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Keyphrases

Hydrocortisone diffusion, *in vitro*—rat intestine
Tyloxapol, effect—cortisol diffusion
Everted sac—technique
Colorimetric analysis
Diffusion rate—tyloxapol concentration function

Base-Catalyzed Hydrolysis of Flavins

Effect of Amines and the Particular Role of Position 3

By DONALD B. McCORMICK and WERNER FÖRY

The base-catalyzed hydrolysis of flavins was investigated in 50 percent water–methanol with excess sodium hydroxide or excess amines at different temperatures. Rate constants for the pseudo first-order cleavage of the pyrimidine portion of the isoalloxazine systems were measured and with sodium hydroxide shown to increase from lumiflavin to 3-ethyllumiflavin to 3-carboxyamido(phenylalanyl)lumiflavin. With amines, the size as well as basicity is of prime importance since the general order in efficacy of the nucleophile for hydrolysis of flavins is ethylamine > diethylamine > triethylamine > benzylamine > tributylamine. In general, alkylation of the 3-imino function of the flavin increases its lability because of inability to ionize and undergo resonance stabilization of the anionic tautomers. Moreover, the special case where a 3-carboxyamido function is present allows intramolecular polarization, *via* hydrogen bonding of the amide hydrogen to 4-carbonyl oxygen, which causes a marked increase in hydrolysis rate in sodium hydroxide. From the temperature dependencies of the rates of hydrolysis, the calculated values for energies of activation indicate the decrease expected upon 3-alkylation and further upon intramolecular enhancement with the 3-carboxyamido group. The calculated values for entropies of activation are large and negative in accord with considerable loss of degrees of freedom in the activated complexes formed in the bimolecular mechanism.

FLAVINS CAN undergo hydrolytic decomposition of their isoalloxazine nucleus, particularly in alkaline solution. Kuhn and Rudy (1) isolated urea and 1,2-dihydro-1,6,7-trimethyl-2-keto-3-quinoxaline carboxylic acid from the degradation of lumiflavin in alkaline medium. These results were confirmed by Surrey and Nachod (2) and extended by Svobodova (3) and Wada *et al.* (4) who noted several other unidentified products. Farrer and MacEwan (5) studied the kinetics for hydrolysis of riboflavin over a wide range of pH and showed the reaction to be general acid-base catalyzed. More recently Guttman and coworkers have examined the influence of complexing agents in decreasing the rates for hydrolysis of flavins (6–8) and the intermediates formed following cleavage of the pyrimidine portion of the isoalloxazine system (9–11).

Since most of the earlier studies on the hydrolysis of flavins involved only the use of hydroxyl ion as the attacking species, it seemed desirable to investigate more thoroughly the effects of size and basicity of other nucleophiles such as amines. Also the rate of hydrolysis of a 3-substituted flavin in aqueous sodium hydroxide had been observed to be faster than that of unsubstituted flavin (8), but the primary cause for the difference had not been reported. Furthermore, though such compounds as purines which complex with flavin by planar overlap of their ring systems (12–14) cause a decrease in rate of hydrolysis of flavin (6, 7), certain other types of molecular as-

Received January 2, 1968, from the Graduate School of Nutrition and Section of Biochemistry and Molecular Biology, Cornell University, Ithaca, NY 14850
Accepted for publication February 15, 1968.
This investigation was supported in part by research grant AM-04585 from the National Institutes of Health and by funds from the State University of New York.

sociations could be expected to facilitate the hydrolysis rate.

The purposes of the present investigation were to circumscribe more thoroughly the efficacy of amines in the base-catalyzed hydrolysis of flavins, to ascertain the effect of substitution in position 3 of the flavin nucleus, and to indicate how intramolecular polarization can enhance the rate of hydrolysis.

Lumiflavin and its analogs were chosen as they are much less susceptible to photolytic decomposition (15) and because the absence of a polyhydroxy side chain precludes certain complicating behavior observed with riboflavin (7). A solution of 50% water–methanol was used as solvent to be compatible with solubility of both flavin and the larger aliphatic amines. The rate of loss of the flavin yellow color ar 450 mμ was used as a satisfactory index for cleavage of the pyrimidine portion of the flavins (6). Rate constants were determined for hydrolysis effected by sodium hydroxide as well as by a series of amines, the relative effectiveness of which is shown to be dependent upon size and basicity. Alkylation of position 3 in the flavin increases the rate for hydrolysis as resonance stabilization through tautomerization is lost. A further increase in rate is caused by interaction of amide-linked amino acid ester in this position due to polarization of flavin carbonyl 4 *via* hydrogen bonding with the amide function. The temperature dependency for hydrolysis in aqueous sodium hydroxide was measured for lumiflavin to compare with the energy of activation previously reported (6) and additionally for ethylamine-catalyzed hydrolyses of lumiflavin and its 3-substituted analogs the latter of which have lower values for activation energies. The entropies of activation then were calculated and found to be large and negative and thereby reflect greater loss of translational and rotational freedom in the activated complexes of 3-substituted flavins, especially with a 3-carboxyamido function.

EXPERIMENTAL

Materials—Lumiflavin (7,8,10-trimethylisoalloxazine) was synthesized according to Hemmerich (16). The 3-ethyl and 3-carboxymethyl derivatives were prepared following the general method of Hemmerich (17) by alkylation of lumiflavin in *N,N*-dimethylformamide with potassium carbonate and the appropriate iodide. The 3-carboxymethyllumiflavin was activated with *N,N'*-carbonyldiimidazole after the procedure of Staab *et al.* (18) to yield the flavin imidazolium amide which was then reacted with L-phenylalanine methyl ester according to the method of Anderson and Paul (19) to form the 3-carboxyamido(phenylalanyl)lumiflavin [3-carboxyamido-(L-phenylalanine methyl ester)-7,8,10-trimethylisoalloxazine].

Usual reagents and other chemicals were obtained from commercial sources.

Methods—Flavins were dissolved in methanol and diluted with water plus base such that the final concentrations of reactants in 25 ml. were 5×10^{-5} *M* flavin and base at a specified concentration, usually 0.1 *M*, in 50% (v/v) water–methanol. These aerobic solutions were heated at a specified temperature controlled by a rheostat and heating mantle or a Haake model F constant temperature circulator. Aliquots of 2 ml. were taken at intervals and the concentration of remaining flavin determined by measuring the absorbance at 450 mμ in a Beckman DU spectrophotometer.

Infrared spectra of flavin compounds were run in potassium bromide pellets (1 mg. flavin per 300 mg. KBr) with a Perkin-Elmer Infracord spectrometer set for slow scan.

Calculations—Under the conditions employed (excess base and water) for the pseudo first-order hydrolysis of the pyrimidinoid portion of the flavin ring system, the apparent rate constants, *k*, were determined by multiplying by 2.303 the initial slopes of lines obtained in plots of log A_{450} *versus* min. where

$$\log A_{450} = \left(\frac{k}{2.303}\right) t + \text{constant} \quad \text{(Eq. 1)}$$

The heats of activation, ΔHa, were calculated from the rate constants at different temperatures using the differential expression of the Arrhenius equation integrated between limits

$$\log \frac{k_2}{k_1} = \left(\frac{\Delta Ha}{2.303R}\right) \frac{T_2 - T_1}{T_2 T_1} \quad \text{(Eq. 2)}$$

The entropies of activation, ΔSa, were calculated from a rate constant at a particular temperature and the heat of activation, where from the Eyring equation

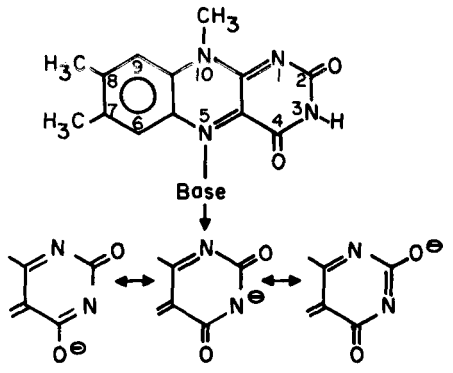
$$\Delta Sa = 2.303R \log \frac{kNh}{RT} + \frac{\Delta Ha}{T} \quad \text{(Eq. 3)}$$

RESULTS AND DISCUSSION

Rate constants for the sodium hydroxide-catalyzed hydrolysis of flavins are given in Table I. From the rate constants at 75 and 50° which indicate the thermal dependency for hydrolysis of lumiflavin, the energy (heat) of activation was calculated to be 20.2 Kcal. which is in good agreement with the value of 20.0 reported previously (6). The entropy of activation was calculated to be −16 e.u. (cal. deg.$^{-1}$) which is reasonable for a bimolecular reaction where decrease in entropy is expected in passing to an activated complex with loss of translational and rotational freedom before rupture of the flavin ring system. Alkylation of the 3-imino function of a flavin

TABLE I—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF 5×10^{-5} *M* FLAVIN WITH 0.1 *M* SODIUM HYDROXIDE IN 50% WATER–METHANOL

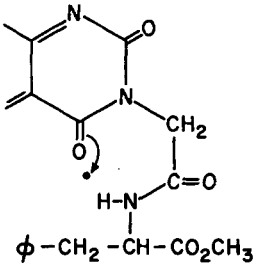
Flavin	°C.	*k*, Min.$^{-1}$
Lumiflavin	75	2.58×10^{-2}
	50	2.69×10^{-3}
3-Ethyllumiflavin	50	1.38×10^{-1}
3-Carboxyamido(phenylalanyl)-lumiflavin	5	1.02



Scheme I

leads to a considerable increase in the rate of hydrolysis as can be seen by comparing the rate constants for lumiflavin and its 3-ethyl derivative, both at 50°. Flavins unsubstituted in position 3 can undergo tautomerization between the 3-imino and 2- and 4-carbonyl functions. In the alkaline medium used for hydrolysis, the isoalloxazine system is ionized, as it is well above its pKa near 10 (20). Thus, as shown in Scheme I, lumiflavin exists as resonance-stabilized anionic forms in strongly basic solution and is less susceptible to nucleophilic attack.

Because the acidic proton in position 3 is lacking, ionization cannot occur with 3-alkylated flavins. The pyrimidine portion of their isoalloxazine systems is more electron deficient and hydrolysis resulting from nucleophilic attack occurs more readily. The exceedingly rapid rate for hydrolysis of 3-carboxyamido(phenylalanyl)lumiflavin in aqueous sodium hydroxide even at 5° must reflect an additional cause for lability. Not only is this 3-substituted flavin not able to form resonance-stabilized anions, but also space-filling molecular models indicate the possibility for intramolecular interaction of the flavin carbonyl functions with the amino acid group. As with lumiflavin and 3-ethyllumiflavin, increasing the concentration of sodium hydroxide increases the rate of hydrolysis of the 3-carboxyamido derivative in agreement with the bimolecular mechanism for hydrolysis. Hence, a unimolecular process such as would occur if the amide nitrogen served as the nucleophile can be excluded as a single-step mechanism. However, this does not exclude such a mechanism as hydrolysis of a function followed by an intramolecular attack by the generated nucleophile. As shown by the infrared spectra in Fig. 1, the band for the 4-carbonyl stretch vibration at 1710 cm.$^{-1}$ in lumiflavin and at 1700 cm.$^{-1}$ in 3-ethyllumiflavin is considerably diminished in the 3-carboxyamido derivative, while the amplitude of the 2-carbonyl band remains approximately the same near 1660 cm.$^{-1}$ for all three compounds. These assignments agree well with those reported earlier for lumiflavin and other derivatives (21). Therefore, it appears that the hydrogen of the amide function intramolecularly bonds to the 4-carbonyl oxygen and, as shown in the formula below, thereby polarizes this function in a manner favorable for hydrolysis.



The decomposition of lumiflavin as a function of time in 0.1 *M* bases at 75° is shown by the data in Fig. 2. A "hard" base such as sodium hydroxide is most effective in the base-catalyzed hydrolysis, but facile degradation is also effected with small aliphatic amines. Molecular size of the amine is quite important as ethylamine (pKb = 3.25) is somewhat less basic than diethylamine (pKb = 2.90), but the rate of hydrolysis with the former is somewhat greater. Further decrease in hydrolysis rate is seen with the larger triethylamine (pKb = 3.25) and more yet with tributylamine (pKb = 3.50). Also the latter is more basic than benzylamine (pKb = 4.70), but its greater size leads to a slightly decreased rate. Aniline (pKb = 9.42) is too weak a base and *N,N*-diethylaniline is too large to be effective. Thus the

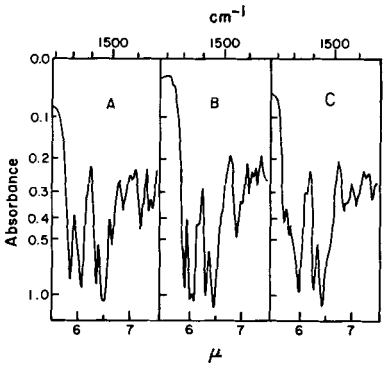


Fig. 1—Infrared spectra of flavins in the carbonyl absorption region. Key: A, lumiflavin; B, 3-ethyllumiflavin; C, 3-carboxyamids(phenylalanyl)lumiflavin.

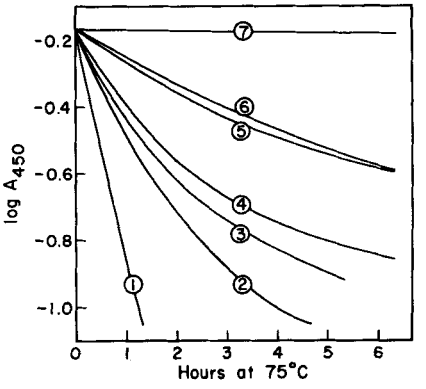


Fig. 2—The decomposition of lumiflavin in 50% water–methanol at 75° with 0.1 M bases. Key: sodium hydroxide; 2, ethylamine; 3, diethylamine; 4, triethylamine; 5, benzylamine; 6, tributylamine; 7, aniline or N,N-*diethylaniline.*

TABLE II—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE HYDROLYSIS OF 5×10^{-5} *M* FLAVIN WITH 0.1 *M* AMINES IN 50% WATER–METHANOL AT 75°

Flavin	Amine	k, Min.$^{-1}$ × 10^3
Lumiflavin	Ethyl-	11.5
	Diethyl-	10.1
	Triethyl-	7.94
	Tributyl-	2.99
	Benzyl-	3.38
3-Ethyllumiflavin	Ethyl-	16.3
	Diethyl-	10.8
	Triethyl-	15.4
	Tributyl-	2.07
	Benzyl-	2.17
3-Carboxyamido-(phenylalanyl)lumiflavin	Ethyl-	9.21
	Diethyl-	4.61
	Triethyl-	4.60
	Tributyl-	0.92
	Benzyl-	0.99

steric factors which limit the approach of an amine as nucleophile are of major importance even when basicity is favorable. As the hydrolysis proceeds, accumulation of intermediate breakdown products leads to deviation from pseudo first-order kinetics with a curving of lines after 1 or 2 hr.

Rate constants for the initial pseudo first-order amine-catalyzed hydrolysis of flavins are listed in Table II. As with lumiflavin, the relative effectiveness of the amine as nucleophile for hydrolysis of the 3-substituted flavins is markedly dependent upon size. An additional steric restriction imposed by the 3-alkyl group may be manifest, as a greater proportional decrease in hydrolysis rate is seen in going from the triethyl- to tributylamine with 3-ethyllumiflavin than with lumiflavin. Also as with sodium hydroxide, the rates for hydrolysis of 3-ethyllumiflavin with small amines is somewhat greater than found for hydrolysis of lumiflavin. As explained before, lack of the acidic hydrogen on position 3 of 3-alkyl flavins causes greater lability toward hydrolytic degradation. The absolute rates for amine-catalyzed hydrolysis of 3-carboxyamido(phenylalanyl)lumiflavin are lower than those for lumiflavin or its 3-ethyl analog.

The heats and entropies of activation for the hydrolysis of flavins with ethylamine are presented in Table III. Values of ΔHa and ΔSa for lumiflavin with this base are similar in magnitude to the respective values obtained with sodium hydroxide as given above. The energies (heats) of activation required for ethylamine-catalyzed hydrolysis of the 3-alkyl flavins are lower and their entropies of activation more negative than lumiflavin. Especially noteworthy is the very large ΔSa value calculated for 3-carboxyamido(phenylalanyl)lumiflavin. This appears to reflect the large orientation factors expected in this bulky, intramolecularly hydrogen-bonded compound upon forming an activated complex with the amine.

TABLE III—HEATS AND ENTROPIES OF ACTIVATION FOR THE HYDROLYSIS OF 5×10^{-5} *M* FLAVIN WITH 0.1 *M* ETHYLAMINE IN 50% WATER–METHANOL

Flavin	ΔHa, Kcal.	ΔSa, e.u.
Lumiflavin	22.2	−12
3-Ethyllumiflavin	16.1	−29
3-Carboxyamido-(phenylalanyl)lumiflavin	5.8	−60

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Keyphrases

Flavins—base catalyzed hydrolysis
Hydrolysis, flavins-amines, position 3 effect
Rate constants—flavin hydrolysis
Lumiflavin and derivatives—synthesis
UV spectrophotometry—analysis
IR spectrophotometry—structure