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Association of a Reduced Riboflavin Derivative with Purines, Pyrimidines and Nicotinamide

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Hydrogen bonding of a reduced riboflavin derivative in chloroform has been studied by infrared spectra measurements. The self-association of the reduced form of riboflavin tetraacetate is much stronger than that of the oxidized form. The reduced form can also form hydrogen bonded complexes with some aminopurines, aminopyrimidines, aminopyridines and nicotinamide derivatives. In the bonding with aminopyridines the contribution of a proton transfer must be predominant. At partial reduction states the formation of radicals, semiquinone forms, was detected with the aid of the electron spin resonance. The concentration of free radicals, however, is about 1 per cent of dissolved riboflavin tetraacetate. The infrared spectra at various stage of reduction can be interpreted by the sum of the two individual spectra of the fully reduced and oxidized forms.

In previous papers selective formation of hydrogen bonding between the oxidized forms of riboflavin derivatives and adenine compounds has been reported.[1-3] The association of oxidized riboflavin is thought to be relevant to the intra- and intermolecular interaction of flavin adenine dinucleotide[4-10] and to have an important role in keeping the structure of FAD.[10] The isoalloxazine ring of riboflavin takes hydrogenated and dehydrogenated forms and hence it can be an electron carrier in a respiratory chain. The present work will show the effect of hydrogenation on the association of a riboflavin derivative with other compounds such as purines, pyrimidines and nicotinamide. The results might help to interpret the nature of previously reported complexes like FMN-$FMNH_2$ and NAD^+-$FMNH_2$.[11-15],*1

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Experimental

Materials. Riboflavin-2′,3′,4′,5′-tetraacetate and 3-*N*-methylriboflavin-2′,3′,4′,5′-tetraacetate were prepared by the method described previously.[2] 9-Ethyl adenine, 1-cyclohexyluracil, 2′3′-benzylidine-5′-tritylguanosine, 2′,3′-benzylidine-5′-tritylcytidine and 4-aminopyrimidine were purchased from Cyclo Chemical Co., Los Angeles. The other chemicals were obtained from Tokyo Kasei Co. Some of them were used after recrystallization from proper solvents and the others were used without further purification. Chloroform and chloroform-d (Showa Denko Co., Kawasaki) were employed as solvents for the infrared measurements. Chloroform was dried with phosphorous pentoxide and purified by distillation. Chloroform-d was dried by passing through an alumina gel column.

The chloroform solutions of reduced riboflavin derivatives were prepared by shaking the solutions of the oxidized forms with an aqueous solution saturated with hydrosulfite. The solutions were then dried with calcium chloride. Completion of reduction was checked by visible-ultraviolet spectra, disappearance of absorptions at 450 and 350 mμ and appearance of a strong band at 300 mμ. Partial oxidation of the reduced form was done by bubbling oxygen gas in the solution of the reduced form or by mixing it with the solution of the oxidized form. Reduced *N*-methylnicotinamide was prepared from *N*-methylnicotinamide chloride by the method of Rafter and Colowick.[16] Hydrogenation at the 4-C position was confirmed by NMR measurement.

Procedures. Infrared spectra were measured with a Perkin-Elmer 621 spectrophotometer for chloroform solutions in fused quartz cells ranging from 1 to 25 mm in thickness. The spectra in the region 1800—1500 cm^{-1} were obtained by the use of potassium bromide cells with variable thickness. To avoid oxidation by atmospheric oxygen the solutions of the reduced samples were prepared in a dry box filled with nitrogen gas.

Electron spin resonance spectra were taken with a JEOL P-10 spectrometer. The concentration of free radicals was determined by comparison of the integrated area of signals with that of a standard solution of diphenyl picryl hydrazyl (Nakarai Chemical Co., Kyoto). Standard 0.01M solution was made up in chloroform. The visible-ultraviolet spectra were recorded with a Hitachi 124 spectrophotometer.

Determination of Association Constants. The self-association constant of RH was calculated from concentration dependence of a monomer band based on the theory described previously.[17] Eight spectra were recorded at different concentrations between 10^{-2} and 10^{-4}M.

Association constants between A and B can be estimated from the optical density change of the association or nonbonded band of A plotted against the mole ratio of mixing. The tangent lines (Fig. 4) show the optical density change which would occur when all of A bond to B. From the location of the cross point of the two lines, the numbers of moles, m and n, in the following equilibrium can be determined.

$$mA + nB \rightleftarrows A_mB_n$$

The association constant K in the equilibrium may be written as

$$K = \frac{C_{AB}}{C_A{}^m C_B{}^n} \tag{1}$$

where C_A, C_B and C_{AB} are the concentrations of monomer A, monomer B and the complex respectively. The length of L from the cross point to the base line is proportional to C_{0A}, the initial concentration of A, and the height l of the observed curve is proportional to mC_{AB}, the concentration of A which is used for the complex formation. Thus the following relations hold

$$\frac{C_{0A}}{L} = \frac{C_A}{L-l} = \frac{mC_{AB}}{l} \tag{2}$$

Similar relations are written for the component B

$$\frac{C_{0B}}{L} = \frac{C_B}{L-l} = \frac{nC_{AB}}{l} \tag{3}$$

The initial concentration C_{0A} and C_{0B} are related to the total concentration C_0 as follows.

$$C_{0A} = \frac{m}{m+n}C_0, \quad C_{0B} = \frac{n}{m+n}C_0 \tag{4}$$

From the relations (1), (2), (3) and (4) it follows that

$$K = \frac{(m+n)^{m+n-1}\dfrac{l}{L}}{m^m n^n \left(1-\dfrac{l}{L}\right)^{m+n} C_0{}^{m+n-1}} \tag{5}$$

The association constants determined by the above method are only reliable within the order of magnitude.

Results and Discussion

Self - association of Reduced Riboflavin Tetraacetate. *Fully Reduced Form.* The infrared spectra of RH and MeRH are given in Fig. 1. The spectrum of fully reduced RH shows very broad strong bands in the 3 μ region contradicting with the sharp N_3–H stretching band of the oxidized form R. MeRH whose oxidized form does not show any absorption in the 3 μ region also gives a broad band around 3400 cm^{-1}. These evidences indicate that the broad bands come from the hydrogenated NH groups at the N_1 and N_5 positions of the reduced isoalloxazine ring. The carbonyl

*1 The abbreviations used are; FMN and $FMNH_2$, the oxidized and reduced forms of flavin mononucleotide; NAD^+ and NADH, the oxidized and reduced froms of nicotinamide adenine dinucleotide; R and RH, the oxidized and reduced forms of riboflavin-2′,3′,4′,5′-tetraacetate; MeR and MeRH, the oxidized and reduced forms of 3-*N*-methylriboflavin-2′,3′,4′,5′-tetraacetate; FAD, flavin adenine dinucleotide; NA, nicotinamide; A, 9-ethyladenine; U, 1-cyclohexyluracil; G, 2′,3′-benzylidine-5′-tritylguanosine; C, 2′,3′-benzylidine-5′-tritylcytidine; NMR, nuclear magnetic resonance; ESR, electron spin resonance; DPPH, diphenyl picryl hydrazyl.

16) G. W. Rafter and S. P. Colowick, *J. Biol. Chem.*, **206**, 773 (1954).

17) Y. Kyogoku, R. C. Lord and A. Rich, *J. Amer. Chem. Soc.*, **89**, 496 (1967).

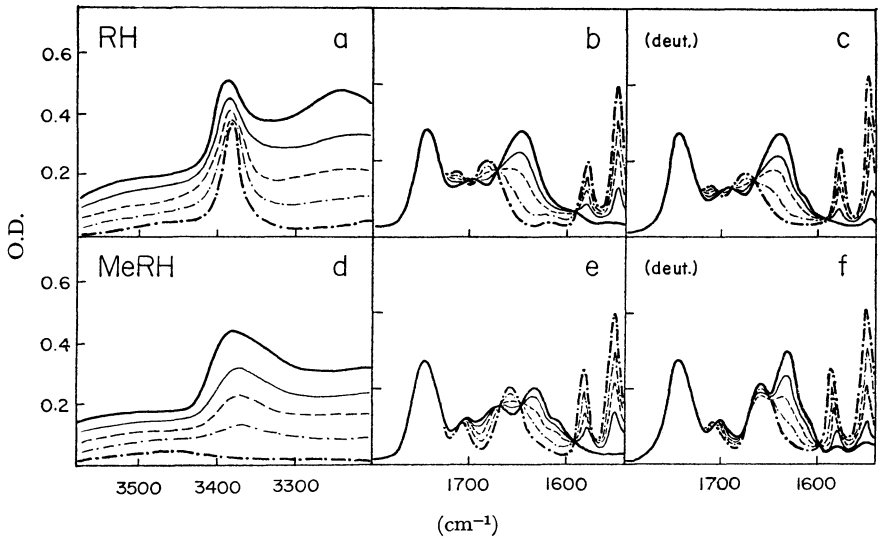
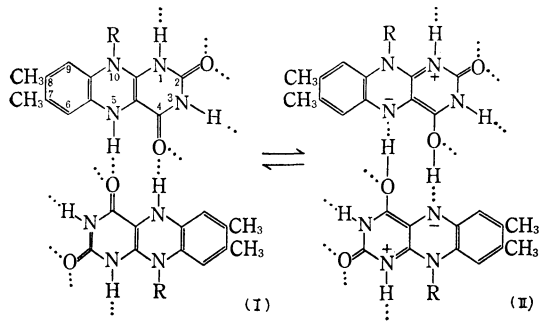


Fig. 1. Infrared spectra of the chloroform solutions of riboflavin tetraacetate (a, b, c) and *N*-methylriboflavin tetraacetate (d, e, f) in the reduced and oxidized forms. The concentration of the solution is 4×10^{-3}M, and the path length is 5 mm for the 3500—3200 cm^{-1} region and 0.5 mm for the 1800—1500 cm^{-1} region. Bold solid line, fully reduced state; bold broken line, fully oxidized state; other three fine lines, partially reduced and oxidized states.

stretching bands at 1715 and 1685 cm^{-1} of R move to lower frequency and the ring stretching bands at 1585 and 1550 cm^{-1} disappear by hydrogenation. The spectral feature of the 6 μ region shows a remarkable change in the conjugated system of the isoalloxazine ring.

The broad strong bands in the 3 μ and 6 μ regions indicate the strong self-association of RH. The fully reduced isoalloxazine ring has three NH and two carbonyl groups and thus four sites can be used for cyclic hydrogen bonds with another RH molecule. Therefore the self-association constant must be at least four times, *i. e.* about 600M^{-1}, as big as that of R which has two sites for the cyclic dimer formation. From the concentration dependence of the 3400 cm^{-1} band of RH the self-association constant was roughly estimated to be $10^3-10^4M^{-1}$. The ability of the formation of hydrogen bonds of the N_1H and N_5H sites are much stronger than that of the N_3H group. This is understandable because the p*K* values of the N_1 and N_5 protons are around 6.5 whereas that of N_3 is 10.[8] The reduced form is a weak acid. The lower frequency shift of the carbonyl stretching band shows the increase of single bond nature in the associated reduced form. The contribution of the proton transfer form (II) may not be so small.



Partially Reduced State. From the potentiometric, spectral and magnetic resonance observations several investigators concluded that riboflavin is reduced and oxidized in two one electron steps and pass through a semiquinoid state.[12,13,18,19] The infrared spectra of RH and MeRH at successive oxidation states from full reduction to full oxidation are given in Fig. 1. The strong NH band of RH gradually decreases in intensity as the per cent of oxidation is increased. Gradual shifts and intensity changes of absorption bands are also observed in the 1800—1500 cm^{-1} region and isosbestic points are seen at 1670 and 1590 cm^{-1}. It is clear from the results that the infrared spectra at intermediate oxidation are explained by the sum of the spectra of two states, fully oxidized and reduced states, and any new band due to transient intermediate could not be observed. In the visible-near infrared spectrum of the FMN-$FMNH_2$ mixture in aqueous solution two bands were reported in addition to the bands due to FMN and $FMNH_2$, one with

18) R. Kuhn and T. Wagner-Jauregg, *Ber.*, **67**, 361 (1934).

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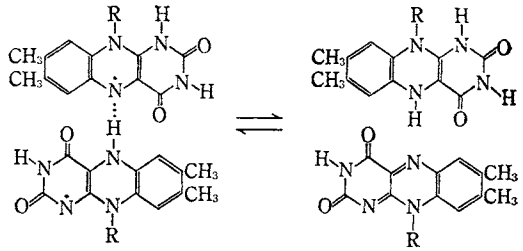
Fig. 2. Electron spin resonance spectra at 50% reduction of 1×10^{-2}M riboflavin tetraacetate in chloroform (a), plus 1×10^{-2}M 9-ethyladenine (b). The spectra were taken at the same instrument setting. Field modulation, 100 kc.

a maximum absorption near 570 mμ and another with a peak at 900 mμ.[12,13] The 570 mμ band was thought to be due to semiquinone, since it increases in intensity to a maximum at about half oxidation together with the increase of the signal intensity of ESR. The 900 mμ band was assigned to the dimer of semiquinone[12] or to the charge transfer complex of FMN-$FMNH_2$.[13] In the present investigation, however, no absorption could be seen in the wavelength region longer than 500 mμ even for the 0.01M solution of RH at 50% oxidation in 1 cm cell. The visible-near infrared spectra of the partially oxidized state did not show the presence of a intermediate species.

ESR was measured for the 0.01M solution of R at 50% reduction and a signal was observed (Fig. 2). From a comparison of the integrated area of the signal with that of DPPH, the concentration of free radicals was calculated to be 1×10^{-4}M, 1% of dissolved R. The signal amplitude did not change one hour after the preparation of the solution. Oxidation of RH by molecular oxygen or by mixing it with R produces radicals, semi-



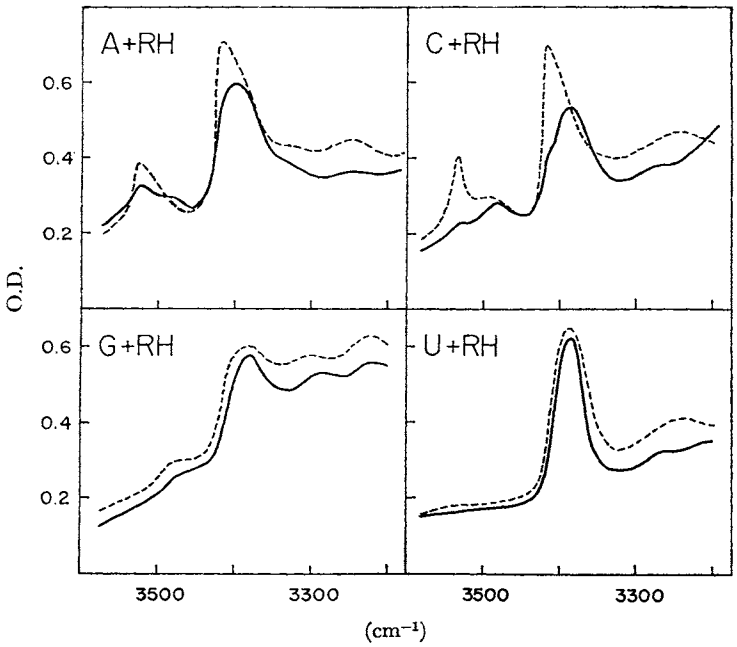


Fig. 3. Infrared spectra of the equimolar mixture solution of reduced riboflavin tetraacetate and one of the A, U, G and C compounds in chloroform. The total concentration is 8×10^{-3}M and the path length is 5 mm. Solid line, observed spectrum; dotted line, calculated sum of the individual spectrum of components.

quinone, which interact to give the fully reduced and oxidized forms as shown above.

Association with Purines, Pyrimidines and Other Related Compounds. *Interaction with 9-Ethyladenine.* Riboflavin tetraacetate formed a hydrogen bonded complex with adenine more strongly than with themselves.[1,2] The infrared spectra of the 1 : 1 mixture of RH and A also show us strong binding of A with RH (Fig. 3). The symmetric and antisymmetric stretching bands of the non-bonded amino group at 3415 and 3525 cm^{-1} become weaker than the calculated sum of the individual spectrum which would be realized if there is no interaction between them. An association band is visible at 3470 cm^{-1}, which is due to the stretching vibration of the partially bonded amino group. The intensity of the non-bonded and association bands changes as a function of the mole ratio of mixing. They reach a maximum or a minimum in the 1 (RH): 3 (A) region (Fig. 4). RH and A form a complex which consists of three A and one RH molecules in average. The ratio is not unreasonable because RH has four sites to which A can bond with cyclic hydrogen bonds. By an application of the procedure described in the preceding section the association constant of the complex formation was estimated to be 10^7 $(l/mol)^3$ with an assumption $m=1$ and $n=3$.

The intensity of absorption bands also changes as a function of the extent of reduction. The broad band of RH gradually decreases in intensity and the band due to the free N_3H group of R at 3380 cm^{-1} becomes sharp as the extent of oxidation increases (Fig. 5). However, the intensity change of the bands of A at 3415 and 3525 cm^{-1} are not parallel with that of the N_3H group of R. This might be explained by the fact that a RH molecule can bond with three A molecules. Contribution of the intermediate state to the RH-A binding does not seem to be important, since an additional band due to the semiquinone-A complex could not be observed. In fact the concentration of radicals was determined by ESR measurement to be 1 per cent of the dissolved RH at 50% reduction and is not affected by the presence of A.

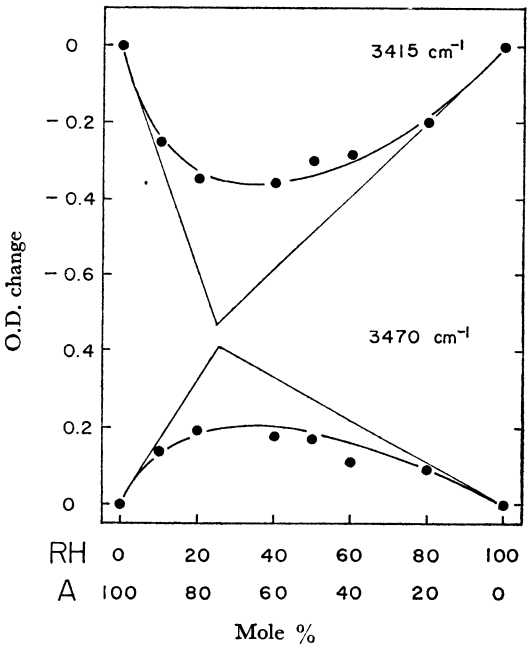


Fig. 4. Change in the optical density of the association band at 3470 cm^{-1} and the non-bonded band at 3415 cm^{-1} as a function of the mole ratio of fully reduced riboflavin tetraacetate and ethyladenine. The optical density of the pure solution is adjusted to zero.

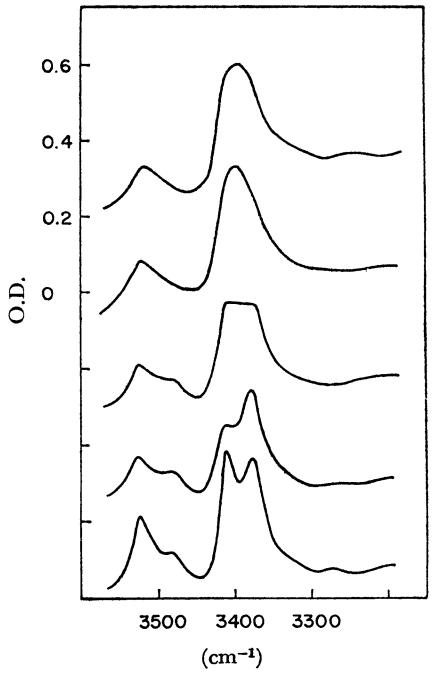
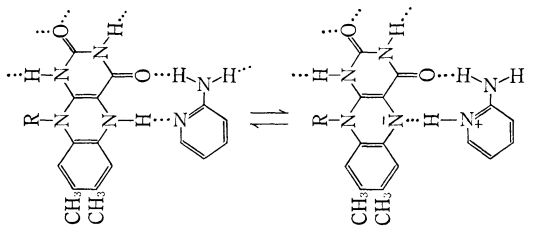


Fig. 5. Infrared spectra of the equimolar mixture solutions of riboflavin tetraacetate and ethyladenine at successive reduction states. Bottom spectrum, fully oxidized state; top spectrum, fully reduced state. The total concentration is 8×10^{-3}M and the path length is 5 mm.

Interaction with Other Compounds. Riboflavin tetraacetate selectively bonded to A and did not interact with other nucleic acid purine and pyrimidine derivatives.[1,2] Whether such specificity still holds for the association of RH was examined. The infrared spectra of the mixtures with G and U show almost the same spectra as the calculated sum of each individual (Fig. 3). A remarkable change, however, is observed in the spectrum of the mixture with C. The symmetric and antisymmetric stretching bands of the free amino group of C at 3415 and 3525 cm^{-1} almost disappear on the addition of RH. The selective binding found

for the oxidized form cannot be observed in the association of the reduced form. To find necessary conditions for the association with RH, several compounds listed in Table 1 were examined for their ability of binding with RH. Association constants were roughly estimated by the variation method described previously. A two + sign in the table shows that the compound interacts with RH as strongly as with A at an association constant in the order of 10^7 (l/mol).[3] A three + sign means stronger complex formation with a constant bigger than 10^8. Weaker bonding than that of A is shown by a one + sign. For all of the associations m and n were assumed 1 and 3 respectively.

From the table it can be concluded that both the amino group and the nitrogen atom in the heterocyclic ring are necessary but are not enough for the association. Acidity or basicisity of the donor and acceptor sites of proton may be associated with the interaction. The pK_a of 4-aminopyrimidine is 5.71, whereas that of 2-aminopyrimidine is 3.54.[20] The latter must be harder to be protonated than the former. This accounts for the fact that 2-aminopyrimidine does not associate with RH. 2-Amino-, 3-amino-, 4-aminopyridines have the pK_a's of 6.86, 5.98 and 9.14 respectively.[20] On the other hand the protons at the N_1 and N_5 positions of RH dissociate around 6.5.[21] RH is a weak acid. Therefore the contribution of proton transfer is expected in the bonding between RH and 2- or 4-aminopyridine. In fact the ring stretching mode at 1600 cm^{-1} of deuterated 2-aminopyridine completely disappears on the addition of RH. A remarkable change in the ring of 2-aminopyridine was shown. On the other hand the ring stretching band of deuterated A at 1610 cm^{-1} still remains in the mixture solution with RH (Fig. 6). In the association of A with RH the ring system is not so affected by hydrogen bonding to the ring nitrogen atoms.



Association with Nicotinamide. As shown in Table 1 and Fig. 7 nicotinamide strongly interacts with RH. The symmetric and antisymmetric stretching bands of the amino group of NA at 3525 and 3410 cm^{-1} decrease in intensity. Although the amino group of NA is used for the association with RH, the scheme shown in the case of the RH-aminopyridine complex is not applicable. The ring nitrogen atom of aminopyridine is the acceptor site of proton in the RH-aminopyridine complex. Reduced N-methylnicotinamide whose nitrogen atom is blocked with a methyl group is expected not to interact with RH. However it shows an interaction with RH. Moreover the nitrogen atom of a nicotinic acid derivative is a weaker base than that of pyridine by 1—2 pK units (*ca.* pK_a 2—3).[22] The spectrum of NA in the 1800—1500 cm^{-1} region shows the carbonyl stretching band at 1680 cm^{-1}, which becomes strong and shifts to lower frequency when RH is added to the solution (Fig. 7). It is clear that a hydrogen bond is formed at the carbonyl group of an amide and the following structure can be proposed for the association.

Table 1. Association of reduced riboflavin tetraacetate with several compounds

Compound		Compound	
A	++	Aniline	—
G	—	Pyridine	—
C	++	Nicotinamide	+
U	—	Isonicotinamide	+
2-Aminopyridine	+++	1-Methyl-4-hydronicotinamide	+
3-Aminopyridine	+	Benzamide	—
4-Aminopyridine	++	*o*-Hydroxybenzamide	—
2-Aminopyrimidine	—	1-Phenylurea	—
4-Aminopyrimidine	+	Urethane	—

The + signs denote the strength of interaction of RH with association constants bigger than 10^8 (l/mol)^3 (+++), around 10^7 (++) and smaller than 10^7 (+).

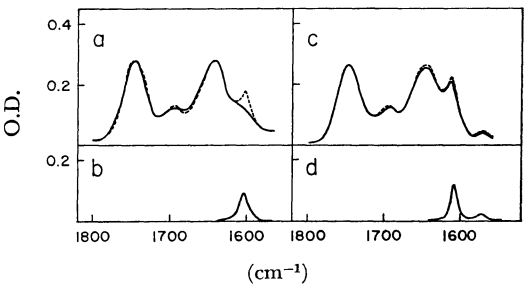


Fig. 6. Infrared spectra of the equimolar mixture solutions of RH and 2-aminopyridine (a), 2-aminopyridine (b), the equimolar mixture of RH and ethyladenine (c) and ethyladenine (d). The compounds are deuterated. The total concentration is 8×10^{-3}M and the path length is 0.5 mm.

20) A. Albert, R. Goldacre and J. Phillips, *J. Chem. Soc.*, **1948**, 2240.

21) P. Hemmerich and J. Spence, "Flavins and Flavoproteins," ed. by M. K. Slater, Elsevier, Amsterdam (1964), p. 82.

22) R. W. Green and H. K. Tong, *J. Amer. Chem. Soc.*, **78**, 4896 (1956).

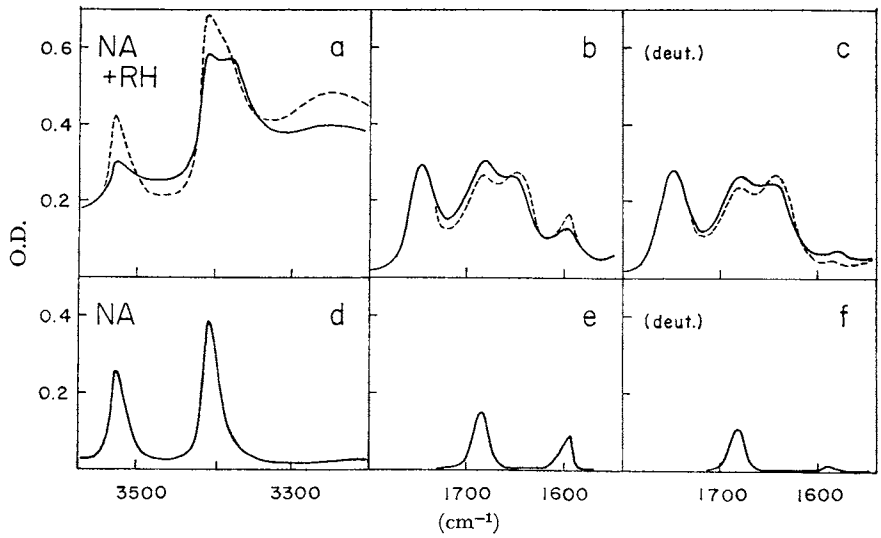
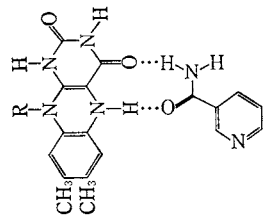


Fig. 7. Infrared spectra of nicotinamide (d, e, f) and the equimolar mixture solutions with reduced riboflavin tetraacetate (a,b,c). Solid line, observed spectra; dotted line, calculated sum of the individual spectrum. The concentration of each solute is 4×10^{-3}M and the path length is 5 mm for the 3500—3200 cm^{-1} region and 0.5 mm for the 1800—1550 cm^{-1} region.



The amide group is almost planar, and there is resonance between the nitrogen and the carbonyl group which may be represented as N–C=O⇌ N^+=C–O^-. In fact, infrared studies of nicotinamide-metal ion complexes suggest strongly that the oxygen atom in amides acts as a donor toward electron acceptors.[23,24] The pyridine ring increases the electron density at the oxygen atom of the amide group by inductive and resonance effects. Therefore the acceptor strength for proton at the oxygen atom of NA is expected to be greater than those of urea derivatives. This might be the reason why NA derivatives interact with RH and aliphatic urea derivatives do not.

Nicotinamide adenine dinucleotide in the oxidized form is thought to form a charge transfer complex with $FMNH_2$.[11,14] The visible spectrum of the mixture showed a broad band at 700 mμ which has been thought to be a charge transfer band. An additional absorption at 520 mμ of the mixture of $FMNH_2$ - *N*-methylnicotinamide iodide is also thought to be due to a charge transfer complex.[14] Any absorption, however, could not be detected in the longer wavelength region than 500 mμ in the chloroform system. It is doubtful whether the association found in the present experiment corresponds to the charge transfer complex of NAD^+-$FMNH_2$ or not.

Any interaction could not be detected between reduced *N*-methylnicotinamide and R. Radda and Calvin have also failed to observe the association between NADH and FMN which might occur under the catalysis of enzyme.[25] Although RH was found to be able to form strong hydrogen bonds with several compounds, the association of RH is probably of no physiological importance.

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23) K. Nagano, H. Kinoshita and A. Hirakawa, *Chem. Pharm. Bull.* (Tokyo), **12**, 1198 (1964).

24) F. R. Nordmeyer and H. Taube, *J. Amer. Chem. Soc.*, **88**, 4295 (1966).

25) G. K. Radda and M. Calvin, *Biochemistry*, **3**, 384 (1964).