PAPER CHROMATOGRAPHY OF FLAVIN ANALOGUES*

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INTRODUCTION

One of the most commonly used and sensitive means for purification and detection of flavins is paper chromatography. HUENNEKENS AND FELTON note that as little as 0.01 μg of flavin can be detected by this technique when the paper chromatograms are viewed under ultraviolet light[1]. In his review on flavin coenzymes[2], BEINERT points out some of the chromatographic methods which have evolved in the study of flavins. Many recent investigations have used paper chromatography in the purification and estimation of riboflavin and its biological derivatives. The paper chromatographic separation of riboflavin from its natural coenzyme forms, flavin mononucleotide and flavin adenine dinucleotide, has been investigated by CRAMMER[3], YAGI[4], and more recently by WHITE AND LINCOLN[5] and by TRAVIS AND ROBINSON[6]. Similar solvents and resolution techniques have been used by WHITBY[7] and by HUENNEKENS *et al.*[8] for the detection of heretofore unknown flavin compounds in biological materials. The paper partition chromatography of riboflavin decomposition products was investigated by HAIS AND PECAKOVA[9].

The synthesis of flavin analogues for ascertaining exact relationships of structure to biological activity is continuing as an active area of research. FORTER AND KARRER studied the behavior of some twenty synthetic flavins upon paper chromatography[10]. However, recent advances in the syntheses of new flavins has increased considerably both the total number of such analogues and the availability of flavins bearing particular substituents which permit more extensive delineation of the contribution of structure to physico-chemical behavior, especially as regards mobility with solvents on paper chromatograms. A need for such extension of information on the chromatographic properties of flavin analogues has stemmed from studies of the flavin specificities of enzymes, particularly flavokinase[11-14]. The present paper represents a more comprehensive compilation of data on the paper chromatography of flavins and the correlation of structure to R_F values.

EXPERIMENTAL

Procedure

Spots of 5 μl volume containing 2 μg of flavin were applied in a darkened room**

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** Considerable care must be taken to avoid light when working with flavin solutions which are readily photodecomposed, especially in alkaline solvents.

at 2 cm intervals along a line 5 cm from the bottom of sheets of Whatman No. 1 paper, usually 40 cm in height and 50 cm in width. The air-dried sheets were stapled into cylinders and placed upright in glass chromatogram jars, usually 45 cm in height and 25 cm in width, which contained 300 ml of solvent previously equilibrated with the internal atmosphere. The jars were kept in the dark at 25° while solvent was allowed to ascend to within approximately 5 cm of the top of the paper. The chromatograms were removed, the solvent front marked, and the paper dried in a current of air generated in a Reco oven. The flavin spots were marked while their location was observed under ultraviolet light and, whenever possible, by observation under incandescent light as well. Most such flavin compounds exhibit a yellow to orange color in white light and a more intense, yellowish green fluorescence under an ultraviolet lamp.

All flavins were run as duplicates at least twice in the solvents used. Suitable standards, *e.g.* riboflavin, also were included on each chromatogram.

Solvents

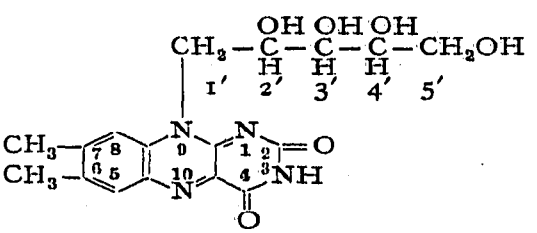
The three solvents chosen for their differing pH and polarity are those which, as reported by HUENNEKENS AND FELTON[1], have been found repeatedly satisfactory in the resolution of flavins and their naturally occurring forms:

S_1 = 5% aqueous Na_2HPO_4
S_2 = *n*-butyl alcohol–acetic acid–water (4:1:5, upper phase)
S_3 = phenol–*n*-butyl alcohol–water (160:30:100, lower phase).

The alkaline solvent, S_1, was used by CARTER[15] in a study of the paper chromatography of purine and pyrimidine nucleotides and has been utilized in the chromatography of flavins and their nucleotides by DIMANT *et al.*[16] and by TRAVIS AND ROBINSON[6]. The acid, more organic S_2 has been used for flavin chromatography by several workers including HAIS AND PECAKOVA[9], WHITBY[7], YAGI[4], and DIMANT *et al.*[16]. Solvent S_3, also used by HUENNEKENS and his coworkers[1,8,16], generally permits the greatest migration of non-phosphorylated flavins.

RESULTS AND DISCUSSION

The correlation of R_F values with structure of a flavin may be considered in terms of substituent functions in the different positions of the parent molecule, riboflavin:



Substitution in the side chain

The effects of side chain substitution at position 9 of the alloxazine ring on R_F values of flavins can be seen in Table I. The R_F values obtained for 6,7-dimethylalloxazine (lumichrome), 6,7,9-trimethylisoalloxazine (lumiflavin), and 6,7-dimethyl-9-(1′-D-ribityl)-isoalloxazine (D-riboflavin) are in good agreement with those reported by

TABLE I

EFFECTS OF SUBSTITUTION IN THE SIDE CHAIN (POSITION 9)

Compound	R_F in solvent system*		
	S_1	S_2	S_3
6,7-Dimethyl substituted			
Alloxazine	0.05	0.62	0.88
9-Methylisoalloxazine	0.15	0.40	0.95
9-Phenylisoalloxazine	0.11	0.68	0.87
9-Formylmethylisoalloxazine	0.15	0.40	0.88
9-(β-Hydroxyethyl)-isoalloxazine	0.26	0.48	0.92
9-(1″-D-Ribityl)-isoalloxazine	0.30	0.28	0.78
9-(1″-D-Arabityl)-isoalloxazine	0.36	0.29	0.80
9-(1″-L-Arabityl)-isoalloxazine	0.37	0.29	0.80
9-(1″-D-Lyxityl)-isoalloxazine	0.35	0.34	0.78
9-(1″-L-Lyxityl)-isoalloxazine	0.33	0.30	0.79
9-(1″-L-2-Deoxylyxityl)-isoalloxazine	0.32	0.47	0.91
9-(1″-D-Sorbityl)-isoalloxazine	0.36	0.24	0.75
9-(1″-D-Dulcityl)-isoalloxazine	0.38	0.21	0.73
9-(1″-D-Rhamnityl)-isoalloxazine	0.36	0.40	0.87
6,7-Dichloro substituted			
9-(1″-D-Ribityl)-isoalloxazine	0.28	0.45	0.75
9-(1″-D-Arabityl)-isoalloxazine	0.36	0.48	0.77
9-(1″-L-Lyxityl)-isoalloxazine	0.32	0.43	0.72
9-(1″-D-Xylityl)-isoalloxazine	0.32	0.41	0.75
9-(1″-D-Sorbityl)-isoalloxazine	0.34	0.36	0.67
9-(1″-D-Dulcityl)-isoalloxazine	0.39	0.36	0.70
9-(1″-D-Mannityl)-isoalloxazine	0.37	0.43	0.70

* Solvent systems: S_1 = 5% aqueous Na_2HPO_4; S_2 = *n*-butyl alcohol–acetic acid–water (4:1:5, upper phase); S_3 = phenol–*n*-butyl alcohol–water (160:30:100, lower phase).

HUENNEKENS AND FELTON[1]. As shown by FORTER AND KARRER[10] for some of the flavins listed in Table I, increasing the number of hydroxyl groups on the side chain causes an increase of the R_F values in a more aqueous solvent, *e.g.* 5% Na_2HPO_4. This may be seen in the proportionally higher values found in the series where position 9 bears no substituent, a methyl group, a β-hydroxyethyl group, a pentityl chain, a 5″-methylpentityl chain, and a hexityl chain, respectively. It is interesting to note, however, that the analogue with a methyl group in this position moves more rapidly in aqueous Na_2HPO_4 and phenol–butanol–water, but less rapidly in butanol–acetic acid–water, than does the unsubstituted 6,7-dimethylalloxazine. This observation is in line with the generally greater solubility of the former flavin, but emphasizes the fallacy of an unqualified assumption that an increase in the number of alkyl groups on the flavin structure elicits a decrease in R_F values in aqueous solvents and an increase in organic solvents[10]. Substitution of position 9 with a relatively hydrophobic phenyl group results in an analogue which has a low R_F value in aqueous Na_2HPO_4, but which value is higher than lumichrome. Less difference in R_F values is seen for longer polyhydroxy chain lengths, *e.g.* L-arabityl *versus* D-dulcityl, where the relative effect of one more alcoholic function is less than at shorter chain lengths. Changes in configuration of a hydroxyl group about only one epimeric center result in minor changes in mobility in the three solvents. The R_F values for D- and L- forms of a

glycityl chain, *e.g.* D-arabityl and L-arabityl, are not significantly different. As expected, a similarity in relative mobilities of those of the above dimethylglycityliso-alloxazines tested previously in a butanol–formic acid–water system[10] was found with the butanol–acetic acid–water solvent used here. The series of pentityl and hexityl flavins with chloro substituents in positions 6 and 7 of the ring have R_F values which are close to but significantly different from their corresponding dimethyl analogues. The dichloro flavins are slightly less water soluble.

Terminal phosphorylation in the side chain

The effects of an orthophosphate ester at the terminal 5′-hydroxymethyl group of the side chain can be seen in Table II. The gross effect of the highly polar phosphonic acid residue is to increase sizably the mobilities of flavins in aqueous solvents, especially in the alkaline Na_2HPO_4 system where effective ionization and salt formation of the flavin phosphate occurs. Moreover, a decrease is found in partially organic

TABLE III

EFFECTS OF TERMINAL PHOSPHORYLATION IN THE SIDE CHAIN (POSITION 5′)

Compound	R_F in solvent system*		
	S_1	S_2	S_3
5′-Monophosphate substituted isoalloxazines			
6,7-Dimethyl-9-(1′-D-ribityl)-	0.56	0.06	0.09
6,7-Dimethyl-9-(1′-D-arabityl)-	0.61	0.09	—
6,7-Dichloro-9-(1′-D-ribityl)-	0.54	0.12	—
6-Methyl-9-(1′-D-ribityl)-	0.64	0.06	—

* Solvent systems used are described under Table II.

solvents, especially in acid conditions where ionization of the phosphate ester is suppressed. These variations in mobility of riboflavin 5′-monophosphate with polarity of solvent have been demonstrated previously[2]. As with the non-phosphorylated D-arabityl compared to D-ribityl flavin, the 5′-monophosphate of the former has slightly higher R_F values than riboflavin 5′-monophosphate, both in aqueous Na_2HPO_4 and in butanol–acetic acid–water. The 5′-monophosphate of 6,7-dichloro-9-(1″-D-ribityl)-isoalloxazine has a slightly lower R_F value in the Na_2HPO_4 but higher in the butanol–acetic acid system than does riboflavin-5′-monophosphate; again this parallels the comparative values for the free flavins.

Substitution in the benzenoid ring

The effects of substitutions in positions 5, 6, and 7 of the benzenoid portion of the isoalloxazine structure can be seen in Table III. The significant variations in R_F values obtained by differential substitution in the aromatic ring portion of flavins is explainable in terms primarily of the differing solubility properties of the substituents *per se* and secondarily to the effects which such functional groups have directly on the electromeric structure and, hence, indirectly on the polarizability of groups in the alloxanoid portion. The solubility conferred by particular substituents in the benzene ring mainly relates directly to the expected solvophilicity of these groups. Their

TABLE III

EFFECTS OF SUBSTITUTION IN THE BENZENOID RING (POSITIONS 5, 6 AND 7)

Compound	R_F in solvent system*		
	S_1	S_2	S_3
Unsubstituted isoalloxazines			
9-(1′-D-Ribityl)-	0.46	0.17	0.75
9-(1′-D-Sorbityl)-	0.53	0.17	0.67
6-Substituted isoalloxazines			
6-Methyl-9-(1′-D-ribityl)-	0.38	0.21	0.76
6-Methoxy-9-(1′-D-ribityl)-	0.30	0.20	0.75
6-Carboxy-9-(1′-D-ribityl)-	0.17	0.20	0.30
6-Ethyl-9-methyl-	0.12	0.74	0.90
6-Chloro-9-methyl-	0.07	0.50	0.53
7-Substituted isoalloxazines			
7-Methyl-9-(1′-D-dulcityl)-	0.49	0.19	0.71
7-Methyl-9-(1′-D-mannityl)-	0.46	0.21	0.73
5,6-Disubstituted isoalloxazine			
5,6-Dimethyl-9-(1′-D-ribityl)-	0.24	0.28	0.78
6,7-Disubstituted isoalloxazines			
6,7-Dimethyl-9-(1′-D-ribityl)-	0.30	0.28	0.78
6,7-Dichloro-9-(1′-D-ribityl)-	0.28	0.45	0.75
6,7-Dibromo-9-(1′-D-ribityl)-	0.16	0.50	—
6-Methyl-7-fluoro-9-(1′-D-ribityl)-	0.29	0.36	0.77
6:7-Benzo-9-(1′-D-ribityl)-	0.11	0.91	—
6,7-Dimethoxy-9-methyl-	—	0.40	0.95
6:7-Tetramethylene-9-methyl-	0.12	0.54	0.91

* Solvent systems used are described under Table I.

positions in the benzene ring appear to be relatively unimportant. For example, whether the methyl substituents are in both positions 6 and 7, as in riboflavin, or in both positions 5 and 6, as in isoriboflavin, have negligible effects on the comparative migrations of these flavins in all three solvents employed. Also, the relative mobilities of the 6-methyl and 6,7-dimethyl derivatives of 9-(1′-D-ribityl)-isoalloxazine are comparable to the relative mobilities of the 7-methyl and 6,7-dimethyl-flavins which bear a dulcityl side chain (*cf.* Table I). Therefore, presence of methyl groups in positions 5, 6 and 7 are approximately equivalent with respect to solubility in the chromatographic solvents. In a comparable series of symmetrical 6,7-disubstituted flavins, *e.g.* dimethyl, dichloro, and dibromo analogues of 9-(1′-D-ribityl)-isoalloxazines, the R_F values are a reflection of the solubilities of these flavins in the solvents. Lack of substitution or substitution with different groups in either position 6 or in both 6 and 7 positions produces flavins which have the expected differences in R_F values.

Substitution in the alloxanoid ring

The effects of substitution in positions 2 and 4 of the alloxanoid portion of the isoalloxazine structure can be seen in Table IV. No great effect of substituents in po-

TABLE IV

EFFECTS OF SUBSTITUTION IN THE ALLOXANOID RING (POSITIONS 2 AND 4)

Compound	R_F in solvent system*		
	S_1	S_2	S_3
1-(D-Ribityl)-2,3-diketo-6,7-dimethyl-1,2,3,4-tetrahydroxyquinoxaline	0.42	0.47	0.73
2-Substituted-6,7-dimethylisoalloxazines			
2-Thio-9-((r″-D-ribityl)-	0.30	0.30	0.96
2-Imino-9-(r″-D-ribityl)-	0.20	0.30	0.78
2-Benzylazino-9-(r″-D-ribityl)-	0.28	0.09	0.96
2-Phenylamino-9-(r″-D-ribityl)-	0.30	0.30	0.92
2-(β-Hydroxyethylamino)-9-(r″-D-ribityl)-	0.28	0.34	0.85
2-Morpholino-9-(r″-D-ribityl)-	0.22	0.41	0.94
2-Methylmercapto-9-(r″-D-ribityl)-	0.29	0.31	0.94
2-Substituted-6,7-dimethoxyisoalloxazine			
2-Thio-9-methyl-	—	0.41	0.96
4-Substituted-6,7-dimethylisoalloxazine			
4-Imino-9-(r″-D-ribityl)-	0.10	0.59	0.88

* Solvent systems used are described under Table I.

sition 2 can be seen from R_F values obtained using aqueous Na_2HPO_4; greater differences are apparent in the partially organic, acid solvents. The polarization effects in position 2 of these analogues are relatively similar, whereas their solubilities in organic solvents are quite dissimilar. Even under the apparently mild conditions of chromatography used, the alkaline pH of Na_2HPO_4 permits nucleophilic displacement of the sulfur in the 2-thio analogue to form riboflavin. This effect has been noted before[14,17]. The resultant continuous production of riboflavin gives rise to a less defined spot on paper chromatograms where the 2-thio and 2-oxy compounds overlap. Suitable stability and separation of both these flavins is found with phenol–butanol–water as solvent. Replacement of the 4-carbonyl of riboflavin with a 4-imino function markedly alters the mobility of this flavin in all three solvents. This finding may be explained by the altered tautomerizable nature of the latter compound about positions 3 and 4. The 4-imino flavin has a decreased ability to form an enamino tautomer analogous to the polar, ionized enol structure of riboflavin induced by an electrophilic agent. Studies of metal chelation by flavins[18] and, more recently, the binding of this flavin to flavokinase[14] substantiate this finding.

SUMMARY

A comprehensive paper chromatographic study has been made of the mobilities of some fifty flavin analogues in three solvent systems which are different with respect to pH and polarity. Specific alterations in the structures of flavins influence the solubility characteristics and have a direct and generally predictable effect on R_F values.

1. Increasing the number of hydroxyl groups on the side chain increases the solubility of flavins in aqueous solvents. Relatively smaller differences are seen among longer chains than shorter ones.

2. Phosphorylation of the hydroxymethyl group terminal to the side chain greatly increases the mobility of flavins in alkaline, aqueous solvents and decreases their mobility in acid, organic solvents.

3. The solubility and resultant mobility conferred by particular substituents in the benzenoid portion of the isoalloxazine ring mainly relate to the known solubility of the substituent group. Their positions in the benzene ring of flavins appear to be relatively unimportant.

4. Effects of substitution in position 2 of the alloxanoid portion of the isoalloxazine ring appear similar in an alkaline, aqueous solvent, but dissimilar in acid, organic solvents. A 4-imino function markedly alters the mobilities in all three solvents.

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