EVIDENCE INDICATING A CHEMICAL REACTION BETWEEN HYDROXYLAMINE AND RIBOFLAVIN*

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(Received for publication, July 13, 1955)

The observation that hydroxylamine inhibits xanthine oxidase activity through interference with the dehydrogenase portion of the enzyme (1) led us to determine whether hydroxylamine forms a chemical complex with riboflavin or its biological derivatives, riboflavin-5-phosphate (FMN) and flavin adenine dinucleotide (FAD). These studies were stimulated by those of Kaplan and his group, who explained hydroxylamine inhibition of alcohol dehydrogenase activity (2, 3) by demonstrating that diphosphopyridine nucleotide (DPN) forms a chemical complex with hydroxylamine (4, 5). Spectrophotometric data reported in this paper indicate the formation of a chemical complex among riboflavin, FMN, FAD, and hydroxylamine.

EXPERIMENTAL

All analyses were carried out with a Beckman DU spectrophotometer. Riboflavin solutions were prepared daily or every other day and stored in the dark. All other reagents were freshly prepared before use, being adjusted to the desired pH with KOH.

RESULTS AND DISCUSSION

The absorption spectrum of riboflavin in the presence and absence of hydroxylamine is presented in Fig. 1. No differences were observed throughout the visible range. However, the minimum at 240 m\(\mu\) was exaggerated in the presence of hydroxylamine, similar changes in optical density being observed in the case of FMN and FAD. The point of maximal difference was 245 m\(\mu\) for riboflavin and FMN and 240 m\(\mu\) in the case of FAD. Obviously, such an alteration in optical density is not indicative of the riboflavin-hydroxylamine complex but represents the summation of the absorption of the remaining riboflavin and the riboflavin-hydroxylamine complex. That this change in the absorption spectrum of riboflavin in the presence of hydroxylamine is not due to the high ionic strength of the salt solution resulting from the neutralization of the hydroxylamine employed is demon-

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Illustrated by the observation that an equivalent or higher concentration of potassium or sodium chloride, per se, had no effect on the absorption spectrum of riboflavin (unpublished data).

Earlier studies, although consistently demonstrating spectral alterations at 245 m\(\mu\), were unsatisfactory, owing to the inability to reproduce the magnitude of difference under supposedly constant conditions. Subsequently, it was discovered that the reaction between hydroxylamine and riboflavin was rather sluggish, equilibrium not being established until 20 to 30 minutes after the mixing of the reaction components (Fig. 2). When optical density measurements were carried out after equilibrium had been established, reproducible results were consistently obtained. It is of interest that the reaction rate curve of the hydroxylamine-riboflavin reaction is similar to that obtained in the case of hydroxylamine inhibition of
xanthine oxidase activity (1). In the latter case, inhibition was not maximal until 30 to 40 minutes after the initiation of the enzymatic reaction. This is roughly the period of time necessary for the hydroxylamine reaction to reach equilibrium and is a further indication that hydroxylamine inhibits xanthine oxidase activity by complex formation with the riboflavin portion of the enzyme. Further evidence demonstrating a chemical

![Graph](image.png)

**Fig. 2.** Rate of formation of the hydroxylamine-riboflavin complex; reversibility of the reaction. •, riboflavin (1.33 × 10⁻⁵ M) + hydroxylamine (0.5 M), final pH 11.1; ○, riboflavin (3.33 × 10⁻⁶ M) + hydroxylamine (0.5 M), final pH 11.1. Freshly prepared hydroxylamine hydrochloride (1 M) was adjusted to pH 11.5 with KOH and immediately added to an appropriate solution of riboflavin in 0.03 M pyrophosphate. The pH of the riboflavin solution containing no hydroxylamine and the reagent blank were adjusted accordingly. The initial readings began 1 to 2 minutes after the mixing of the hydroxylamine and the riboflavin. At the point designated by the arrow, the pH of the contents in the cuvette containing the higher concentration of riboflavin and its reagent control was adjusted to 7.3 with a minute quantity of 5 N HCl. The dilution was kept constant in both cases and did not exceed 3 per cent. Control readings were made at 270 m, an area unaffected by hydroxylamine reaction with riboflavin. These values remained constant throughout.

reaction between riboflavin and hydroxylamine is the observation that increasing concentrations of riboflavin in the presence of a constant level of hydroxylamine (Fig. 2), or increasing concentrations of hydroxylamine in the presence of a constant level of riboflavin (Fig. 3), resulted in a corresponding increase in complex formation. The effect of pH on the formation of the riboflavin-hydroxylamine complex is presented in Fig. 4. At pH 7 to 8, no complex formation was observed. However, as the pH was increased, changes in optical density were observed in the presence of hydroxylamine.

There appears to be some discrepancy between the inhibition of xanthine
oxidase at pH 8 (1) and the relative absence of a reaction between riboflavin and hydroxylamine at this pH (Fig. 4). Differences between enzymatic and non-enzymatic reactions are, however, not uncommon and undoubtedly reflect the ability of the apoenzyme to control local conditions on the protein surface.

![Graph](image)

**Fig. 3.** Effect of hydroxylamine concentration on the formation of the riboflavin-hydroxylamine complex. Final concentration of the riboflavin was $2.7 \times 10^{-6}$ M. Freshly prepared hydroxylamine hydrochloride was adjusted to pH 10.5 with KOH immediately before use. 0.03 M pyrophosphate served as the buffer. All cuvette contents were mixed and allowed to stand for 30 minutes before reading. The values are the difference in optical density between cuvettes containing riboflavin and those containing riboflavin and hydroxylamine. Control readings made at 270 m$\mu$ were unaltered throughout the experiment.

That the hydroxylamine riboflavin complex is freely reversible is demonstrated in Fig. 2. The lowering of the pH of a riboflavin-hydroxylamine mixture at equilibrium from pH 11.1 to 7.3 by the addition of a minute quantity of acid (this point is indicated by the arrow) resulted in the virtual elimination of the decrease in optical density at 245 m$\mu$ produced at the higher pH by hydroxylamine.

No unequivocal explanation of the mechanism of the chemical reaction between hydroxylamine and riboflavin is here proposed, but certain points of interest are presented for consideration. The reaction between hy-
hydroxylamine and riboflavin occurs only in an alkaline medium, the spectroscopic effect increasing in magnitude with increasing pH (Fig. 4). The absence of any marked alteration in the slope of the curve at pH 10 could lead one to surmise that the ionization of the very weakly acidic group of the —CO—NH— grouping of the isoalloxazine ring (pKₐ 9.8 (6), 10.2 (7)) is unimportant as related to the study at hand; i.e., the ionized group

![Figure 4](https://www.jbc.org/content/journal/jbc/250/1/387)

**Fig. 4.** Effect of pH on the reaction of hydroxylamine with riboflavin. Final concentration of riboflavin and hydroxylamine was 2.7 × 10⁻⁴ M and 0.25 M, respectively. Freshly prepared hydroxylamine hydrochloride was adjusted to the desired pH immediately before use. 0.03 M phosphate served as the buffer at pH 6 to 8, 0.03 M pyrophosphate being used from pH 9 to 11. All the samples were read 30 to 40 minutes after the mixing of the reaction components. The values are the difference in optical density at 240 μm between cuvettes containing riboflavin and those containing hydroxylamine and riboflavin. Control readings made at 270 μm were unaltered throughout the experiment.

neither participates in nor hinders the formation of the hydroxylamine-riboflavin complex. This is probably due to the fact that the proportion of riboflavin ionized even at pH 11 to 12 would be of such a low order of magnitude as to have no measurable effect on the hydroxylamine-riboflavin ratio. Hydroxylamine in basic solutions forms an anion (Equations 1 and 2) which can react with aldehydes and ketones (8, 9).

\[
\text{NH}_2\text{OH} + \cdot\text{OH} = \text{−NHOH} + \text{HOH} \tag{1}
\]

\[
\text{−NHOH} = \text{NH}_2\text{O}^- \tag{2}
\]
Oxime formation involves a nucleophilic attack on a positively charged carbon atom forming a C—N bond. Since the cyanide ion, like the hydroxylamine ion, adds more readily to carbonyl groups than does the undissociated molecule (8, 10), the effect of −CN on the absorption spectrum of riboflavin was studied (Fig. 5). Cyanide addition produces changes in the riboflavin spectrum, in the region of 245 mμ, similar to those obtained in the case of hydroxylamine. The reaction appears to be somewhat more profound, as differences were also observed in the region of 300 mμ; no alterations were observed in the visible range. In the case of the cyanide-riboflavin complex, equilibrium is reached in less than 1 minute after the reaction components are mixed, compared to the 25 to 30 minutes needed in the hydroxylamine studies. Secondly, the reaction between −CN and riboflavin is dependent on the pH. No reaction takes place at pH 7.3 (unpublished data). However, at pH 11.3 the spectral alterations as re-

![Figure 5. Effect of cyanide on the absorption spectrum of riboflavin.](http://www.jbc.org/)

**Fig. 5.** Effect of cyanide on the absorption spectrum of riboflavin. ○, riboflavin (3.33 × 10⁻⁴ M) in 0.03 M pyrophosphate (pH 11.2); X, riboflavin (3.33 × 10⁻⁴ M) and sodium cyanide (0.5 M) in 0.03 M pyrophosphate, pH 11.3. Freshly prepared sodium cyanide (1 M) in 0.03 M sodium pyrophosphate was added to an equal volume of riboflavin (6.67 × 10⁻⁵ M) in 0.03 M pyrophosphate; final pH 11.3. The riboflavin control, in 0.03 M pyrophosphate, was adjusted to pH 11.3 with NaOH. Readings began 30 minutes after the addition of the cyanide to the riboflavin.
corded in Fig. 5 occur. Finally, the cyanide reaction appears to be irreversible. The lowering of the pH from 11 to 7, at which cyanide is essentially undissociated, caused no change in optical density in the region of 245 m\(\mu\). This is in contrast to the freely reversible hydroxylamine-riboflavin reaction. The ability of two negatively charged groups, i.e. \(-\text{NH}_2\) and \(-\text{CN}\), to produce similar absorption changes of the spectrum of riboflavin in the region of 245 m\(\mu\) would seem to indicate a common point of action or attack.

As a result of various studies (6, 11) it is apparent that there is at least one intermediate in the reduction of riboflavin and its derivatives. This semireduced compound is a semiquinoid or quinhydrone type of substance and is thought to have a formula similar to Scheme I. Thus it can be seen that the formation of any oxime or cyanhydrin-like compound, involving the carbon at position 4, could have a controlling influence on the formation of this quinhydrone. Such a reaction would prevent the normal enzymatic reduction of FAD.

Theoretically, the substrates used in the xanthine oxidase studies, xanthine and hypoxanthine (1), could, under the proper conditions, undergo a similar type of reaction with either hydroxylamine or cyanide. At the substrate and antagonist concentrations employed, such a reaction does not account for the enzymatic inhibition observed (1). In view of these findings this facet was not explored.

SUMMARY

1. The addition of high concentrations of hydroxylamine to aqueous solutions of riboflavin, riboflavin-5-phosphate, and flavin adenine dinucleotide produced a marked decrease in optical density in the region of 245 m\(\mu\) in the case of riboflavin or FMN and 240 m\(\mu\) in the case of FAD. The remaining portion of the spectrum was unaltered by hydroxylamine addition.

2. The rate of formation of the hydroxylamine-riboflavin complex, as measured by the decrease of optical density at 245 m\(\mu\), was found to be dependent on the pH.
3. The reaction between hydroxylamine and riboflavin was found to be slow, reaching equilibrium 20 to 30 minutes after mixing the reaction components.

4. Within limits, the degree of reaction appears to be proportional to the concentration of hydroxylamine and riboflavin.

5. The reaction between hydroxylamine and riboflavin was found to be freely reversible, as determined by altering the pH of the reaction.

Addendum—Since this paper was submitted for publication, spectrophotometric studies were carried out employing a crystalline xanthine oxidase preparation generously supplied by Mr. R. C. Bray of the Chester Beatty Research Institute, London, England. Incubation of hydroxylamine with enzyme preparation, in the absence of substrate, caused no change in the enzymatic spectrum. Likewise, the incubation of substrate (formaldehyde), in an excess of oxygen, produced no spectral alterations. Incubation of hydroxylamine and substrate with enzyme produced spectral alterations at 240 mp similar to those observed in the case of the non-enzymatic reaction between hydroxylamine and FAD. However, there were additional spectral alterations in the area of 250 to 280 mp, indicating that the mechanism of hydroxylamine inhibition of xanthine oxidase is more than a simple reaction of inhibitor and cofactor.

BIBLIOGRAPHY
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