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FEB 8 1966

MOLECULAR COMPLEXES OF FLAVINS AND PHENOLS. IV SEMIQUINONE HYDROIODIDE COMPLEXES*

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SUMMARY

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We have found that hydroiodic acid can reduce isocyanazine derivatives to the semiquinone level. Crystals of a solid material, probably an HI complex of the semiquinone hydroiodide, containing approximately 100% unpaired spin content, can be obtained from such solutions. The semiquinone hydroiodide is also found to form stable EPR complexes with various phenols. These may be isolated in crystalline form and also contain approximately 100% unpaired spin concentration.

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This work was supported in part by the United States Air Force Cambridge Research Laboratory (Contract No. AF19(628)414) and by the United States Atomic Energy Commission (Contract No. AT(11-1)908).

Taken in part from a dissertation submitted by Darrell E. Fleischman in partial fulfillment of the requirements for the Ph.D. degree at the University of Arizona.

Darrell E. Sloan Foundation Fellow.

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IN NUCLEAR SCIENCE AND ENGINEERING

FEB 11 1963

Introduction

Molecular complexes involving oxidized flavins are well known (1,2,3,4,5) and, on the basis of potentiometric data, Harbury et al. (1) have demonstrated the existence of rather stable complexes between fully reduced flavins and a variety of interactants.

Evidence also exists which suggests that flavin semiquinones can form stable complexes. For example, a series of highly colored intermediates have been isolated as crystalline solids by Kuhn and Ströbele (6) by various treatments of araboflavin solutions, in sodium hydroxide, which had been reduced by dithionite. Riboflavin reduction appeared to result in the formation of similar intermediates. On the basis of paramagnetic susceptibility and oxygen uptake in the course of reoxidation, the solids were identified as molecular complexes of the flavin semiquinone with flavin of varying degrees of oxidation and protonation. A red intermediate isolated from acidic solution, for example, was thought to be a complex of one mole of protonated semiquinone with one mole of completely reduced flavin. Similar observations were subsequently reported by other workers (7,8).

More recently, a stable free radical was obtained as a solid by Mahler and Brand (9) upon the addition of ethanol to a mixture of solid riboflavin and NADH. On the basis of the properties of this material, it was concluded that it differed from the radical produced by the reduction of riboflavin by other chemical methods and that both riboflavin and NADH were necessary for its formation.

In the present paper we will present evidence that a high concentration of flavin semiquinone is present in solutions of riboflavin in hydroiodic acid and that a molecular complex of the semiquinone hydroiodide and hydroiodic acid can be isolated from the solution in crystalline form. The interaction of the semiquinone in such solutions with phenolic compounds to form crystalline molecular complexes will also be discussed.

Experimental

Materials. Riboflavin was obtained from the California Corporation for Biochemical Research and was used without further purification. The procedures employed in the synthesis of lumiflavin and 4-methylisoalloxazine have been described previously. (10) Naphthalenediols were obtained from Eastman and recrystallized from suitable solvents. 47% hydriodic acid stabilized with 1% hypoarsenious acid, obtained from Mallinckrodt, was used.

Optical spectra. Visible and near infrared spectra were obtained with the use of the Cary Model 14 spectrophotometer. Spectra of suspensions of solids in mineral oil were obtained by the opal glass technique of Shibata (5,11).

Electron paramagnetic resonance (EPR) spectra. EPR spectra were recorded in derivative presentation using a Varian V-4501 spectrometer equipped with 100 kc modulation. Spin concentrations were assumed to be proportional to signal width squared times peak-to-peak height, and a diphenylpicrylhydrazyl standard was employed.

Semiquinone crystals. A concentrated solution of riboflavin in an ethanol - 47% HI mixture was permitted to evaporate at room temperature. Crystals in the form of pink platelets, formed over several days.

Lumiflavin was dissolved in 12N HCl, and 47% HI was added dropwise. A dark red-brown solid precipitated immediately. The latter was separated and dried under high vacuum in the presence of P_2O_5 . Various attempts at recrystallization were unsuccessful. Anal. Calculated for $C_{13}H_{14}N_4O_2I_2$: C, 30.49; H, 2.73; I, 49.58. Found: C, 28.04; H, 2.31; I, 50.04.

Saturated solutions of riboflavin in 47% hydriodic acid were prepared and filtered. To the filtrates were added ethanolic solutions of various naphthols and naphthalenediols. Black (red in thin section) hexagonal platelets formed after the solutions had been permitted to stand for a few hours. These were separated and dried under high vacuum in the presence of P_2O_5 . Crystals were obtained in this manner from solutions containing β -naphthol, 2,3-naphthalenediol, 1,7-naphthalenediol, 2,7-naphthalenediol, 1,5-naphthalenediol and 1,4-naphthalenediol. Various attempts to recrystallize these materials were unsuccessful. Analysis of the riboflavin - 2,7-naphthalenediol crystals gave the following results. Anal. Calculated for $C_{27}H_{29}N_4O_8I$: C, 48.82; H, 4.37; I, 19.11. Found: C, 46.58; H, 4.59; I, 19.88. Analysis of the riboflavin - 1,7-naphthalenediol crystals yielded the following. Anal. Found: C, 46.46; H, 4.60; I, 20.00.

Stoichiometry of crystals. The crystals were weighed and dissolved in dilute aqueous sodium thiosulfate, and the quantity of naphthalenediol and/or flavin determined from the optical densities at 326 m μ and 450 m μ . The quantity of iodide was obtained by difference.

Results and Discussion

When hydriodic acid is added to solutions of various isoalloxazines in hydrochloric acid ($> 1N$), the solutions become deep red and a dark precipitate forms, either immediately or after partial evaporation of the solvent. The visible absorption spectrum of a solution of riboflavin in 47% HI resembles the spectrum of the flavin semiquinone in acid (Figure 1). From the optical density at 490 m μ in this spectrum, the semiquinone concentration can be calculated to be 30% of the total flavin present (12).

EPR spectra of solutions of flavins in concentrated HI are identical to those obtained for the semiquinone species. For example, the EPR spectrum of a dilute solution of 9-methylisoalloxazine in a hydrochloric acid-hydriodic acid mixture is shown in Figure 2. This should be compared with Figure 6 of reference 10.

On the basis of these observations, we can conclude that hydriodic acid will reduce flavin to the semiquinone level.

If solutions of riboflavin in 47% HI-ethanol mixtures are permitted to evaporate slowly, flat, transparent pink crystals form. These may be as large as several millimeters across, and usually have well-developed faces and edges. The crystals display a very intense, narrow and structureless EPR absorption in the $g = 2.00$ region (Figure 3). The crystals darken gradually upon standing in air for long periods of time.

The EPR absorption of a carefully dried sample of this material was compared to a diphenylpicryl hydrazyl standard. The crystals were found to possess approximately 1×10^{21} unpaired spins per gram. Such measurements probably have order of magnitude validity.

The EPR spectrum of a single crystal of the material was found to be independent of orientation and to display no hyperfine structure.

Spectrophotometric analysis (see Experimental section) of the solid obtained by adding hydroiodic acid to a hydrochloric acid solution of lumiflavin indicated a flavin: iodide ratio of 1:2 (the actual value obtained was 1:1.9, as an average of two independent determinations). The elemental analysis (see Experimental) is in good agreement with this stoichiometry, indicating a fairly pure material. One might postulate a molecular complex between HI and RH_2^+I^- (semiquinone hydroiodide) as a rationalization of this ratio and of the observed high level of paramagnetism. With respect to the latter, a sample of this material was found to contain 1.6×10^{21} spins per gram. If the composition is as assumed, this would correspond to 8×10^{23} spins per mole, that is, to approximately 100% unpaired spin content.

The absorption spectrum of a mineral oil suspension of this material (Figure 4) displays an intense, broad absorption band with a maximum near 500 mμ. It seems quite reasonable to attribute this band to a broadening of the long wavelength absorption band which the semiquinone has in acid solutions (see Figure 1).

When hydroiodic acid solutions of riboflavin which also contain hydroquinone are permitted to evaporate slowly, black crystals form which are visually indistinguishable from riboflavin hydrochloride-hydroquinone complex crystals (5). Such crystals are also very intensely paramagnetic (as compared with the rather low paramagnetism of the hydrochloride complex) with a signal in the $g = 2.00$ region. The EPR spectrum of what appeared to be a single crystal of this material is reproduced in Figure 5. This spectrum was found to be independent of crystal orientation, in contrast to what was previously found for the hydrochloride complex (13).

When solutions of riboflavin and 2,3-naphthalenediol in hydroiodic acid are evaporated, black, hexagonal platelets form. These crystals also give an intense, narrow, structureless EPR absorption in the $g = 2.00$ region. Similar hexagonal crystals can be obtained from hydroiodic acid solutions of riboflavin containing other naphthalenediols or β -naphthol.

Spectrophotometric analysis (see Experimental section) of a sample of crystals thus obtained from a solution of riboflavin and 2,7-naphthalenediol in a hydroiodic acid-ethanol mixture indicated a flavin:diol:1-d.i.d. ratio of 1:1.2:1.3 (average of three independent determinations). We feel that this indicates a 1:1:1 stoichiometry. Solvent trapping could account for high iodide value (which was obtained by difference), and it may be assumed that the high naphthalenediol value is due to an excess of the diol in the solution from which the crystals were grown. This would be consistent with observations made in the course of our study of flavin hydrochloride-phenol complexes (13,14). These hold solvent tenaciously and initially possess a high flavin:diol ratio which approaches 1:1 upon repeated recrystallization. Unfortunately, it was not possible to recrystallize the hydroiodide complexes. The elemental analysis (see Experimental) is in agreement with this stoichiometry, again indicating a reasonable purity of the crystals.

When crystals of the phenol complexes or of the semiquinone hydroiodide-HI complexes are dissolved in dilute aqueous thiosulfate solution, the visible absorption spectra of the resulting solutions are indistinguishable from the spectra of the corresponding pure flavins. This indicates that there has been no significant decomposition of the flavin associated with complex formation.

The unpaired spin concentrations of the flavin-phenol-HI complexes are uniformly quite high. For example, a calibration of the riboflavin-2,3-naphthalenediol complex gave a value of 2.1×10^{21} unpaired spins per gram. If the formula of this material is assumed to be $(\text{riboflavin})_1(\text{diol})_1(\text{HI})_1$, this corresponds to 1×10^{24} spins per mole or, again, to approximately 100% unpaired spin content.

It seems reasonable to conclude, therefore, that these materials are molecular complexes of the flavin semiquinone hydroiodide and the phenolic component.

Optical spectra of suspensions of the phenol complexes (Figure 6) display an intense, broad absorption band near 500 mμ. The exact position of this band is found to vary slightly with the phenolic moiety. However, the variation in position is only of the order of 25 mμ. There is not the same sort of variation in position, shape and intensity which is observed with the long wavelength bands of the hydrochloride complexes (5). Furthermore, the band in a hydroiodide complex may differ markedly in position and intensity from that in the corresponding hydrochloride complex. Thus, as in the case of the semiquinone hydroiodide-hydroiodic acid complex, this band can probably be attributed to a broadening of the semiquinone band with an additional slight shifting due to interaction with the phenolic component.

Suspensions of all of these complexes, as well as of the hydrochloride complexes and of the hydrochloride and hydroiodide salts of oxidized flavins, were found to display extremely broad absorption bands in the near infrared region (between 9000 and 20,000 Å). The nature of these absorptions is not clear at present.

The paramagnetism of all of the hydroiodic acid complexes was found to diminish only very slowly upon standing in air over a period of weeks. Thus, complexing appears to stabilize the semiquinone. This conclusion would be consistent with the observation of Mahler and Brand (9) that a radical-presumably of flavin-is stabilized in the presence of NADH, and also with the observation of Hemmerich et al. (15) that metal chelation stabilizes the flavin radical in solution. These observations may have biological significance in terms of the functioning of the flavin semiquinone in flavoprotein enzyme catalysis (16).

Fig. 1

7.8 gauss

FIG. 3

FIG. 4

FIG. 5

Fig 6