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MOLECULAR COMPLEXES OF FLAVINS: A COMPARISON OF FLAVIN–INDOLE AND FLAVIN–PHENOL INTERACTIONS

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SUMMARY

1. Molecular complex formation between flavins and indoles in neutral and acid solution has been studied. One to one complexes are formed, many of which can be isolated as crystalline solids. Visible spectra of complex solutions show more or less broadening of the flavin absorption and tailing into the long-wavelength region. New bands are present in the visible spectra of the solids, on the long-wavelength side of the flavin absorption.

2. Complex stabilities are in the range of 10–1000 M^{-1}. Charge-transfer forces do not seem to be involved in stabilizing the indole complexes, but may be involved in stabilizing the neutral naphthalenediol complexes. Measurements of thermodynamic parameters for complex formation in acid solution indicate that hydrophobic forces may be involved in stabilization.

3. Complex formation is shown to inhibit the photoproduction of flavin radicals in acid solution, probably by quenching the excited triplet state.

4. Comparisons between the flavin–indole and the flavin–phenol systems are made.

INTRODUCTION

As a consequence of our interest in the redox chemistry of riboflavin, we have carried out extensive investigations of molecular complex formation between flavin and phenol derivatives[1–4]. Various other studies have shown that indoles form molecular complexes with biologically important compounds such as quinones[5], thiamine[6], pyridine nucleotides[7] and flavins[8–11]. Inasmuch as an indole (tryptophan) furnishes one of the important side-chain groups in proteins, and several physiologically active indoles are known (*e.g.*, serotonin, indoleacetic acid), we felt it would be of some interest to carry out a quantitative comparison between the indole and the phenol complexes of the flavins.

ISENBERG AND SZENT-GYÖRGYI[8,9] and HARBURY AND FOLEY[10] first detected spectral changes in FMN–tryptophan solutions which suggested complex formation. Further evidence for such complexes was provided by temperature-jump studies of SWINEHEART[12]. MASSEY AND GANTHER[13] have demonstrated spectral changes, indicative of complex formation, upon addition of indole-2-carboxylic acid to solutions of D-amino-acid oxidase (a flavoprotein enzyme). Interactions between the

lowest triplet state of flavins and indole (in its ground electronic state) have been suggested by the photochemical investigations of RADDA AND CALVIN[14] and RADDA[15] and by EPR studies of SHIGA AND PIETTE[16].

EXPERIMENTAL

Riboflavin and FMN were obtained from Calbiochem and were used without further purification. Lumiflavin was prepared as previously described[17]. Indole derivatives were obtained from Aldrich and from Eastman and were not subjected to further treatment. The naphthalenediols (Eastman) were purified by boiling with an aqueous suspension of Norite followed by recrystallization from water.

Solutions of the complexes were examined as soon as possible after being prepared. Efforts were made to minimize exposure to light.

Crystalline complexes of protonated flavins and indoles were obtained by mixing equimolar quantities of each material dissolved separately in acid and in alcohol, respectively. Crystals formed upon slow evaporation of the solvent. Crystalline neutral complexes of lumiflavin and indoles were obtained by heating equimolar quantities of each compound in distilled water, followed by slow cooling of the solution in the presence of a strip of aluminum foil. Crystals formed on the aluminum strip.

Absorption spectra were obtained using the Cary Model 14. Absorbance measurements for stability constant determinations, for measurements of stoichiometry in solution and for quantitative analysis of crystalline complexes were made using the Zeiss PMQ II spectrophotometer. All optical measurements were made at room temperature using 1-cm cells, except where otherwise stated. Many of the procedures used were the same as in our earlier work[1-4].

Optical absorption spectra of the crystalline complexes suspended in mineral oil were obtained as previously described[1-4].

A Varian V-4501 spectrometer equipped with 100-kcycles field modulation was used to record EPR spectra. Samples were illuminated with a 500-W tungsten lamp. Heat-absorbing filters and a water filter were used to remove the infrared radiation.

Temperature-dependence measurements of the absorption spectra of flavin–indole and flavin–naphthalenediol complexes in acid solutions were made by placing the samples in a cryostat and following the spectral changes on a Cary Model 11 spectrophotometer. Temperatures were measured using a copper–constantan thermocouple.

RESULTS AND DISCUSSION

We have successfully grown nicely formed crystals of complexes of lumiflavin with various indoles from both acid and neutral solutions. It has not been possible to crystallize the corresponding riboflavin complexes.

Elemental analysis of crystals of a lumiflavin–tryptophan complex grown from 50 % (v/v) ethanol–HCl and dried to constant weight was consistent with a 1:1:1 stoichiometry between the flavin, tryptophan and HCl, if it was assumed that the hydrochloride of tryptophan was involved and that 1 molecule of water was tightly

bound. Analysis: Calculated for $C_{24}H_{28}N_6O_5Cl_2$: C, 52.27; N, 15.24; H, 5.12; Cl, 12.86; O, 14.51. Found: C, 51.60; N, 15.48; H, 5.13; Cl, 12.17; O (by difference), 14.60.

Elemental analysis of a crystalline lumiflavin–3-methylindole complex isolated from neutral solution was consistent with a 1:1 stoichiometry between the flavin and the indole. Analysis: Calculated for $C_{22}H_{21}N_5O_2$: C, 68.19; H, 5.46; N, 18.08; O, 8.26. Found: C, 67.97; H, 5.50; N, 18.32; O (by difference), 8.21.

The above elemental analyses are consistent with measurements made by dissolving the complex crystals in excess solvent (which caused dissociation) and determining the flavin and indole concentrations spectrophotometrically. The molar ratio, tryptophan: lumiflavin, for the lumiflavin–tryptophan acid complex was found in this way to be 0.91 and the molar ratio, 3-methylindole: lumiflavin, for the lumiflavin–3-methylindole neutral complex, was 0.74.

Measurements of the stoichiometry in solution[1-4] gave the results shown in Table I. Again we see a 1:1 relationship between flavin and indole.

TABLE I

STOICHIOMETRY OF FLAVIN–INDOLE COMPLEXES IN SOLUTION

Complex	*Solvent*	*Molar ratio (indole:flavin)*
Riboflavin–tryptophan	Acid*	1.19
Riboflavin–3-methylindole	Acid*	1.06
FMN–indole	Neutral**	0.86
FMN–3-methylindole	Neutral**	1.10
FMN–tryptophan	Neutral**	1.07

* 12 M HCl–abs. ethanol (1:1, v/v).
** Abs. ethanol–0.1 M phosphate buffer (pH 6.8) (3:7, v/v).

It is interesting to compare these results to those obtained with the phenol complexes[1-4]. In the latter case, although a 1:1 stoichiometry was observed for acid complexes in both the solid state and in solution, and also for neutral complexes in solution, the solid neutral complexes had a 1:2 flavin–phenol ratio.

The crystals of the complexes are more highly colored than is the flavin itself. In Fig. 1 are shown optical spectra of several of the solid complexes. It is evident that new bands are present in the complexes on the long-wavelength side of the flavin absorption. This is particularly apparent in the acid tryptophan and carbazole complexes and in the neutral 3-methylindole complex. However, these bands are at considerably shorter wavelengths than are similar bands found in the flavin–phenol complexes[1-4]. Also, there is no marked dependence of the position of the band on the specific indole derivative employed, such as was found with the phenols. It is not at all certain whether or not these absorptions should be designated as being of the charge-transfer type.

In Fig. 2 are shown absorption spectra, in the long-wavelength region, of several of the complexes in acid solution (spectra of complexes in neutral solution generally show a slight broadening of the long-wavelength flavin band toward the red (see ref. 11). It is apparent that, in contrast to the case of the phenol complexes[1-4], no new absorption bands are present. One merely observes a long tailing into the red. Presum-

ably, if charge-transfer absorptions are present, they must be buried beneath the main flavin absorption.

The spectral properties of flavin–indole complexes, particularly in acid solution, are complicated by the fact that a more or less rapid decomposition of the indole

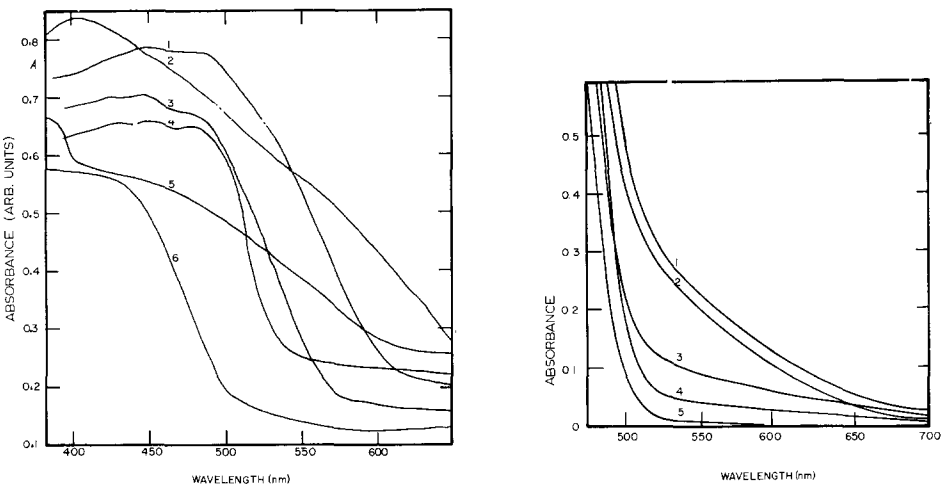


Fig. 1. Absorption spectra at room temperature of several crystalline lumiflavin–indole complexes dispersed in mineral oil. Baselines have been shifted for clarity. 1, lumiflavin–3-methylindole (neutral); 2, lumiflavin–tryptophan (acid); 3, lumiflavin–indole (neutral); 4, lumiflavin alone (neutral); 5, lumiflavin–carbazole (acid); 6, 9-methylisoalloxazine hydrochloride.

Fig. 2. Absorption spectra of flavin–indole complexes at room temperature in acid solution. 1, riboflavin–tryptophan; 2, lumiflavin–tryptophan; 3, lumiflavin–1,2-dimethylindole; 4, riboflavin; 5, lumiflavin.

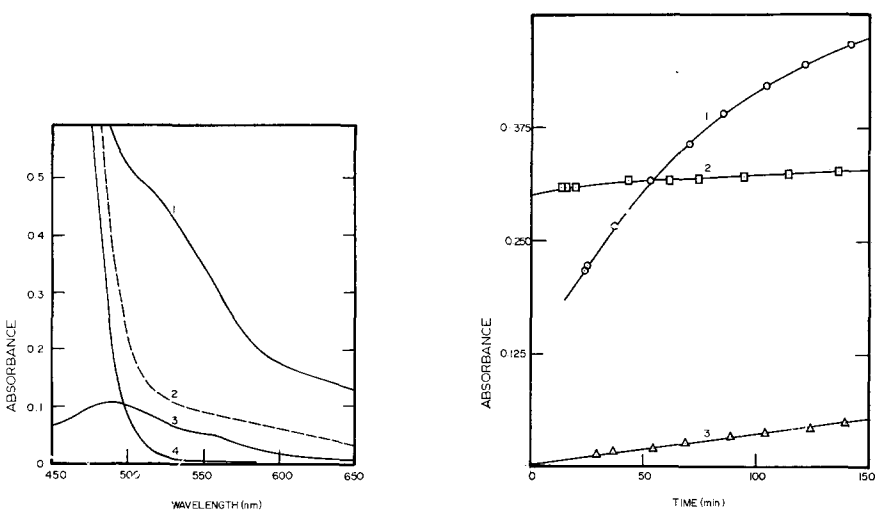


Fig. 3. Absorption spectra changes of lumiflavin–1,2-dimethylindole complex in acid solution on standing for 24 h. 1, lumiflavin–1,2-dimethylindole (after); 2, lumiflavin–1,2-dimethylindole (before); 3, 1,2-dimethylindole alone (after); 4, lumiflavin alone.

Fig. 4. Absorbance *vs.* time plots at room temperature of flavin–indole complexes in acid solution. 1, riboflavin–indole; 2, riboflavin–3-methylindole; 3, indole.

occurs producing compounds which absorb in the long-wavelength region. Further, this decomposition seems to be accelerated by the presence of flavin. In Figs. 3 and 4 are shown some results which illustrate this. The indole derivatives were found to vary considerably in their decomposition rates. Indole itself was the most rapid of the compounds studied here; tryptophan and 3-methylindole were the most stable. We have not further investigated the nature of this decomposition.

Stability constants for various flavin–indole complexes in acidic and neutral solutions are given in Tables II and III. The indole complexes are generally somewhat more stable than are the phenol complexes[1-4], although it should be noted that the solvents used in the measurements with indoles contain ethanol, whereas completely aqueous solvents were used in the phenol measurements. However, the presence of ethanol destabilizes the complexes. This is shown in Table II, and also in Table IV, in which stability constants for several flavin–phenol complexes are shown in the presence and in the absence of ethanol.

A comparison of complexes of various flavin derivatives with a given indole in acid solution reveals the following order of stabilities: FMN $\simeq$ lumiflavin $>$ riboflavin. This differs from our previous results[1-4] with the flavin–phenol system in which riboflavin complexes were more stable than the corresponding FMN complex.

TABLE II

APPARENT STABILITY CONSTANTS OF FLAVIN–INDOLE COMPLEXES IN ACID SOLUTION

Complex	*Solvent*	*K* (M^{-1})
Riboflavin–2-methylindole	Abs. ethanol–12 M HCl (6:4, v/v)	603
Lumiflavin–1,2-dimethylindole	Abs. ethanol–12 M HCl (6:4, v/v)	284
Riboflavin–1,2-dimethylindole	Abs. ethanol–12 M HCl (6:4, v/v)	268
Riboflavin–3-methylindole	Abs. ethanol–12 M HCl (5:95, v/v)	150
Lumiflavin–3-methylindole	Abs. ethanol–12 M HCl (6:4, v/v)	67.1
FMN–3-methylindole	Abs. ethanol–12 M HCl (6:4, v/v)	60–70*
FMN–tryptophan	Abs. ethanol–12 M HCl (6:4, v/v)	30–40*
Riboflavin–3-methylindole	Abs. ethanol–12 M HCl (6:4, v/v)	35.5
Lumiflavin–tryptophan	Abs. ethanol–12 M HCl (6:4, v/v)	28.2
Riboflavin–tryptophan	Abs. ethanol–12 M HCl (6:4, v/v)	15.3

* Some uncertainty indicated due to the fact that FMN was not completely soluble in the solvent system used.

TABLE III

STABILITY CONSTANTS OF FMN–INDOLE COMPLEXES IN NEUTRAL SOLUTION*

Complex	*Solvent*	*K* (M^{-1})
FMN–tryptophan	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	98.4
FMN–2-methylindole	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	89.6
FMN–3-methylindole	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	48.8
FMN–1,2-dimethylindole	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	45.2

* pH 6.8.

The stabilities of the neutral indole complexes are roughly the same as those of the acid complexes. This is again in contrast to what was found for the phenol complexes[1-4] in which the neutral species are considerably more stable than are the acid species.

TABLE IV

APPARENT STABILITY CONSTANTS OF RIBOFLAVIN–NAPHTHALENEDIOL COMPLEXES IN ACID SOLUTION

Complex	*Solvent*	*K* (M^{-1})	*Ref.*
Riboflavin–1,5-naphthalenediol	Abs. ethanol–12 M HCl (6:4, v/v)	22.0	This work
Riboflavin–2,3-naphthalenediol	Abs. ethanol–12 M HCl (6:4, v/v)	14.9	This work
Riboflavin–2,7-naphthalenediol	Abs. ethanol–12 M HCl (6:4, v/v)	6.1	This work
Riboflavin–2,3-naphthalenediol	6 M HCl	162	1–4
Riboflavin–1,5-naphthalenediol	6 M HCl	111	This work
Riboflavin–2,7-naphthalenediol	6 M HCl	102	This work

TABLE V

STABILITY CONSTANTS OF FMN–NAPHTHALENEDIOL COMPLEXES IN NEUTRAL SOLUTION*

Complex	*Solvent*	*K* (M^{-1})	*Ref.*
FMN–1,4-naphthalenediol	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	230	This work
FMN–1,5-naphthalenediol	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	191	This work
FMN–2,3-naphthalenediol	Abs. ethanol–0.1 M phosphate buffer, (3:7, v/v)	176	This work
FMN–2,7-naphthalenediol	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	124	This work
FMN–1,7-naphthalenediol	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	73	This work
FMN–resorcinol	Abs. ethanol–0.1 M phosphate buffer (3:7, v/v)	33.7	This work
FMN–2,3-naphthalenediol	0.1 M phosphate buffer	242	1–4

* pH 6.8

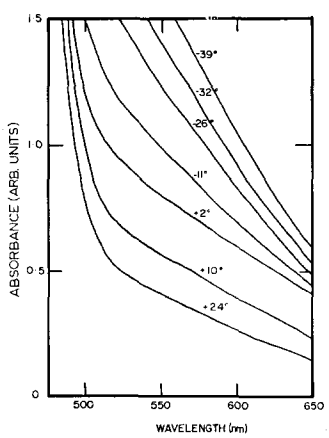


Fig. 5. Temperature dependence of absorption spectra of riboflavin–tryptophan complex in acid solution.

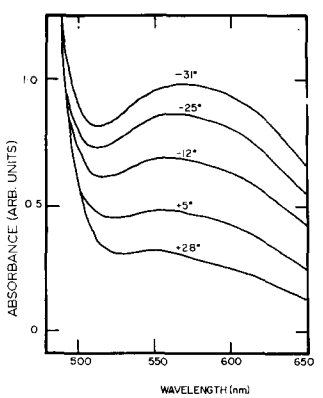


Fig. 6. Temperature dependence of absorption spectra of riboflavin–1,5-naphthalenediol complex in acid solution.

Stability constant data for a number of flavin–phenol complexes in neutral solution are given in Table V. Again, ethanol is found to destabilize the complex, although the effect is probably less marked than in acid solution.

TABLE VI

THERMODYNAMIC PARAMETERS FOR THE FORMATION OF FLAVIN–INDOLE COMPLEXES IN ACID SOLUTION*

Complex	K (M^{-1})	ΔF°_{300} (*kcal/mole*)	ΔH°_{300} (*kcal/mole*)	ΔS°_{300} (*cal/mole·degree*)
Riboflavin–2-methylindole	603	−3.82	−1.02	+9.3
Lumiflavin–1,2-dimethylindole	284	−3.37	−1.26	+7.0
Lumiflavin–3-methylindole	67.1	−2.51	−1.99	+1.7
Riboflavin–3-methylindole	35.5	−2.13	−1.92	+0.7
Lumiflavin–tryptophan	28.2	−1.99	−2.60	−2.0
Riboflavin–tryptophan	15.3	−1.63	−2.22	−2.0

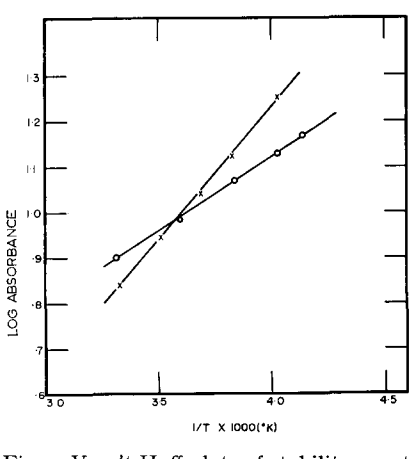
* Solvent in each case was abs. ethanol–12 M HCl (6:4, v/v).

TABLE VII

THERMODYNAMIC PARAMETERS FOR THE FORMATION OF RIBOFLAVIN–NAPHTHALENEDIOL COMPLEXES IN ACID SOLUTION*

Complex	K (M^{-1})	ΔF°_{309} (*kcal/mole*)	ΔH°_{300} (*kcal/mole*)	ΔS°_{300} (*cal/mole·degree*)
Riboflavin–1,5-naphthalenediol	22.0	−1.84	−1.56	+1.0
Riboflavin–2,3-naphthalenediol	14.9	−1.61	−1.90	−1.0
Riboflavin–2,7-naphthalenediol	6.1	−1.08	−1.72	−2.2

* Solvent in each case was abs. ethanol–12 M HCl (6:4, v/v).



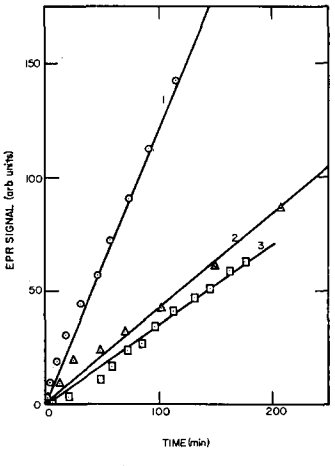


Fig. 7. Van 't Hoff plots of stability constants of flavin complexes in acid solution. ○, riboflavin–1,5-naphthalenediol; ×, lumiflavin–tryptophan.

Fig. 8. Light-induced EPR signal (growth) *vs.* time plots at room temperature of flavin complexes in acid solution. 1, riboflavin; 2, riboflavin–2,3-naphthalenediol; 3, riboflavin–tryptophan.

Lowering the temperature was found to increase the intensity of the absorption in the long-wavelength region for all of the complexes in acid solution. Some typical results are shown in Figs. 5 and 6. Little or no change in absorption was obtained with flavin alone. This demonstrates that the absorption in this region is a consequence of complex formation. Fig. 7 shows some typical Van 't Hoff plots. In Tables VI and VII are found the thermodynamic parameters calculated from these data and from the stability constants.

In our previous work[1-4] we reported the reversible formation of flavin free radicals upon illumination of acid solutions of flavins and phenols with white light. We suggested that this might be due to a light-induced electron transfer from phenol to flavin. We have now reinvestigated this more carefully and find that the presence of phenol or indole actually inhibits flavin radical production by light (Fig. 8).

CONCLUSIONS

We have shown that indoles form molecular complexes with flavins which have spectra, stoichiometries and stabilities similar to the flavin–phenol complexes described earlier[1-4]. Several differences do exist between these two systems, however. Prominent among these is the fact that charge-transfer absorptions are either not present in the spectra of the acid indole complexes, or, if they are present, they are at considerably shorter wavelengths than has been observed for the phenol complexes[1-4]. This indicates that the indoles are poorer electron donors to flavin than are the phenols. This contrasts with the fact that the indole complexes are generally more stable than are the phenol complexes. The foregoing relationships serve to demonstrate the relative unimportance of charge-transfer forces in stabilizing the ground electronic states of the complexes. Further support for this is to be found in the absence of a correlation between complex stability and the expected order of donor abilities of the various indole derivatives, either in acid or in neutral complexes. Thus, FOSTER AND HANSON[5] observed the following order of donor abilities (based upon positions of charge-transfer bands) in indole–chloranil complexes: 1,2-dimethylindole > 2-methylindole > 3-methylindole. We find, in acid solution, the stability order: 2-methylindole > 1,2-dimethylindole > 3-methylindole, and, in neutral solution: 2-methylindole > 3-methylindole > 1,2-dimethylindole.

Previously[1-4], we have suggested that charge-transfer forces may be important in stabilizing the ground states of the neutral flavin–phenol complexes. The data in Table V support this contention. Thus, the order of decreasing stabilities of the FMN–naphthalenediol complexes is approximately the same as the order of decreasing ionization potentials of the phenols[1-4]. The only exception to this is the 1,7-naphthalenediol complex, which is less stable than would be predicted on this basis.

The destabilizing effect of ethanol on all of the complexes demonstrates that solvent interactions play an important role in complex formation. Further insight into this may be obtained by a consideration of the thermodynamic parameters given in Table VI for the acid indole complexes. When there is no charged group present in the donor, we see that entropy is the principal factor in determining complex stability. This would suggest that hydrophobic interactions (*i.e.* solvent ordering) are playing an important role. One would expect, according to this, that the presence of ionizable functions in the complex would tend to reduce complex stability and possibly

also to change the nature of the stabilizing forces. The results with tryptophan as a donor support this. The parameters obtained for the acid naphthalenediol complexes (Table VII) suggest that factors other than solvent structure are important in complex formation with these materials. The data of WILSON[11] for neutral FMN–indole complexes and of CILENTO AND BERENHOLC[18] for neutral flavin–tyrosine complexes in aqueous solution show that the entropy changes are invariably negative and thus that hydrophobic forces are relatively unimportant here as well.

The fact that complex formation with phenols and indoles diminishes the rate of radical formation in illuminated acid solutions of riboflavin indicates that the flavin excited state (probably the triplet) is being quenched by the interaction. This is consistent with the work of RADDA AND CALVIN[14,15], who showed that phenols and indoles inhibit flavin photobleaching in neutral solution and also with the EPR results of SHIGA AND PIETTE[16] on tryptophan quenching of the FMN triplet state.

The precise biological significance of the flavin–phenol and flavin–indole interactions is not at al clear at present. However, it would seem proper to consider molecular complex formation in assessments of the biological action of phenol and indole derivatives and of the binding of flavin to protein.

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