Complex Formation Influence on Reaction Rate II

Hydrolytic Behavior of Riboflavin in Borate Buffer

By DEODATT A. WADKE* and DAVID E. GUTTMAN*

A study was made of the hydrolytic behavior of riboflavin in aqueous solutions buffered with a borate buffer system. It was observed that the vitamin decomposed slower in the presence of the buffered rather than unbuffered solutions of equivalent hydroxide-ion concentration. Rate studies suggested that a reversible 1 : 1 association occurred between riboflavin and a component of the buffer to yield a product which underwent hydrolytic cleavage of the isoalloxazine ring at a slower rate than the parent compound. Similar studies with lumiflavin failed to demonstrate this effect and showed that the ribityl side-chain of riboflavin was involved in the association.

Evidence was presented in a previous communication (1) which demonstrated that the velocity of the base-catalyzed decomposition of riboflavin in aqueous solution was influenced by the incorporation of caffeine in the reaction mixture. The rate was shown to be slowed and dependent on the concentration of caffeine. The effect was rationalized and effectively quantitated by hypothesizing that the riboflavin-caffeine complex, which is known to form in such systems, possessed little or no reactivity toward hydroxide ion and that only the noncomplexed form of the vitamin underwent observable decomposition. It is the objective of this communication to describe a related but somewhat more complex behavior which was observed in solutions of riboflavin buffered with a boric acid buffer. Here an association between the vitamin and a buffer component apparently took place. However, in contrast to the caffeine system, here both the free and combined forms of the vitamin appeared to undergo hydrolytic breakdown but at different rates.

Other workers have shown previously that riboflavin and boric acid do undergo some type of rapid reversible union. Surrey and Nachod (2), for example, demonstrated that aged alkaline solutions of riboflavin exhibited a depressant action on cardiac and visceral muscles and that this was due to the 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid which resulted from hydrolysis of the vitamin. They reported, however, that similar solutions which contained boric acid failed to show any hypotensive activity even after prolonged standing. The reasons for this apparent stabilization were not pursued. Other reports, including those of Takata *et al.* (3) and Frost (4), indicated a reaction by noting that riboflavin was more soluble in aqueous borate solutions than in water alone. Frost also found that the degree and direction of optical rotation exhibited by riboflavin in aqueous solution was altered by the addition of boric acid. He suggested on the basis of this and other evidence that the ribityl side-chain combined with the boron compound in some way. Data obtained in the present investigation support this conclusion and demonstrate that the rate of hydrolysis of the isoalloxazine ring of the vitamin is significantly reduced as a result of association.

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*Present address: Department of Pharmaceutics, School of Pharmacy, State University of New York, at Buffalo, Buffalo.

EXPERIMENTAL

Reagents.—Riboflavin was obtained from Eastman Organic Chemicals. Lumiflavin was prepared by decomposing riboflavin in sodium hydroxide solution (pH 12.0–12.5) in the presence of light. The reaction mixture was acidified (pH 4.5–5.0) and extracted with chloroform. The solvent was removed to obtain lumiflavin which was washed several times with 0.5 *N* hydrochloric acid and finally with distilled water. The melting point was 332–334° with decomposition (uncorrected).

Procedure.—The reaction mixture was prepared by placing 50 ml. of appropriate buffer or sodium hydroxide solution of desired normality in a 100-ml. volumetric flask together with about 25 ml. of distilled water. The flasks were suspended in a Sargent Thermonitor constant temperature bath. Twenty milliliters of riboflavin stock solution was added to each flask and the volume made up to the mark with distilled water. Five-milliliter samples were withdrawn periodically and immediately mixed with 5 ml. of 2 *N* acetic acid to quench the hydrolysis. Samples were analyzed spectrophotometrically at 450 mμ using a Beckman model DU spectrophotometer. All samples were protected from light at all times. In studying the effect of buffer concentration on the hydrolysis of riboflavin and lumiflavin, ionic strength was maintained constant at 0.3 by adding potassium chloride.

RESULTS

A preliminary indication that the hydrolytic behavior of riboflavin was somewhat unusual in borate

buffer was obtained by comparing the pH profile for the reaction determined in unbuffered systems to that obtained using buffers. All solutions exhibited a first-order disappearance of riboflavin, but the rates of disappearance were slower in the buffered solutions than in corresponding unbuffered solutions of identical pH. Figure 1 illustrates this observation. Here, the logarithm of the first-order rate constant determined for a particular solution was plotted as a function of the logarithm of the hydroxide ion concentration. It can be seen that the rate constants for the borate systems are smaller than would be anticipated on the basis of their hydroxide-ion concentration. Subsequent experiments showed that the effect was not due to differences in ionic strength. The velocity of the reaction was, however, specifically influenced by the concentration of boric acid in the buffer. Figure 2 illustrates this effect. Here, for three different pH conditions the experimentally determined rate constants were plotted as a function of borate buffer molarity. It can be seen that initial, relatively small additions of buffer resulted in a rather marked reduction in the magnitude of the rate constant. Subsequent additions had a lesser effect, and each curve approached an asymptotic value. Rate constants at zero buffer concentration were estimated by extrapolation of the curves to the y-axis and determination of the intercept value. Rate constants at infinitely high concentrations of buffer components were estimated from the plots illustrated in Fig. 3. Here rate constant was plotted as a function of the reciprocal of the buffer molarity. The resulting curves were extrapolated to the y-axis and the intercept value determined. The constants representing the concentration extremes and those for intermediate concentrations are tabulated in Table I. Similar experimentation was conducted to study the influence of buffer molarity on the rate of disappearance of lumiflavin from aqueous solution. The results are shown in Table II and in Fig. 4, where a reference curve for riboflavin at the same pH is drawn for comparative purposes. The rate of lumiflavin disappearance was within experimental error, independent of the molarity of buffer. Spectrophotometric determinations confirmed the observation made by Frost (4) that the visible and ultraviolet spectra of riboflavin were no different in solutions buffered with borate than in unbuffered solutions.

DISCUSSION

The nature of the observed dependency of reaction rate of the buffer molarity intuitively suggested that a reversible reaction or interaction between

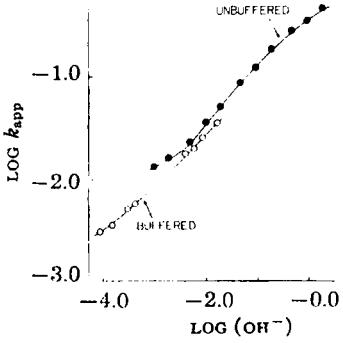


Fig. 1.—The influence of hydroxide-ion concentration on the rate of hydrolysis of riboflavin at 35° C. in the presence and absence of borate buffer.

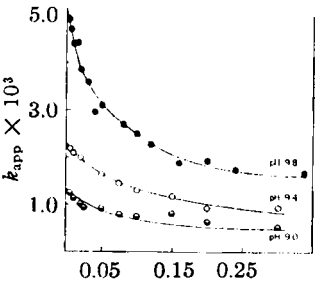


Fig. 2.—The influence of borate buffer concentration on the rate of hydrolysis of riboflavin at different pH values at an ionic strength of 0.3.

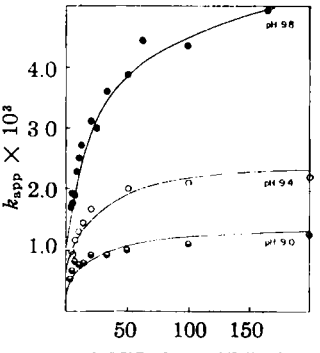
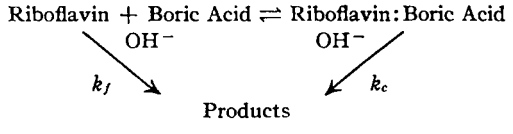


Fig. 3.—The influence of borate buffer concentration on the rate of hydrolysis of riboflavin at different pH values at 35° C. and at an ionic strength of 0.3.

riboflavin and a buffer component occurred to form a new species of riboflavin which could not be differentiated from the original by the assay method employed. Examination of the data also suggested that both the free and combined forms of the vitamin which were in equilibrium underwent hydrolytic cleavage of the isoalloxazine ring but at different rates. The suspected mechanism is summarized by:



As it will be seen, a mathematical model based on this over-all mechanism was consistent with the experimental observations.

A rate expression for the postulated situation can be derived from the following considerations. All solutions exhibited a first-order disappearance of the riboflavin chromophore, *i.e.*,

$$-d(R_t)/dt = k_{app}(R_t) \qquad \text{(Eq. 1)}$$

where (R_t) = stoichiometric concentration of riboflavin, k_{app} = pseudo first-order rate constant, and t = time. If riboflavin and boric acid, or another form of boric acid, underwent a rapid reversible 1:1 association to form a product which was also susceptible to hydrolysis of the ring, then the rate equation becomes

$$-d(R_t)/dt = k_f(R_f) + k_c(R_c) \qquad \text{(Eq. 2)}$$

where k_f = rate constant for the degradation of free riboflavin, (R_f) = concentration of free riboflavin, k_c = rate constant for the degradation of the riboflavin-boric acid product, and (R_c) = concentration of riboflavin-boric acid product. Both (R_f) and (R_c) can be expressed in terms of the stoichiometric concentration of riboflavin and the equilibrium constant for the reversible combination, *i.e.*,

$$(R_f) = (R_t)/[1 + K(B)] \quad \text{(Eq. 3)}$$

and

$$(R_c) = (R_t)K(B)/[1 + K(B)] \quad \text{(Eq. 4)}$$

where (B) = concentration of free boric acid which may be approximated by the stoichiometric concentration if it is large compared to the concentration of riboflavin, K = equilibrium constant = $(R_c)/(R_f)(B)$.

The rate equation can then be written

$$-d(R_t)/dt = (R_t)\{k_f/[1 + K(B)] + k_cK(B)/[1 + K(B)]\} \quad \text{(Eq. 5)}$$

The dependency of rate constant on buffer concentration is thus predicted by

$$k_{app} = k_f/[1 + K(B)] + k_cK(B)/[1 + K(B)] \quad \text{(Eq. 6)}$$

Equation 6 can be rearranged to yield

$$(k_f - k_{app})/(k_{app} - k_c) = K(B) \quad \text{(Eq. 7)}$$

If the basic assumptions are valid, then a plot of

TABLE I.—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE DISAPPEARANCE OF RIBOFLAVIN IN BORATE BUFFER AT CONSTANT IONIC STRENGTH OF 0.3 AND A TEMPERATURE OF 35° C. ± 0.01° C.

Molarity of Borate	k_{app}, Hr.$^{-1}$ × 10^3
pH 9.0	
0.0	1.3[a]
0.005	1.24
0.01	1.13
0.02	1.01
0.03	0.935
0.05	0.935
0.075	0.795
0.1	0.75
0.15	0.805
0.2	0.64
0.3	0.53
infinite	0.23[a]
pH 9.4	
0.0	2.31[a]
0.005	2.19
0.01	2.09
0.02	1.98
0.05	1.64
0.075	1.43
0.1	1.30
0.15	1.17
0.2	0.92
0.3	0.96
infinite	0.65[a]
pH 9.8	
0.0	5.14[a]
0.003	4.9
0.006	4.7
0.01	4.34
0.016	4.42
0.02	3.85
0.03	3.58
0.04	2.97
0.05	3.1
0.08	2.69
0.1	2.51
0.12	2.27
0.16	1.88
0.2	1.93
0.24	1.74
0.336	1.67
infinite	1.05[a]

[a] Value was obtained by extrapolation.

TABLE II.—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE DISAPPEARANCE OF LUMIFLAVIN IN BORATE BUFFER AT pH 9.8 ± 0.05, AT CONSTANT IONIC STRENGTH OF 0.3 AND A TEMPERATURE OF 35° C. ± 0.01°C.

Molarity	k_{app}, Hr.$^{-1}$ × 10^3
0.005	1.97
0.01	1.88
0.02	2.03
0.03	2.23
0.05	2.25
0.1	2.16
0.2	1.77
0.3	1.88

$(k_f - k_{app})/(k_{app} - k_c)$ *versus* the concentration of boric acid should yield a straight line with a slope of K. In treating the data for such a plot, the value of k_f used was that obtained by extrapolating rate constant to zero buffer concentration where free riboflavin would be the primary species. The value of k_c used was that obtained by extrapolating the rate constant to infinitely high buffer concentrations where the combined form of riboflavin would be expected to predominate. The concentration of boric acid was not used in testing this relationship. Rather, the total molarity of buffer was used. This was necessary because the exact nature of the boron-containing reactant is not known. The molarity of buffer does, however, provide a first-order reflection of the concentration of boric acid, borate ion, and other forms of boric acid, therefore, they can be used to gain an indication of whether the relationships under consideration are valid. The plot which tests the relationship expressed by Eq. 7 is illustrated in Fig. 5 and represents the data obtained for the pH

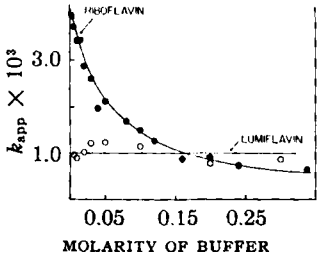


Fig. 4.—The influence of borate buffer concentration on the rates of riboflavin and lumiflavin hydrolysis at a pH value of 9.8, ionic strength of 0.3, and a temperature of 35° C.

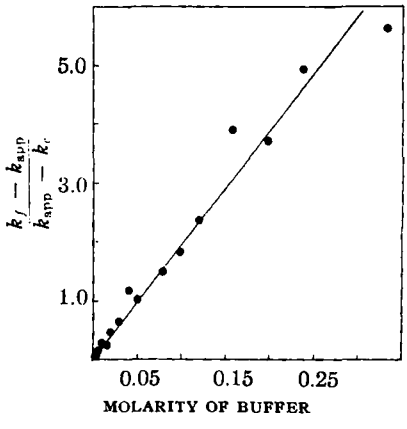


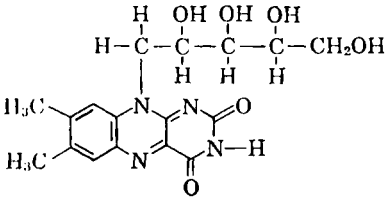
Fig. 5.—A plot of the relationship expressed by Eq. 7 (see text) for riboflavin at a pH value of 9.8, constant ionic strength of 0.3, and at 35° C.

TABLE III.—EQUILIBRIUM CONSTANTS FOR THE RIBOFLAVIN-BORIC ACID ASSOCIATION AT DIFFERENT pH VALUES AND AT A TEMPERATURE OF 35° C. ± 0.01° C.

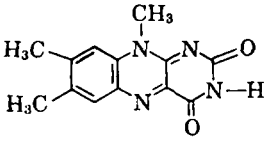
pH	K, L. mole^{-1}
9.0	10.0
9.4	14.5
9.8	19.5

condition of 9.8. It can be seen that excellent linearity over a wide range of buffer molarity was followed. The equilibrium constant which can be graphically estimated from this plot is not a thermodynamic value, but can be considered as an apparent value. This relationship was also tested with the data collected at the two other pH conditions. In both cases the expected linearity was achieved. The apparent equilibrium constants calculated from such plots are presented in Table III as a function of the pH condition. The graphical tests provide support for the model which was assumed, and indicate that such a model is reasonable and consistent with the observed behavior.

Numerous reports (5, 6) have shown that riboflavin undergoes charge-transfer interactions with a number of nucleophilic agents. The absence of changes in the spectral characteristics of the vitamin in the presence of borate buffer precludes this occurrence in the present study. That the ribityl side-chain was involved in a combination with a species of boric acid is apparent from the observation that, in contrast to riboflavin (I), the rate of reaction of lumiflavin (II) was not influenced by buffer concentration. The observed reduced rate of reaction exhibited by riboflavin in borate buffer must then be due to its partial and easily reversible conversion to a derivative formed by the combination of boric acid with the side-chain.

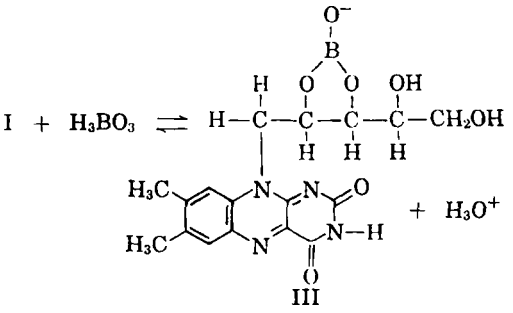


I. Riboflavin



II. Lumiflavin

On the basis of various reports on the reactivity of boric acid with polyhydroxy compounds (7, 8) we suggest that a borate ester might have formed in a manner similar to that represented by III. Such an



III

ester would be expected to be less susceptible toward hydroxide-ion attack on the ring because of a repulsive effect arising from the formal negative charge carried by the side-chain. The small decrease in the value of the apparent equilibrium constant with a decrease in pH can be considered as indirect support for such an effect. Protonation of the anionic group would occur as the pH was decreased and would result in a decreased repulsion and subsequent decrease in the apparent equilibrium constant determined in this way.

REFERENCES

(1) Guttman, D. E., THIS JOURNAL, **51**, 1162(1962).
(2) Surrey, A. R., and Nachod, F. C., *J. Am. Chem. Soc.*, **73**, 2336(1951).
(3) Takata, R., Nagata, T., and Shimamoto, S., *J. Fermentation Technol.*, **27**, 184(1946); through *Chem. Abstr.*, **44**, 5975(1950).
(4) Frost, D. C., *J. Biol. Chem.*, **145**, 693(1942).
(5) Isenberg, I., and Szent-Gyorgyi, A., *Proc. Natl. Acad. Sci. U. S.*, **44**, 857(1958).
(6) Pullman, B., and Pullman, A., *ibid.*, p. 1197.
(7) Sciarra, J. J., and Elliott, D., THIS JOURNAL, **49**, 115 1960).
((8) Riegelman, S., and Fischer, E. Z., *ibid.*, **51**, 207 (1962).