Miles, D. W., and Urry, D. W. (1968), *J. Biol. Chem. 243,* 4181.
Sarma, R. H., and Kaplan, N. O. (1970a), *Biochemistry 9,* 557.
Sarma, R. H., and Kaplan, N. O. (1970b), *in* Pyridine Nucleotide-Dependent Dehydrogenases, Sund, H., Ed., New York, N. Y., Springer-Verlag, p 44.
Sarma, R. H., Ross, V., and Kaplan, N. O. (1968), *Biochemistry 7,* 3052.
Shifrin, S., and Kaplan, N. O. (1960), *Advan. Enzymol. 22,* 337.
Vinogradov, S. N., and Linnell, R. H. (1971), Hydrogen Bonding, Cincinnati, Ohio, Van Nostrand-Reinhold, p 75.
Weber, G. (1957), *Nature (London) 180,* 1409.

Intramolecular Hemiacetal Formation in 8-Formylriboflavine†

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ABSTRACT: Reports in the literature suggest that the flavine site of *Chromatium* cytochrome c_{552} contains a covalent adduct in which a cysteine residue is linked to 8-formyl-FAD in a thiohemiacetal linkage. This indication of a biological function for 8-formylflavines and the lack of published information on their chemical and physical properties prompted the present study of three 8-formylflavine analogs. It was found that a ribityl hydroxyl group forms a stable hemiacetal with the 8-formyl group in 8-formylriboflavine. Acid hydrolysis of 8-formylriboflavine converts it from a species which gives a negative 2,4-dinitrophenylhydrazine test to one which gives a positive test. On standing in aqueous solution it reverts to the original species. 8-Formyltetraacetylriboflavine and 8-formyl-3-methyllumiflavine have hydroquinone absorption spectra characterized by maximal absorption at 520 and 392 nm, while reduced 8-formylriboflavine has little or no absorption in this spectral region. All three flavine analogs give identical absorption spectra when overreduced with $TiCl_3$ in 6 N HCl. The oxidation-reduction potential of 8-formylriboflavine is −0.159 V, which is raised after acid hydrolysis to −0.090 V. The respective oxidation-reduction potentials of 8-formyltetraacetylriboflavine and 8-formyl-3-methyllumiflavine are −0.006 and −0.045 V. The circular dichroism spectrum of 8-formylriboflavine is drastically altered with respect to riboflavine, whereas the spectrum of 8-formyltetraacetylriboflavine is very similar to that of tetraacetylriboflavine. Studies with molecular models suggest that the 5′-hydroxyl group is involved in hemiacetal formation.

Studies on the covalently bound flavine of *Chromatium* cytochrome c_{552} (Hendricks *et al.*, 1972, Kenney *et al.*, 1972, 1973) strongly suggest that the flavine (FAD) is bound by way of a thiohemiacetal linkage to a cysteine residue in the polypeptide chain. Since the flavine component of this adduct is 8-formyl-FAD, a biological role is established for 8-formylflavines. Although 8-formylflavines have been synthesized and used as intermediates in the syntheses of other 8α-substituted flavines (Salach *et al.*, 1972; McCormick, 1970), there is little or no information in the literature on their chemical and physical properties. Information of this kind is of value for further studies of the *Chromatium* flavine.

This paper reports a comparison of the physical and chemical properties of three 8-formylflavine analogs. The results show that in the case of 8-fRF,[1] a side-chain hydroxyl group (most likely that in the 5′ position) forms a hemiacetal with the 8-formyl group. This intramolecular interaction profoundly affects the oxidationdshreduction potential and circular dichroic (CD) properties of the 8-formyl-substituted isoalloxazine ring.

Experimental Section

Flavine Analogs. 8-FRF was synthesized by acid hydrolysis (6 N HCl, reflux for 2 hr) of 8α-dibromotetraacetylriboflavine. The dibromo compound was prepared by prolonged heating of tetraacetylriboflavine with excess bromine in the presence of dibenzoyl peroxide (Ghisla *et al.*, 1970). 8-fRF was also synthesized according to the procedure outlined by McCormick (1970). In the early part of this work, the samples of 8-fRF used were gifts from Dr. G. Blankenhorn, University of California, Davis, Calif., and from Dr. Peter Hemmerich, University of Konstanz, Germany.

8-Formyl-3-methyllumiflavine was synthesized as described by Salach *et al.* (1972). The sample of 3-methyllumiflavine used in this synthesis was a gift from Dr. S. Ghisla, the University of Michigan. 8-fAc_4RF was observed to be a degradation product of 8α-cysteinyltetraacetylriboflavine upon prolonged storage of the thioflavine in aqueous solution (1 week or longer). Purification of the formylflavine involved preparative high-voltage electrophoresis (pH 1.6) and subsequent descending paper chromatography in solvent A (see below).

Homogeneity of all flavine analogs was monitored by thin-layer chromatography on cellulose plates (Eastman Kodak 13255), using 1-butanol-acetic acid-H_2O, either 4:2:2, v/v (solvent A) or 4:1:5, v/v (upper layer) (solvent B), for development.

Methods. Anaerobic spectrophotometric titrations with dithionite solutions were performed under helium in a glass titration cell slightly modified from the design of Burleigh *et al.*

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[1] Abbreviations used are: 8-fRF, 8-formylriboflavine; 8-fAc_4RF, 8-formyltetraacetylriboflavine.

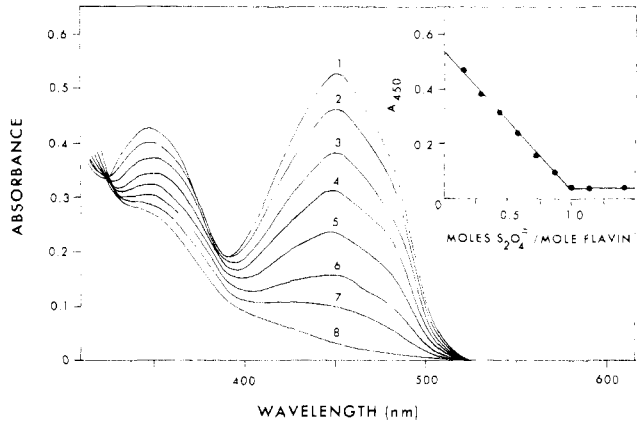

FIGURE 1: Anaerobic dithionite titration of 8-fRF in 0.1 M sodium phosphate (pH 7.0). Curve 1 is the spectrum before the addition of dithionite, curves 2–7 are the intermediate spectra during reduction, and curve 8 is the spectrum after the addition of 1.1 mol of dithionite/mol of flavine. The inset shows the graphical plot of this titration.

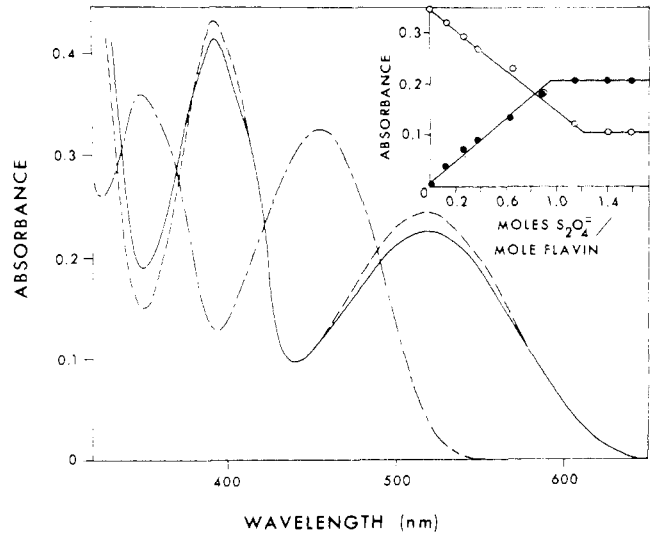

FIGURE 2: Absorption spectral properties of 8-fAc_4RF in its oxidized form (- · -), after reduction by light-EDTA (- - -), and after the addition of 1.4 mol of dithionite/mol of flavine (——) in 0.1 M sodium phosphate (pH 7.0). The *inset* shows the absorption at 450 nm (○) and at 540 nm (●) *vs.* the amount of dithionite added.

(1969). Dithionite solutions were standardized by anaerobic spectrophotometric titrations of 3-methyllumiflavine solutions. Absorption spectra were recorded using a Cary 14 spectrophotometer. 8-Formylflavine concentrations were estimated using the molar absorption coefficient of 9000 M^{-1} cm^{-1} at 450 nm (Salach *et al.*, 1972).

Oxidation–reduction potential measurements were obtained by reductive, anaerobic spectrophotometric titrations of solutions of the appropriate 8-formylflavine in the presence of either indigodisulfonate ($E_{m,7} = -0.116$ V) or indigotetrasulfonate ($E_{m,7} = -0.046$ V) (Clark, 1960) at 25°, as previously described (Edmondson and Singer, 1973). Under conditions in which the reduced 8-formylflavine had an absorption maximum at 520 nm, dye reduction was measured at 630 nm, while flavine reduction was measured at 540 nm, after correction for the contribution of the oxidized dye to the absorbance.

Flavine photoreductions were performed by irradiation of the appropriate flavine solution in the presence of 0.05 M EDTA and 0.1 M P_i (pH 7.0), in an anaerobic quartz Thunberg cuvet at room temperature. The samples were irradiated at a distance of 10 cm from two 15-W fluorescent lamps.

CD spectra were measured at 25° with a Jasco UV-5 instrument equipped with a Sproul SS-10 modification. Cylindrical quartz cells of 10-mm path length were used in all measurements.

Results

Qualitative Studies. The initial observation that differentiated 8-fRF from either 8-fAc_4RF or 8-formyl-3-methyllumiflavine was its reactivity with 2,4-dinitrophenylhydrazine. 8-fRF forms a hydrazone only on boiling the aldehyde with the reagent in 2 N HCl while the latter two formylflavines form hydrazones at room temperature. The reactivity of 8-fRF toward 2,4-dinitrophenylhydrazine parallels that of the other two formylflavine analogs if it is first refluxed with 6 N HCl.

In an effort to clarify this unexpected behavior, 8-fRF was refluxed in the presence of 6 N HCl and, at intermittent times, samples were taken for thin-layer chromatography in solvent B. The flavine migration changed from an R_F value of 0.42–0.37 during refluxing. This change was complete in 90 min. The species migrating with the lower R_F value gave a positive 2,4-dinitrophenylhydrazine test, whereas that initially present gave a negative test. The flavine solution was then dried *in vacuo* and redissolved in H_2O. Chromatography at this point indicated that this procedure had resulted in substantial reversion (~70%) to the original form of the flavine, since the major spot had an R_F value of 0.41 and the 2,4-dinitrophenylhydrazine test was negative.

It was also observed that 8-fRF is very stable to storage in aqueous solution at room temperature in the dark, while 8-formyl-3-methyllumiflavine and, to a lesser extent, 8-fAc_4RF gradually decompose, forming substantial amounts of 8-hydroxy- and 8-carboxyflavines under the same conditions. These products were identified by high-voltage electrophoresis and by thin-layer chromatography. The mechanism and rate of this breakdown have not been investigated, but these observations emphasize the need to use freshly prepared solutions of 8-formylflavine, in order to avoid contamination with breakdown products.

Oxidation–Reduction Properties. Reduction of 8-fRF by dithionite results in a 2-electron uptake and a reduced spectrum very similar to that of other 8α-substituted flavines (Edmondson and Singer, 1973) (Figure 1). Identical spectral properties were observed upon photoreduction in the presence of EDTA. Atmospheric oxidation of the reduced flavine produced by either method restores the original absorption spectrum.

The absorption spectrum of 8-fAc_4RF, after reduction by dithionite or by the light-EDTA procedure, was very different from that of reduced 8-fRF (Figure 2). The spectral properties of reduced 8-formyl-3-methyllumiflavine (not shown) were identical with those of reduced 8-fAc_4RF. Reduction of acid-hydrolyzed 8-fRF gave an absorption spectrum (not shown) with a band at 520 nm, the intensity of which was only 20–30% of that given by 8-fAc_4RF. The spectrum of reduced 8-fAc_4RF in Figure 2 shows absorption maxima at 520 and at 392 nm, with molar absorption coefficients of 6700 and 11,900, respectively. The spectral properties of these reduced flavines are quite different from those of any known flavine (Hemmerich *et al.*, 1971) and most probably reflect the conjugation of the 8-formyl group with the isoalloxazine ring system. Alternate possibilities, such as flavine semiquinone or dimer formation, are eliminated since the molar absorption coefficients of spectral bands are not influenced by concentration over a 50-fold range and the reduced flavines do not give any electron spin resonance (esr) signals.

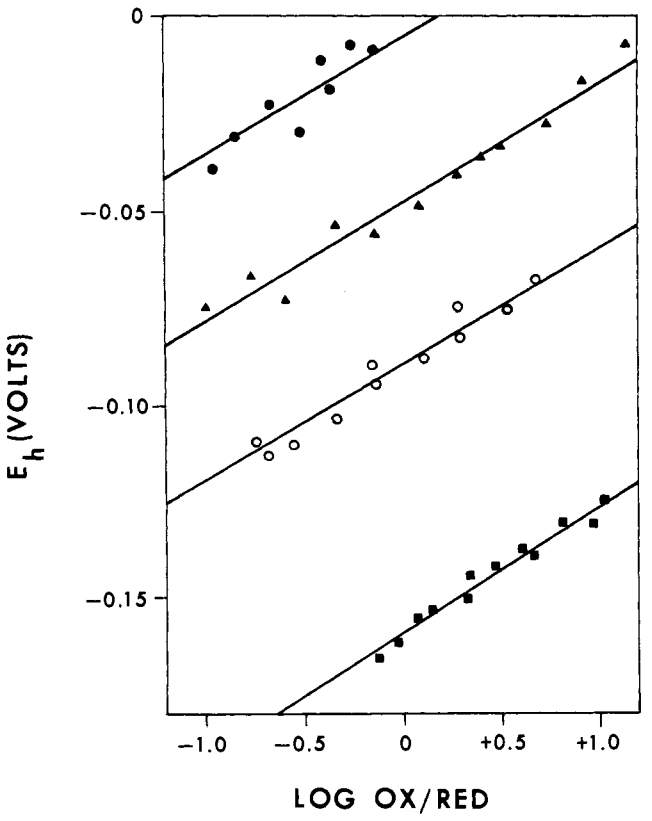

FIGURE 3: Oxidation–reduction titrations of 8-formylflavine analogs in 0.1 M sodium phosphate (pH 7.0) at 25°, and in the presence of either indigodisulfonate or indigotetrasulfonate: (■) 8-fRF, (○) 8-fRF after acid hydrolysis, (▲) 8-formyl-3-methyllumiflavine, and (●) 8-fAc$_4$RF. The solid lines are for a theoretical $N = 2$ at each of the various $E_{m,7}$ values.

Figure 2 shows some differences in extinction when 8-fAc$_4$RF is reduced by dithionite or by the light-EDTA procedure. As illustrated in the inset, on titration with dithionite the absorbance at 540 nm reached a maximum before the 450-nm band reached a minimum. Well-defined isosbestic points at 489, 422, 370, and 335 nm were observed during photoreduction. These isosbestic points, however, were not as well defined during titration with dithionite. These data suggest the formation of an additional flavine species upon reduction by dithionite which is likely to be a sulfite adduct of the 8-formyl group. On thin-layer chromatography, however, the dithionite-reduced 8-fAc$_4$RF (1.5 mol of dithionite/mol of flavine) showed the same mobility as the untreated compound. This observation indicates that modification of the flavine aldehyde is probably a reversible process and supports the idea that residual sulfite (a product of the oxidation of dithionite) forms an adduct with the 8-formyl group.

The fact that the 540-nm absorbance was maximal before complete bleaching of the 450-nm band (Figure 2) suggests that the unsubstituted flavine aldehyde has a higher redox potential than the flavine aldehyde–sulfite complex. It also shows that addition to the 8-formyl group disrupts the conjugation responsible for the long-wavelength absorption. This explains why during the reduction of 8-fRF no 520-nm band is seen (Figure 1). On the basis of these chemical and spectral observations, it seemed likely that if the formyl group is complexed in 8-fRF, it should have a lower oxidation–reduction potential than either 8-fAc$_4$RF or 8-formyl-3-methyllumiflavine.

The data in Figure 3 provide evidence for this hypothesis. Anaerobic titration of 8-fRF with dithionite in the presence of indigodisulfonate and analysis of the data (Edmondson and Singer, 1973) showed an oxidation–reduction potential of −0.159 V. This value is close to the potentials of other 8α-substituted flavines (Edmondson and Singer, 1973) but is much lower than the potentials of other 8-formylflavine analogs. The oxidation–reduction potentials of 8-fAc$_4$RF, 8-formyl-3-methyllumiflavine, and acid-hydrolyzed 8-fRF were obtained by anaerobic titration of each flavine with dithionite in the presence of indigotetrasulfonate. To avoid spectral overlap with the reduced flavine, dye reduction was monitored at 630 nm. Because of aldehyde addition by residual sulfite or, in the case of 8-fRF, partial reversion to a dinitrophenylhydrazine negative species, flavine reduction was monitored by the increase in absorbance at 540 nm, after correction for the absorbance due to the oxidized dye. The plots (Figure 3) were consistent in all cases with a 2-electron reduction. The oxidation–reduction potential for "uncomplexed" 8-fRF is −0.090 V, for 8-formyl-3-methyllumiflavine, −0.045 V, and for 8-fAc$_4$RF, −0.006 V. On the basis of the known potentials of their unsubstituted analogs, one would expect that "uncomplexed" 8-fRF would have an oxidation–reduction potential similar to that of 8-fAc$_4$RF. The difference observed shows that ribityl side-chain modification has a greater influence on the oxidation–reduction potentials of 8-formylflavines than on those of 8-methylflavines.

CD Studies. The above chemical and oxidation–reduction studies clearly show that in 8-formylflavines in which the ribityl hydroxyl groups are either missing or are acetylated, the 8-formyl group is chemically reactive. Where ribityl hydroxyl groups are present (8-fRF), the formyl group exhibits anomalous behavior. Any visible CD bands exhibited in riboflavine arise from interaction of the optically inactive isoalloxazine ring with the optically active ribityl side chain (Tollin, 1968; Edmondson and Tollin, 1971). If the anomalous behavior of 8-fRF is the result of hemiacetal formation between the 8-formyl group and a ribityl hydroxyl group, a large difference between the CD spectra of riboflavine and 8-fRF would be expected. Changes in optical activity would result from two effects: (a) the generation of an optically active carbon at the 8α position and (b) the rigidity of the ribityl side chain, induced by attachment to the isoalloxazine ring at two positions (N-10 and 8α). The former effect may not be as important as the latter, since hemiacetal formation could occur from either side of the ring, yielding both optical isomers of the asymmetric 8α carbon.

The CD spectra in Figure 4 show that 8-fRF exhibits considerably more optical activity than does riboflavine, in agreement with the postulate of an intramolecular hemiacetal bond. The spectrum of an 8-fRF sample (not shown), in which 70% was estimated to be in the hemiacetal form, was 61% as intense in the 450-nm region as the spectrum shows in Figure 4. The CD spectrum of 8-fRF shows positive bands at 505 and at 296 nm and more intense negative bands at 452, 370, and 320 nm (Figure 4). The previous finding that the intensity of flavine dichroic bands in the 450-nm region is sensitive to the degree of interaction of ribityl hydroxyl groups with the isoalloxazine ring (Edmondson and Tollin, 1971) is further substantiated, as the 452-nm band is the most intense of the CD bands in the visible spectral region. The CD spectrum of 8fAc$_4$RF (not shown) is very similar in shape and intensity to that of tetraacetylriboflavine (Edmondson and Tollin, 1971), indicating similar side-chain mobility for both flavine analogs.

Reduction of 8-Formylflavines in Acid Media. The cation flavoquinone spectrum of 8-fRF in 6 N HCl (Figure 5), with a maximum at 373 nm and a shoulder at 420 nm, is quite similar to those of 8-fAc$_4$RF and 8-formyl-3-methyllumiflavine. Reduction with approximately a stoichiometric amount of $TiCl_3$ gave a species with an absorption maximum at 475 nm, which is attributed to the cation semiquinone species (Figure 5), since

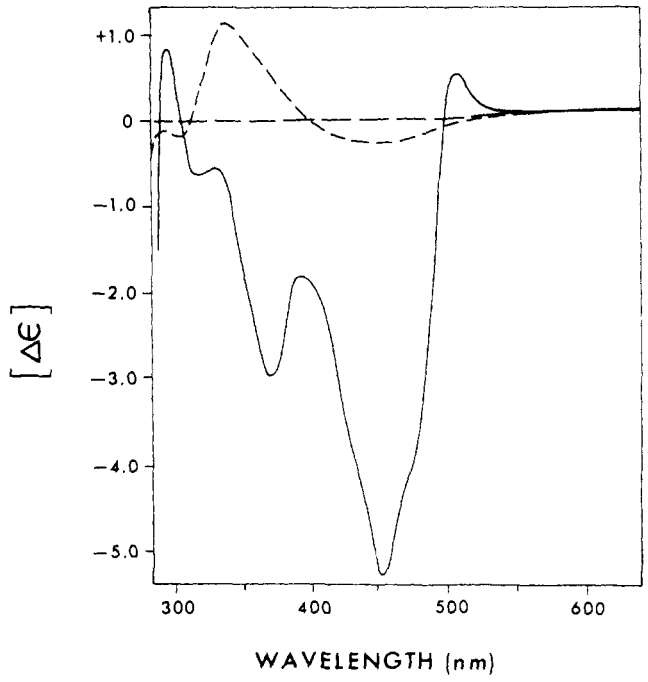

FIGURE 4: Circular dichroism spectra of riboflavine (- - -) and 8-fRF (—) in 0.1 M sodium phosphate (pH 7.0) at 25°.

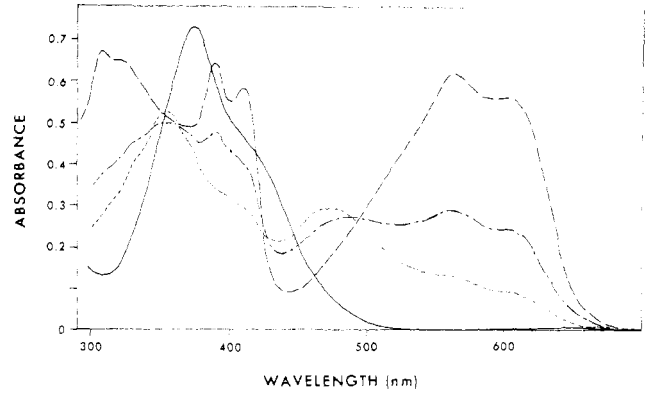

FIGURE 5: Absorption spectra of 8-fRF in 6 N HCl: (—) oxidized form, (···) after the addition of approximately 1 equiv of $TiCl_3$, (- · -) after the addition of slightly more than 1 equiv of $TiCl_3$, and (- - -) after the addition of excess (~1 mM) $TiCl_3$.

other 8α-flavine semiquinone cations exhibit similar spectral properties (Salach *et al.*, 1972). Further reduction with $TiCl_3$ decreased the absorption at 475 nm, while appreciable absorption developed in the 550- to 600-nm region (Figure 5). Addition of excess $TiCl_3$ to any of the three 8-formylflavine analogs produced a reduced flavine species with absorption maxima at 605, 565, 410, and 390 nm. These spectral characteristics are due to the 8-formylflavine hydroquinone cation, as the intermediate semiquinone species has a quite different absorption spectrum (Figure 5). This unusual spectrum for a flavine hydroquinone cation is not due to complex formation with Ti^{3+} or Ti^{4+}, since anaerobic addition of HCl to a dithionite-reduced neutral solution of 8-fRF produced a spectrum identical with that obtained with excess $TiCl_3$ at the same acid (2 M) concentration.

The absorption band at 565 nm is dependent on acid concentration, as shown in Figure 6A. A molar absorption coefficient of 24,700 is estimated at infinite hydrogen-ion concentration

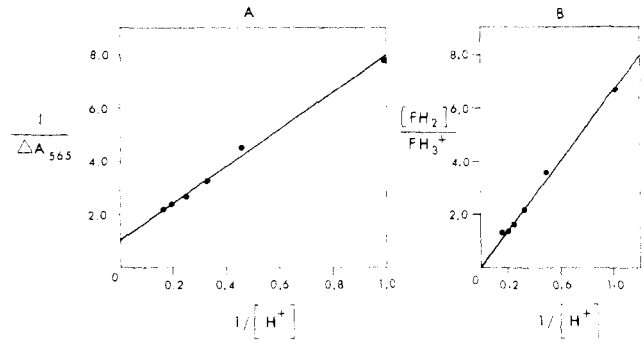

FIGURE 6: (A) Benasi–Hildebrand plot of the absorbance at 565 nm at different HCl concentrations for 8-fRF reduced with excess (~1 mM) $TiCl_3$. (B) Plot of FH_2 to FH_3^+ (where F = 8-fRF) *vs.* $1/(H^+)$ using the extrapolated extinction of 24,700 (A) to calculate FH_3^+ concentration.

from the Benasi–Hildebrand plot (Benasi and Hildebrand, 1949). If one assumes the 565-nm absorption to be a measure of only the flavohydroquinone cation, the calculated ratio of $[8\text{-fRFH}_2]$ to $[8\text{-fRFH}_3^+]$ *vs.* $1/[H^+]$ gives a linear plot, with the intercept at the origin (Figure 6B), as expected for a single proton ionization. The slope (6.69) is equal to the dissociation constant, indicating a pK of −0.825.

The identical spectral properties shown by all three 8-formylflavine analogs in their hydroquinone cation forms show that the intramolecular hemiacetal of 8-fRF is hydrolyzed under these conditions. In agreement with this conclusion, the CD spectrum of 8-fRF, reduced with $TiCl_3$, shows little or no optical activity. The hemiacetal bond is still intact in the oxidized form in 6 N HCl at ambient temperature, since the CD spectrum shows two negative bands in the visible spectral region, with maxima at 440 and at 370 nm, and with intensities ($\Delta\epsilon$) of −3.5 and −9.0 M^{-1} cm^{-1}, respectively. For comparison, the CD spectrum of the riboflavine cation shows a single positive band at 395 nm with an intensity of +6.3 M^{-1} cm^{-1} (Tollin, 1968).

Discussion

The unusual properties of 8-fRF documented in this paper, as compared with other 8-formylflavines lacking free ribityl hydroxyl groups, strongly suggest intramolecular hemiacetal formation between a ribityl hydroxyl group and the 8-formyl group. CD spectral data and thin-layer chromatographic results show the bond to be quite stable in the oxidized form of the flavine in both neutral and acidic aqueous solution at ambient temperature. Absorption spectral data indicate that the bond is stable in the flavine hydroquinone form at neutral pH (Figures 1 and 2), but not under acidic conditions (Figure 5). While the 8-fRF hydroquinone cation has little or no optical activity and has the same spectral properties as the other 8-formylflavine analogs, the neutral form shows a positive CD band at 370 nm, with an intensity three times that of reduced riboflavine (which has a broad, negative band at 335 nm).

These results may be rationalized if one considers that the electron-deficient, oxidized isoalloxazine ring promotes hemiacetal formation by decreasing the electron density of the 8-formyl group. Reduction of the flavine increases the electron density and thus labilizes the hemiacetal bond. A neutral, aqueous environment is not sufficient to hydrolyze the bond, which is hydrolyzed only under acidic conditions.

The question as to which of the four ribityl hydroxyl groups participates in the hemiacetal bond is still open for further investigation. Molecular models (Corey–Pauling type) indicate that the 5′-hydroxyl group is involved, since sterically it is the

only one close enough to the 8-formyl group to form the hemiacetal bond. A molecular model, built in this manner, shows the ribityl side chain to be held in a very rigid conformation, with the rotation of the 2′- and 4′-hydroxyl groups severely restricted.

Such rigid conformation is, in fact, evident from the CD spectrum of 8-fRF (Figure 4). The intensities observed are as great as those observed for flavoenzymes (Edmondson and Tollin, 1971), in which the flavine is tightly bound to an asymmetric protein environment. The near-ultraviolet dichroic bands are of interest in that they are not readily apparent in the absorption spectrum (Figure 1). The wave number difference between the 370- and 320-nm bands (4250 cm^{-1}) makes it unlikely that they are vibronic components of the same electronic transition. Previous CD (Edmondson and Tollin, 1971) and magnetic CD (Tollin, 1968) results, as well as molecular orbital calculations (Fox *et al.*, 1967; Song, 1968), support the existence of two electronic transitions in this spectral region for unsubstituted flavines. Further work is required to assess the influence of 8 substitution on the electronic transitions in this spectral region.

The unusual absorption spectra of the reduced cationic forms of 8-formylflavines can serve as an intrinsic property for their detection since no other flavine analog tested shows this spectral behavior. Previous observations (Walker *et al.*, 1971) that overreduction of 8α-cysteinylriboflavine or of 8α-hydroxyriboflavine by $TiCl_3$ in acid media yields a blue color (λ_{max} 565–605 nm) could not be confirmed with carefully purified preparations of these flavines. It seems likely that these previous observations were due to the presence of 8-fRF in the samples.

The last point to be discussed is the relevance of these observations to the flavine thiohemiacetal of *Chromatium* cytochrome c_{552}. The enzyme-bound flavine is in the dinucleotide form (Kenney *et al.*, 1973) and thus the 5′-ribityl hydroxyl group is substituted. It is probable, therefore, that the relative stability of the 8α-flavine–cysteine bond is not the result of mixed acetal formation, for steric reasons, with other hydroxyl groups of the ribityl chain. Instead, the thiohemiacetal bond in the *Chromatium* flavine peptide is probably stabilized by interaction with a tyrosyl residue (Kenney *et al.*, 1973). The interaction of the ribityl side chain with the isoalloxazine ring is also less in the *Chromatium* flavine peptic, as compared with 8-fRF, as judged from the relative intensities of their CD bands in the 450-nm region (Figure 4) (Kenney *et al.*, 1973).

Acknowledgments

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References

Benasi, H. A., and Hildebrand, J. H. (1949), *J. Amer. Chem. Soc. 71,* 2703.

Burleigh, B. D., Jr., Foust, G. P., and Williams, C. H., Jr. (1969), *Anal. Biochem. 27,* 530.

Clark, W. M. (1960), Oxidation-Reduction Potentials of Organic Systems, Baltimore, Md., William and Wilkins.

Edmondson, D. E., and Singer, T. P. (1973), *J. Biol. Chem. 248,* 8144.

Edmondson, D. E., and Tollin, G. (1971), *Biochemistry 10,* 113.

Fox, J. L., Laberge, S. P., Nishimoto, K., and Forster, L. S. (1967), *Biochim. Biophys. Acta 136,* 544.

Ghisla, S., Hartmann, U., and Hemmerich, P. (1970), *Angew. Chem., Int. Ed. Engl. 9,* 642.

Hemmerich, P., Ghisla, S., Hartmann, U. and Müller, F. (1971), *in* Flavins and Flavoproteins, Kamin, H., Ed., Baltimore, Md., University Park Press, pp 83–103.

Hendricks, R., Cronin, J. R., Walker, W. H., and Singer, T. P. (1972), *Biochem. Biophys. Res. Commun. 46,* 1262.

Kenney, W. C., Edmondson, D., Seng, R., and Singer, T. P. (1973), *Biochem. Biophys. Res. Commun. 52,* 434.

Kenney, W. C., Walker, W. H., Kearney, E. B., Seng, R., and Singer, T. P. (1972), *Z. Naturforsch. B 27,* 1069.

McCormick, D. B. (1970), *J. Heterocycl. Chem. 7,* 447.

Miles, D. W., and Urry, D. W. (1968), *Biochemistry 7,* 2791.

Salach, J., Walker, W., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S., and Hartmann, U. (1972), *Eur. J. Biochem. 26,* 267.

Song, P. S. (1968), *Int. J. Quantum Chem. 2,* 463.

Tollin, G. (1968), *Biochemistry 7,* 1720.

Walker, W. H., Kearney, E. B., Seng, R. L., and Singer, T. P. (1971), *Eur. J. Biochem. 24,* 328.