(2×10^{-5}M); b) R (2×10^{-5}M) and A (2×10^{-2}M);
c) R (2×10^{-5}M), A (2×10^{-2}M) and CB (2×10^{-2}M).

Recently the complex crystals of riboflavin and adenosine monophosphate[18] and of riboflavin and 8-bromoadenosine[19] have been obtained. Although their detailed structure have not yet been reported, such structure as shown above can be expected in those complex crystals.

In dilute solution of organic solvents the existence of the ring stacked complex is less possible as seen by analogy to cases of nucleic acid purine and pyrimidine complexes. The NMR study showed the absence of stacking in the chloroform solution of 9-ethyladenine and 1-cyclohexyluracil,[20] although purine derivatives in the vertical interaction caused by hydrophobic bonds are predominant in the aqueous solution at higher concentration.[21] It is generally believed that there is some kind of interaction between isoalloxazine and adenine moieties of the FAD molecule.[11–15] Not only the stacked model but also the coplanar interaction in the molecule must be taken into consideration.

Fluorescence Spectra. Fluorescence spectra of the 2×10^{-5}M chloroform solution of riboflavin tetraacetate are given in Fig. 5. When the same amount of ethyladenine was added to the solution, there was no change in the fluorescence spectrum of riboflavin tetraacetate. As the concentration of ethyladenine was increased, however, decisive quenching was detected. Sixty per cent quenching was observed for the solution containing 2×10^{-5}M riboflavin tetraacetate and 2×10^{-2}M ethyladenine. The other nucleic acid base derivatives gave smaller quenching effect than ethyladenine did (Table 1). The solubility of the C compound is less than 0.01M and the result obtained by the addition of its 0.01M solution is given in the table.

Table 1. Quenching of the fluorescence of riboflavin tetraacetate and *N*-methylriboflavin tetraacetate by some purine and pyrimidine derivatives in chloroform solution

(The figures in the table are values relative to the intensity of the fluorescence of the pure solution at 25°C. Those in parentheses are intensities relative to that of pure solution at 50°C. The error is estimated less than 2%.)

Quencher 2×10^{-2}M each	Riboflavin tetraacetate 2×10^{-5}M		*N*-Methylriboflavin tetraacetate 2×10^{-5}M	
	25°C	50°C	25°C	50°C
None	100	95 (100)	100	96 (100)
A	40	43 (45)	76	70 (73)
G	60	36 (38)	67	40 (42)
C*	92		100	
U	92		100	
BrU	93		101	
CB	99		101	
A+U	45		82	
A+BrU	50		85	
A+CB	48		83	

* 1×10^{-2}M

The quenching appears to be related to the formation of the complex with ethyladenine through hydrogen bonds, because *N*-methylriboflavin tetraacetate shows much smaller quenching effect when it is mixed together with ethyladenine. The number of the complex molecules at a given concentration can be calculated from the association

18) K. Uehara, T. Mizoguchi, S. Hosomi, T. Fujiwara and K. Tomita, *J. Biochem.*, **64**, 589 (1968).
19) D. Voet and A. Rich, private communication.
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21) M. V. Schweizer, S. I. Chan and P. O. P. Ts'o, *J. Am. Chem. Soc.*, **87**, 5241 (1965).

constant determined by the infrared measurements. In the binary solution of riboflavin tetraacetate (2×10^{-5}M) and ethyladenine (2×10^{-2}M) seventy two per cent of the riboflavin tetraacetate molecules are bound to ethyladenine. Since the association constant for the complex formation of R-U, R-C and R-G are estimated by the infrared measurements to be in the order of magnitude of 10M^{-1}, about ten per cent of riboflavin tetraacetate must be bound to those base derivatives. The amount of quenching is in the same order of the percentage of the riboflavin tetraacetate molecules used for the complex formation. This is another evidence showing that the quenching is mainly caused by the formation of hydrogen bonding.

However, the G compound shows greater quenching effect in both of the mixture solutions of R and MeR than those expected from the infrared measurements. Ethyladenine also shows greater quenching effect than U and C do on the fluorescence of *N*-methylriboflavin which can not form the hydrogen bonded complex. Purine compounds appear to possess a quenching ability without forming a hydrogen bond. It may be caused by a simple collision process between the isoalloxazine and purine rings. To test this idea the temperature dependency of the quenching was measured. The fluorescence of the R-A complex solution at 50°C became stronger than that at room temperature, *i.e.*, the extent of quenching decreased. This may be due to the increase of the number of the free riboflavin molecules at higher temperature. On the other hand the quenching observed for the MeR+A, R+G and MeR+G solutions increased at higher temperature. This phenomenon can be explained by the increase of the thermal collisions of the molecules at higher temperatures.[22]

The intensity of the fluorescence of FAD is about one tenth of that of riboflavin monophosphate.[15] Such big quenching could be caused not only by the vertical interaction of the isoalloxazine and adenine rings[14] but also by the coplanar interaction of the rings in the FAD molecule.

Ultraviolet and Visible Spectra. The spectra of riboflavin tetraacetate and *N*-methylriboflavin tetraacetate in chloroform are given in Fig. 6. Spectra in the same region were recorded for all of the mixture solutions that were examined by the fluorescence measurements. The absorption at 260 mμ of the purine and pyrimidine derivatives solution at 2×10^{-2}M is so strong that the spectra of the mixture solutions could not be recorded below 300 mμ. Neither position nor intensity of the absorption between 300 and 500 mμ of riboflavin tetraacetate changes by the addition of the base derivatives.

The Effects of Barbiturates on the Specific Association. In a previous paper it was reported that ethyladenine forms a stable complex with phenobarbital, the association constant of which is 1200M^{-1}.[8] The equimolar mixture of ethyladenine and phenobarbital gives precipitate, if the concentration of the solution is more than 10^{-3}M. When 10^{-3}M of phenobarbital was added to the chloroform solution containing 10^{-3}M of ethyladenine and riboflavin tetraacetate, phenobarbital-ethyladenine precipitation was still produced. The presence of riboflavin tetraacetate does not affect the formation of the phenobarbital - ethyladenine complex. In such a dilute solution as 10^{-3}M, however, the amount of the riboflavin tetraacetate-ethyladenine complex is negligibly small and most of the ethyladenine molecules exist as monomers when phenobarbital is added to the solution. Therefore 1-ethyl-5-cyclohexenyl-5-methylbarbituric acid whose complex with ethyladenine is quite soluble has been employed. The use of the compound makes it possible for us to see the effect

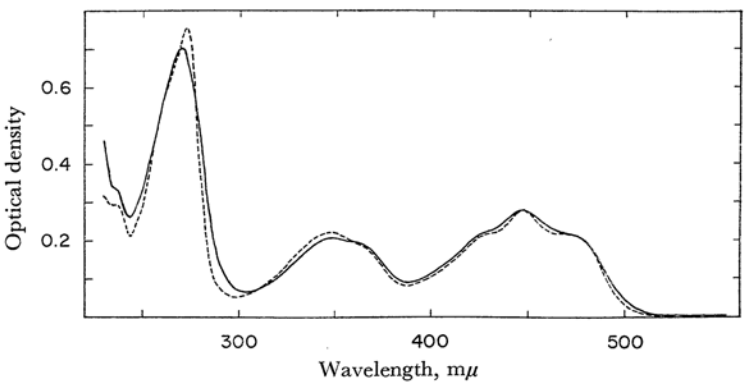

Fig. 6. Ultraviolet and visible spectra of riboflavin tetraacetate (—) and *N*-methylriboflavin tetraacetate (···) in chloroform. The concentration of the solution is 2×10^{-5}M and the path length is 1 cm.

22) K. Yagi, Z. Yoshida and R. Ohata, "Keiko (Fluorescence)," Nankodo, Tokyo (1958), p. 68.

of the addition of barbiturate at a high concentration where the R-A complex is predominant in the solution. The association constant of the CB-A complex has been determined to be 170M^{-1} which is fairly smaller than that of the PB-A complex.

An infrared spectrum was measured with the solution containing equimolar A, R and CB at 0.04M The observed spectrum is different from the calculated sum of the individual spectrum and the strong association bands are observed. But the positions of the association bands of the R-A and the CB-A complexes are almost the same. It is not possible to distinguish which complex is predominant in the mixture solution from the band position and intensity.

In the fluorescence spectrum of the R-A solution the decrease of quenching by the addition of CB was detected. Twenty per cent increase of the fluorescence of the mixture solution, *i.e.*, eight per cent recovery of the original fluorescence of riboflavin tetraacetate was observed, when 2×10^{-2}M solution of CB was added to the mixture solution containing 2×10^{-2}M of A and 2×10^{-5}M of R. The CB-A complex is more stable than the R-A complex and the added CB releases R from the R-A complex. The binding site of A in the R-A complex appears the same as that in the CB-A complex and the triple complex like CB-(R-A) is not predominant. The enhancement of the fluorescence of the R-A solution, however, is not the special activity of CB. 1-Cyclohexyluracil and 1-cyclohexyl-5-bromouracil, which associate with ethyladenine at the constants 100 and 250M^{-1}[5,6] respectively, can also increase the fluorescence of the R-A complex. Their ability to disturb the formation of the R-A complex is not parallel with the inhibitory activity in the respiratory chain, since uracil derivatives do not have such activity. Permeability and solubility differences of these compounds in mitochondria must be taken into account.

Increase of the fluorescence of the MeR+A solution by the addition of CB or uracil derivative was also observed. Thermal motion of the ethyladenine molecule may be reduced by the formation of the complex with these compounds.

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