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MOLECULAR COMPLEXES OF FLAVINS AND PHENOLS. III. FACTORS
INFLUENCING SPECTRA AND STABILITY*†

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INTRODUCTION

In a previous paper (1), we presented evidence that alloxazine and isoalloxazine derivatives, including riboflavin and FMN, form molecular complexes with a variety of phenolic compounds in both acidic and neutral solution. Highly-colored crystalline complexes were isolated from each type of solution. The acidic complexes were found to have a flavin:phenol:HCl ratio of 1:1:1 both in solution and in the crystalline state. Optical absorption bands which were attributed to charge transfer transitions were found in the spectra of all of the solid complexes, and in the spectra of solutions of some of the acid complexes.

In the present paper, stability constants of a number of the complexes are presented, and correlations between complex stabilities, charge transfer band positions and the structures and electronic properties of the components are attempted. Light is thereby shed upon the nature of the bonding in the complexes.

Harbury et al. (2) discussed the effect upon complex stability of the donation of hydrogen to the isoalloxazine nucleus in hydrogen bond formation. In the systems they studied it was found that hydrogen bonded isoalloxazine formed more stable complexes, presumably because of enhancement of the electron affinity of the isoalloxazine. It was there-

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fore of interest to observe the effect upon complex stability of complete protonation of the isoalloxazine. Such effects may have considerable biological importance in view of a suggestion by Harbury et al. (2) that the degree of flavin protonation may vary rather widely among flavoproteins.

In the numerous previous studies of neutral flavin complexes (2,3,4,5) a 1:1 flavin:interactant stoichiometry has been assumed. In the present work the flavin:interactant ratio is determined experimentally for neutral complexes both in solution and in the crystalline state.

EXPERIMENTAL

Materials

Riboflavin, FMN, FAD and isoriboflavin were obtained from the California Corporation for Biochemical Research and were used without further purification.

The procedures employed in the synthesis of lumiflavin and 9-methyl-isoalloxazine have been described previously (6), as have those employed in the preparation of 1,2-naphthalenediol, 1,4-naphthalenediol and the acidic and neutral flavin-phenol complexes (1).

Optical Spectra

Optical spectra were obtained with the use of Cary 14, Cary 11 and Zeiss PMQII spectrophotometers.

Stability Constants

For the measurement of apparent stability constants in acidic solution, a stock solution containing approximately 0.1 M flavin and an appropriate amount of the phenol in 6 N hydrochloric acid was prepared. The flavin concentration in the stock solution was determined by diluting one

milliliter to 1000 ml. with distilled water and observing the optical density of the resultant solution at 450 mμ, where the extinction coefficient of FMN and riboflavin is 1.22×10^{-3} (7). Portions of the stock solution were pipetted into a 5 ml. volumetric flask and diluted to volume with 6 N HCl. The optical densities of the solutions were measured at appropriate wavelengths in 1 cm Cary cells in the Cary 14 spectrophotometer or in the Zeiss PMQII spectrophotometer. In most cases three solutions at each concentration were prepared and observed and the optical densities averaged.

For the calculation of the constants the following equation was employed:

$$[F]_T[P]_T/\Delta D + K[F]_T[P]_T/\epsilon = ([F]_T + [P]_T)/\epsilon + \frac{1}{\epsilon K}, \quad (1)$$

where $[F]_T$ and $[P]_T$ are the total formal concentrations of flavin and phenol derivative, respectively; ΔD is the difference between the measured optical density of the solution of complex and that of a solution containing only flavin of the same formal concentration; ϵ is the difference between the extinction coefficient of the complex and that of the flavin at the wavelength employed, and K is the apparent stability constant of the complex, defined as $[C]/[F][P]$, where $[C]$, $[F]$ and $[P]$ are the actual molar concentrations of the complex, the flavin and the phenol, respectively.

This equation is similar to the Scott form of the Benesi-Hildebrand equation. Its derivation parallels that presented by Hayman (8). However, since it was convenient to use rather high concentrations of both components, the assumption, made in Hayman's derivation, that one component is in large excess was not followed here. Also, it was not assumed here that the term $[C]/\epsilon$ (approximated above by $K[F]_T[P]_T/\epsilon$) is negligible,

as was done by Hayman. The approximation that $[C] = K[F]_T[P]_T$ becomes inaccurate for systems in which K is quite large. For such cases the exact term:

$$\frac{[P]_T + [F]_T + 1/K - \left(([P]_T + [F]_T + 1/K)^2 - 4[P]_T[F]_T \right)^{1/2}}{2\epsilon}$$

was used in place of $K[F]_T[P]_T/\epsilon$. First order values of K and ϵ were obtained by neglecting the second term on the left of equation (1) and plotting $[F]_T[P]_T/\Delta D$ against $[F]_T + [P]_T$. The initial slope is approximately $1/\epsilon K$. In general, the first order plot is a curve. The neglected term is then calculated with the use of the first order values of K and ϵ , and the data is replotted. A straight line is now ideally obtained and second order values of K and ϵ are determined from its slope and intercept.

Stoichiometry in Neutral Solution

Weighed portions of 2,3-naphthalenediol were placed in 10 ml. volumetric flasks. 5 ml. portions of a solution of FMN in 0.2 M pH 6.8 phosphate buffer were pipetted into the flasks and the solutions diluted to volume with buffer. The optical densities of the solutions at 575 $m\mu$ were observed and the optical densities of FMN solutions of equal concentration were subtracted. The coefficient of 2,3-naphthalenediol in the equation $a \text{ FMN} + b \text{ naphthalenediol} \rightleftharpoons \text{FMN}_a\text{naphthalenediol}_b$ was determined from a plot of $\log(\text{optical density})$ vs. $\log(\text{weight of 2,3-naphthalenediol})$.

For the determination of the coefficient of FMN, naphthalenediol solutions were pipetted into flasks containing weighed portions of FMN, and an analogous procedure followed.

Stoichiometry of neutral crystalline complexes.

Crystals of the complexes were decomposed by dissolving in water

and the optical densities of the resulting solutions were observed at 445 $m\mu$ and 295 $m\mu$. From the ratio of optical densities, the flavin-naphthalenediol ratio was calculated.

RESULTS AND DISCUSSION

As has been described previously (1) acidic flavin solutions containing a variety of naphthalenediols display long wavelength absorption bands which are characteristic of neither of the components, and we have suggested that these may be due to charge transfer transitions.

One of the consequences of the Mulliken theory of charge-transfer complexing (9) is that the energy of the charge transfer band should increase approximately linearly with the ionization potential of the donor. The ionization potential of an organic compound is theoretically equal to the negative of the eigenvalue of the highest filled molecular orbital obtained by the Pople self-consistent field (SCF) method (10, 11). The coefficients for the highest filled orbital obtained in this manner for naphthalene are shown in Fig. 1 (Hückel calculations yield qualitatively similar results). In accordance with first order perturbation theory, substitution of electron-donating groups such as hydroxy groups at positions at which the squares of the coefficients are large should raise the energy of an orbital relative to its value in the unsubstituted compound. By this argument, α -substitution of hydroxy groups should raise the energy of the highest filled naphthalene orbital, and hence lower its ionization potential. For the naphthalenediols studied, the order of ionization potentials should therefore be $2,3 = 2,7 > 1,2 = 1,7 > 1,4 = 1,5$. The absorption maxima of the corresponding long wavelength bands of the 9-methylisoalloxazine hydrochloride complexes are as follows (1): 2,3 and 2,7 $< 500 m\mu$; 1,2 and 1,7

= 550 $m\mu$; 1,5 = 590 $m\mu$; 1,4 = 625 $m\mu$. Thus the energies of these bands are found to fall in the order which would be predicted for charge transfer absorptions.

There remains the question of why the band of the 1,4-naphthalenediol complex falls at a shorter wavelength than that of the 1,5-naphthalenediol complex. In order to obtain more precise information, SCF calculations were performed for 1,4-naphthalenediol and 1,5-naphthalenediol, employing parameters found to be suitable for the interpretation of the electronic spectra of α - and β -naphthol (12). The SCF ionization potentials of 1,4-naphthalenediol and 1,5-naphthalenediol were found to be 9.26 ev and 9.46 ev, respectively. Thus the calculated ionization potential of 1,5-naphthalenediol is higher by 0.2 electron volt and the difference is in the direction which would be predicted from the positions of the charge transfer bands. This correlation lends further support to the belief that the observed bands are due to charge transfer.

Spectra of Acidic Naphthalenediol Solutions with Various Flavins.

The visible spectra of acid solutions of 1,4-naphthalenediol with a variety of flavins were observed. The absorption maxima of the charge transfer bands of 9-methylisoalloxazine and lumiflavin complexes were found to be near 625 $m\mu$, those of the riboflavin and isoriboflavin complexes were near 600 $m\mu$ and those of FMN and FAD complexes appeared to fall at somewhat less than 600 $m\mu$ (the exact values are difficult to determine because of overlapping with the flavin absorption). From the similar positions of the charge transfer band absorption maxima, it appears that the substituents on the benzenoid ring of the isoalloxazine nucleus have little influence upon the amount of charge transferred. By implication, it might be argued that these substituents have little effect upon the electron affinity of the isoalloxazine.

On the other hand it appears that the presence of the ribityl side chain does shift the charge transfer band to the blue by at least 25 m μ . It may be argued that part of this effect is an artifact caused by differences in the degree of overlap of the charge transfer band with the 385 m μ flavin band. However, flavin concentrations in these experiments were equal, and in addition it was found that variation of the flavin concentration affected the apparent symmetry of the charge transfer band, but had no noticeable effect upon the position of its maximum. It is possible that the ribityl chain may fold over and interact in some manner with the isoalloxazine ring, perhaps by hydrogen bonding. If a ribityl oxygen accepts a hydrogen from the isoalloxazine ring (a likely candidate would be the hydrogen that goes on in the acid protonation process) in the formation of such a bond, the electron density in the ring would be raised, and a shift of the charge transfer band in the observed direction would be expected.

It is interesting that the addition of the adenosine phosphate group in FAD has no effect upon the position of the charge transfer band.

The charge transfer band maximum of the lumichrome-1,4-naphthalenediol complex is also near 600 m μ . Thus it seems that the alloxazine and isoalloxazine rings may have similar electron affinities.

The molar extinction coefficient at the absorption maximum of the long wavelength band in acidic solutions of riboflavin and 1,4-naphthalenediol was found, in conjunction with stability constant measurements (see below), to be $750 \text{ M}^{-1} \text{ cm}^{-1}$. This is of the order of magnitude usually found for the maximum extinction coefficient of charge transfer bands (13).

Stoichiometry of neutral complexes.

The stoichiometry of the FMN-2,3-naphthalenediol complex in pH 6.3 phosphate buffer was determined from log-log plots as was done in the case of the acid complexes (1). The FMN:2,3-naphthalenediol ratio was found to be 1:1 (Fig. 2).

Spectrophotometric analysis of crystals of the riboflavin-2,3-naphthalenediol and riboflavin-2,7-naphthalenediol complexes (see experimental section) indicated a diol:flavin ratio of 2:1 (the actual values obtained were 1.9:1 and 2.1:1, respectively) in contrast to the 1:1 ratio found in solution, and in the case of the acid complexes in both solution and the solid state. A possible explanation may be that flavin is capable of accepting an electron from two donors. In the solid acid complexes the chloride ion would act as one of the donors whereas in neutral crystals two phenol molecules would be involved. Halide ions have been found to act as charge transfer donors toward pyridinium ion (14). However, attempts to obtain spectral evidence for such interaction with FMN solutions were unsuccessful (15).

Stability Constants

Apparent stability constants of a number of the complexes were determined as described in the experimental section. In these measurements all activity coefficients were assumed to be unity. At the acid concentration employed during the measurement of constants in acidic solution, there exists an equilibrium between protonated and nonprotonated flavin. It is the total flavin concentration which is used in defining the stability constants reported here. It can be shown that if the true stability constant is given by $K = [C]/[FH^+][P]$, where $[C]$ is the complex

concentration, $[P]$ is the phenol concentration and $[FH^+]$ is the concentration of protonated flavin, then

$$[C]/[F]_T[P] = K(1 + K_A/[H^+]) = K',$$

where $[F]_T$ is the total flavin concentration, K_A is the dissociation constant of FH^+ , $[H^+]$ is the hydrogen ion concentration and K' is the measured stability constant. The value of K_A is approximately 1.6 M (1) and for example, if $[H^+]$ is 6 M, then $K' = 1.26 K$. That K' is not strongly pH dependent in concentrated acid may be seen by comparing its values measured in 12 N and 6 N acid.

In Table I the stability constants (K') are summarized. Values are reported to two significant figures. This is probably the degree of precision which is justifiable on the basis of the reliability of the data. The probable error is estimated to be about five per cent.

In Fig. 3 we have given a typical set of plots resulting from a stability constant measurement. It can be seen that the scatter of points is quite negligible. The principal source of error in most instances probably lies in the accuracy of the value of the optical density of the solution of lowest concentration. The point corresponding to this solution is the most highly weighted in the determination of the intercept and initial slope of the curve, and unfortunately the fixing of this point is also subject to the greatest imprecision. However, successively measured values of the optical density of the most dilute solution rarely differed by more than one per cent.

Although it is difficult to unequivocally establish structure-stability correlations, several trends seem apparent. For the constants measured in 12 N HCl, it may be seen that the presence of the ribityl side

Table I. Stability Constants of Flavin-Phenol Complexes

<u>Donor</u>	<u>Acceptor</u>	<u>K', Stability Constant (M⁻¹)</u>	<u>HCl Concentration</u>
2,3-naphthalenediol	FMN	68	12 <u>N</u>
catechol	FMN	0.68	12 <u>N</u>
catechol	9-methylisoalloxazine	2.9	12 <u>N</u>
catechol	riboflavin	3.2	12 <u>N</u>
hydroquinone	riboflavin	2.9	6 <u>N</u>
resorcinol	riboflavin	3.3	6 <u>N</u>
catechol	riboflavin	3.9	6 <u>N</u>
pyrogallol	riboflavin	5.5	6 <u>N</u>
2-methylresorcinol	riboflavin	5.5	6 <u>N</u>
4-methylcatechol	riboflavin	6.2	6 <u>N</u>
phloroglucinol	riboflavin	7.4	6 <u>N</u>
orcinol	riboflavin	9.2	6 <u>N</u>
trimethylhydroquinone	riboflavin	10	6 <u>N</u>
3,4-dimethylphenol	riboflavin	55	6 <u>N</u>
2,5-dimethylphenol	riboflavin	88	6 <u>N</u>
1,4-naphthalenediol	riboflavin	55	6 <u>N</u>
1,7-naphthalenediol	riboflavin	98	6 <u>N</u>
2,7-naphthalenediol	riboflavin	102	6 <u>N</u>
1,5-naphthalenediol	riboflavin	111	6 <u>N</u>
2,3-naphthalenediol	riboflavin	162	6 <u>N</u>
catechol	FMN	10.4	0 (pH 6.8)
2,3-naphthalenediol	FMN	242	0 (pH 6.8)

chain has little effect upon the stability of the complexes, but that the phosphate group significantly diminishes complex stability. It is possible that the ribityl chain folds over and permits the phosphate group to act as a donor of an electron to the isoalloxazine ring in competition with phenol molecules. The fact that the ribityl chain itself does not affect complex stability is not inconsistent with the fact that it does seem to shift the charge transfer bands (see above). As will be seen, there is no clear correlation between complex stability and the position of the charge transfer band.

All of the dihydroxybenzenes form about equally stable complexes in acid, and the addition of methyl groups increases the stability constants perhaps slightly more than the addition of hydroxy groups (e.g. orcinol vs. phloroglucinol). Especially striking is the rather high stability of dimethylphenol complexes. This may suggest that phenol derivatives such as tyrosine would form quite stable complexes, and perhaps even benzene derivatives containing no hydroxy groups (e.g., phenylalanine) might complex strongly. Unfortunately, such compounds induce no significant spectral change in flavin solutions, and so the appropriate stability constants cannot be measured spectrophotometrically.

Perhaps the most significant conclusion which may be drawn from the acid stability constants is that the nature of the bonding in these complexes is complicated. That there is no positive correlation between the donor ability of the phenol and the stability constant is especially apparent in the case of the naphthalenediols. There appears in fact to be almost an inverse correlation between stability constants and the spectral positions of the charge transfer bands (see above). The same trend, though less striking, is to be found among the benzene derivatives. This latter is based upon the degree of broadening of the flavin spectrum into the red.

A possible rationalization for this apparent inverse correlation may lie in the fact that for acid flavin-phenol complexes the ground state no-bond structure has the form DA^+ , while the dative structure is D^+A . Thus as the dative structure becomes more important, i.e., as D becomes a better donor, the total wave function begins to describe a structure in which both components have net positive charges. The increasing coulombic repulsion could more than compensate for the increasing resonance stabilization. Whatever explanation is advanced, it is clear that charge transfer, although incidentally present, is not a major contributing force to the stabilization of the complexes. Therefore, if a "charge transfer complex" be defined as one in which resonance resulting from ground-state charge transfer is a major source of bonding energy, the acid flavin complexes cannot be designated as such.

Perhaps the major source of bonding energy is charge-dipole and charge-induced dipole interaction. In agreement with this, naphthalenediols, which should be especially polarizable, form by far the strongest complexes. The significant contribution of methyl groups to complex stability suggest that hydrophobic forces also may play a part.

It is to be noted that the complexes are appreciably more stable in neutral solution than in acidic solution. A possible explanation for this is that, in the neutral flavin-phenol complexes, the no-bond structure has no charge and the components have opposite charge in the dative structure. Thus, these complexes may be stabilized by coulombic attraction, in contrast to the postulated de-stabilization in the acidic complexes. If this is true, one might expect that in the neutral complex series stabilities may increase with increasing charge transfer. This is currently under investigation.

The increased stability of the neutral complexes is of obvious importance with reference to their possible role in biological systems, since here the pH is approximately neutral.

CONCLUSION

The presence of a charge transfer optical absorption in the spectra of flavin-phenol molecular complexes has been rather firmly established. However, at least in the case of the acid complexes, charge transfer is found to contribute very little to the strength of intermolecular binding. In fact, the evidence shows that while protonation of flavins increases their electron affinity, it actually diminishes the stability of the complexes, and that poor donors form more stable acid complexes than do similar good donors. At the same time it appears that flavins can form quite stable complexes with highly polarizable molecules, even though these may not be exceptionally good electron donors. Thus an interpretation of flavin to protein binding in terms of molecular complexing is given support (1).

On the other hand, charge transfer interactions would be expected to affect the redox properties of the flavin. Therefore, factors which have been found to influence the degree of charge transfer, e.g., hydrogen bonding to isoalloxazine (2), protonation of the isoalloxazine ring and the properties of the donor, may significantly affect the catalytic process.

The ability of flavin to complex simultaneously with two donors may be of importance in the mechanism of action of flavoenzymes. For example, flavin coenzymes may be able to form complexes simultaneously with a protein amino acid and a substrate molecule, or to influence protein tertiary structure by complexing with two amino acid residues.

SUMMARY

1 - The position of the charge transfer band of flavin-naphthalenediol complexes in acidic solution is found to be a monotonic function of the ionization potential of the donor as estimated from molecular orbital theory.

2 - Flavin-phenol complexes in neutral solution are found to have a 1:1 flavin:phenol ratio, while crystalline complexes obtained from such solutions display a 1:2 ratio.

3 - Stability constants of the complexes range roughly from 1 to 250. Neutral complexes are much more stable than acid complexes. Charge transfer does not contribute significantly to the stabilization of the acid complexes.

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LEGENDS FOR THE FIGURES

Fig. 1 HCF coefficients for the highest filled orbital of naphthalene.

Fig. 2. Determination of stoichiometry of FMT-2,3-naphthalenediol complex in phosphate buffer (pH 6.8).

o constant FMT, e constant 2,3-naphthalenediol

Fig. 3 Stability constant of the riboflavin-1,7-naphthalenediol complex in 0.1 N HCl. o first order plot; O second order plot.

FIG. 1

FIG. 2

FIG. 3