ASSAY OF FLAVINES AND LUMAZINES IN THE STUDY OF FLAVINOGENESIS IN *ASHBYA GOSSYPII*

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The separation of ribolumazines from flavines has become an important problem in recent times since complex mixtures of these compounds are encountered in studies of flavine biosynthesis in flavinogenic organisms.

Ashbya gossypii and the related flavinogenic ascomycetes *Eremothecium ashbyii* produce riboflavine and appreciable amounts of the intermediate, in riboflavine synthesis, 6,7-dimethyl-8-ribityllumazine and of its oxidation product 6-methyl-7-hydroxy-8-ribityllumazine[1–4]. Large amounts of flavine coenzymes are formed in *Eremothecium ashbyii*, but neither FAD* nor FMN have so far been reported in *Ashbya gossypii*.

Analytical methods based on paper chromatography are reported in the literature[1,2,5,6]. However they do not appear adequate for quantitative work, particularly if aimed at the estimation of all the compounds in question.

Some procedures using column chromatography have been described more recently[7–9]. Two of them may be used in sequence[9] to resolve mixtures containing the lumazines, FMN and riboflavine. For routine work on broth extracts it seemed desirable to have procedures which allow a ready separation of the lumazines and all flavine coenzymes (FAD included), over a wide range of concentration of each component relative to the others. Indeed the assay of FAD and FMN in *Ashbya gossypii* is hindered by differences of several orders of magnitude in the content of these compounds, riboflavine and the ribolumazines. Conditions for a complete analysis were therefore studied.

The methods developed are reported in the present paper, as well as their application to the study of flavinogenesis in *Ashbya gossypii*.

MATERIALS

Riboflavine was kindly supplied by Hoffmann-La Roche. FMN was from Sigma Chemical Co. FAD was prepared by the procedure of CERLETTI AND SILIPRANDI[10]. 6,7-Dimethyl-8-ribityllumazine and 6-methyl-7-hydroxy-8-ribityllumazine were isolated from *Eremothecium ashbyii* as described by MASUDA[1] and by KUWADA *et al*[3].

Fluorimetric measurements were made either with a Farrand spectrofluorimeter, equipped with two grating monochromators and a photomultiplier tube 1P28, or with a Klett fluorimeter, lamp filter Corning glass 5543 (transmittance band

* Abbreviations used: FAD = flavine-adenine dinucleotide; FMN = flavine mononucleotide.

between 350 and 505 mμ), photocell filter Corning 3385 (transmittance above 475 mμ).

Fluorescent and U.V. absorbing spots were detected on paper chromatograms with the aid of Mineralight 3660 and 3537 U.V. lamps.

Amberlite IRC 50, Rohm & Haas, Philadelphia, U.S.A.; Munktell cellulose powder; and Whatman paper No. 4 were used.

Ashbya gossypii was grown in submerged cultures[11]. The mycelium was obtained by filtration of the broth and flavines and lumazines were extracted from it with 0.01 *M* KCl–(0.03 *M* KH_2PO_4–K_2HPO_4 buffer, pH 6.8) (96:4) at 80°[12].

METHODS AND RESULTS

Throughout this study, the composition of the mixtures and the purity of the isolated compounds were checked by the paper chromatography procedures described in the text.

The quantitative determination of flavine was done by spectrophotometry and/or fluorimetry[12,13]. Lumazines were determined spectrophotometrically[1,3,14]; they were also assayed fluorimetrically, as described in the text.

For quantitative fluorimetric work internal standards of the pure compounds were added to the samples. This procedure overcomes the influence of varying salt concentration on fluorescence[12]. Organic solvents, if necessary, were removed before assay.

The lower limit of sensitivity of the spectrofluorimetric determination was about 0.2 mμ moles/ml of each compound with the equipment used in this research. Paper chromatography detected $2 \cdot 10^{-3}$ μmole flavine and about $1 \cdot 10^{-2}$ μmole lumazines.

Paper chromatography

Good separations were obtained with acidic solvents which had *n*-butanol as the main constituent. To improve resolution, it was necessary to increase the amount of water dissolved in the solvents. This was accomplished by adding alcohols, which mix freely with both water and *n*-butanol, and then saturating again with water. Methanol and *tert.*-butanol were found most suitable. The influence of the amount of *tert.*-butanol in the solvent is shown in Table I. With alkaline solvents, it was observed that, in addition to the nature and amounts of the alcohols, variations in the pH affect the mobility of the various compounds in quite different ways.

As a result of this study, the following solvents were routinely used:

In the acidic range, solvent 3 (Table I).

In the alkaline range a solvent composed of: *tert.*-butanol–33 % (v/w) trimethylamine–H_2O (60:0.95:39.05); pH 10.9.

Cellulose column chromatography

As shown in Fig. 1 chromatography on a Munktell cellulose column resolves mixtures of pure ribolumazines and flavine coenzymes. Elution is performed with solvent 3 (Table I) followed by H_2O to displace FAD. Since FMN and FAD can be determined quantitatively in the presence of each other[12], the analysis is speeded up by washing out both components with water as soon as FMN appears into the alcoholic eluate. Recovery is between 95 and 100 %. The separation is obtained over a wide range of concentration of each component relative to the others. The procedure sepa-

TABLE I

PAPER CHROMATOGRAPHY OF FLAVINES AND LUMAZINES IN ACIDIC SOLVENTS

Chromatograms (on Whatman paper No. 4) were developed by the descending technique at a temperature of 18–22°. The main constituent of the solvents was the upper alcoholic phase of the mixture: *n*-butanol–1 *N* HCl–H_2O (100:2:148). Additions to it were made as shown below.

Solvent No.	*Addition to 100 ml of butanol phase*	R_F				
		6-Methyl-7-hydroxy-8-ribityllumazine	*6,7-Dimethyl-8-ribityllumazine*	*Riboflavine*	*FMN*	*FAD*
1	None	0.01	0.02	0.05	0	0
2	*tert.*-Butanol (40 ml) + 0.01 *N* HCl (2.5 ml) + H_2O (30 ml)	0.06	0.09	0.23	0.01	0
3	*tert.*-Butanol (50 ml) + 0.01 *N* HCl (2.5 ml) + H_2O (40 ml)	0.08	0.11	0.24	0.04	0
4	*tert.*-Butanol (60 ml) + 0.01 *N* HCl (2.5 ml) + H_2O (50 ml)	0.08	0.12	0.25	0.25	0.01
5	Methanol (10 ml) + H_2O (13 ml)	0.14	0.17	0.35	0.11	0.04

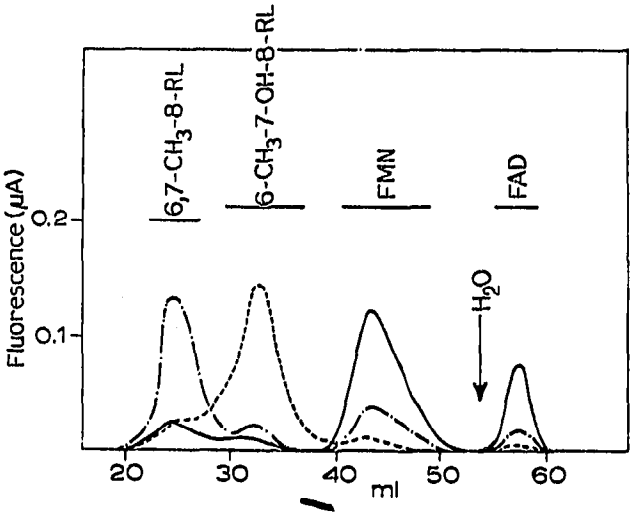

Fig. 1. Separation of flavines and lumazines on a cellulose column. A mixture containing 10 μg each of the following compounds: FMN, FAD, 6,7-dimethyl-8-ribityllumazine (6,7-CH_3-8-RL) and 6-methyl-7-hydroxy-8-ribityllumazine (6-CH_3-7-OH-8-RL), was applied on a column, 200 mm × 75 mm^3, packed with Munktell cellulose powder. Eluant was solvent 3 (Table I) followed by water, at a flow rate of 15 ml/h. 1.5-ml fractions were collected. Ordinate: fluorescence intensity of the eluate in μA: (– – – –) excitation at 340 mμ, emission at 425 mμ; (–·–·–·–) excitation at 415 mμ, emission at 475 mμ; (————) excitation at 445 mμ, emission at 520 mμ. Upper horizontal lines indicate the compounds identified by paper chromatography in the eluate.

rates larger quantities than paper chromatography and is used for the quantitative assay of mixtures and for the isolation of the compounds on a semipreparative scale.

The elution can be followed spectrophotometrically by recording the absorption at 350 mμ, 400 mμ and 450 mμ, corresponding respectively to 6-methyl-7-hydroxy-8-ribityllumazine, 6,7-dimethyl-8-ribityllumazine and the flavines. The absorption spectra of the above compounds show however considerable overlaps, and if separations are not clear cut, spectrophotometric measurements do not permit identification and quantitative estimation of the components in the eluate. Fluorescence measurements are more selective and afford a higher sensitivity. Spectral and emission properties of some of the compounds under study have been reported in the literature[13]. Those lacking were determined on the pure substances and the data are summarized in Table II. Determinations in the eluate were made at the following wavelengths, 6,7-dimethyl-8-ribityllumazine, 415 mμ excitation, 475 mμ emission; 6-methyl-7-hydroxy ribolumazine, 340 mμ excitation, 425 mμ emission; flavines, 445 mμ excitation, 520 mμ emission. The resolving power of fluorimetry is nevertheless not absolute since excitation and emission bands of some of the above compounds show a minor overlap. Emission may therefore be recorded also at wavelengths corresponding to a compound which in reality is not present. This is what happens in the peaks of Fig. 1 for fluorescence at 520 mμ excited at 445 mμ (first and second peak), fluorescence at 475 mμ excited at 415 mμ (second and fourth peak) and fluorescence at 425 mμ excited at 430 mμ (first, third and fourth peak). The same is observed in the first peak of Fig. 2A for the emission at 475 mμ excited at 415 mμ, and for the fluorescence at 520 mμ excited at 445 mμ in the second peak of the same figure. Relative intensities at specific wavelengths however permit a decision about the identity of the compound, which if necessary can be confirmed *e.g.* by paper chromatography.

TABLE II

FLUORESCENCE CHARACTERISTICS OF PURIFIED LUMAZINES AND CONTENT OF FLAVINE COENZYMES AND PRECURSORS IN FLAVINOGENIC AND NON-FLAVINOGENIC STRAINS OF *Ashbya gossypii*

Fluorescence spectra were recorded at pH 6, in 0.01 *M* phosphate buffer.

Compound	*Fluorescence*		*μmoles/g of protein*	
	Maximum of exciting light (mμ)	*Maximum of emission (mμ)*	*In flavinogenic strains*	*In non-flavinogenic strains*
6,7-Dimethyl-8-ribityllumazine	415	475	3.4	0
6-Methyl-7-hydroxy-8-ribityllumazine	287	425	30	0
	342	425		
Riboflavine	370	520[13]	148	0.103
	445	520		
FMN	370	520[13]	0.123	0.115
	445	520		
FAD	370	520[13]	0.065	0.028
	445	520		

Ion exchange chromatography

Previous work has established that flavine mixtures could be resolved readily on the weak cation exchanger Amberlite IRC 50, H form[12,15]. Experiments with the pure compounds showed that on this resin lumazines could also be satisfactorily separated from riboflavine. Flavine coenzymes and 6-methyl-7-hydroxy-8-ribityllumazine are eluted from the resin with water in a first peak, 6,7-dimethylribolumazine in a second. Riboflavine is eluted only by 1 *N* HCl. Recoveries are 95 % for flavine coenzymes and lumazines and about 85 % for riboflavine.

The removal of riboflavine and of 6,7-dimethyl-8-ribityllumazine by this procedure facilitates the subsequent analysis of other components in mixtures where the concentration of the above compounds far exceeds that of the other ones.

Application to flavinogenic and non-flavinogenic strains of Ashbya gossypii

Flavines and lumazines were determined by the methods described above in flavinogenic strains of *Ashbya gossypii*, and in strains which apparently did not accumulate riboflavine, obtained by U.V. irradiation of the mold as described elsewhere[11].

Ion exchange chromatography on Amberlite IRC 50 was used as a preliminary

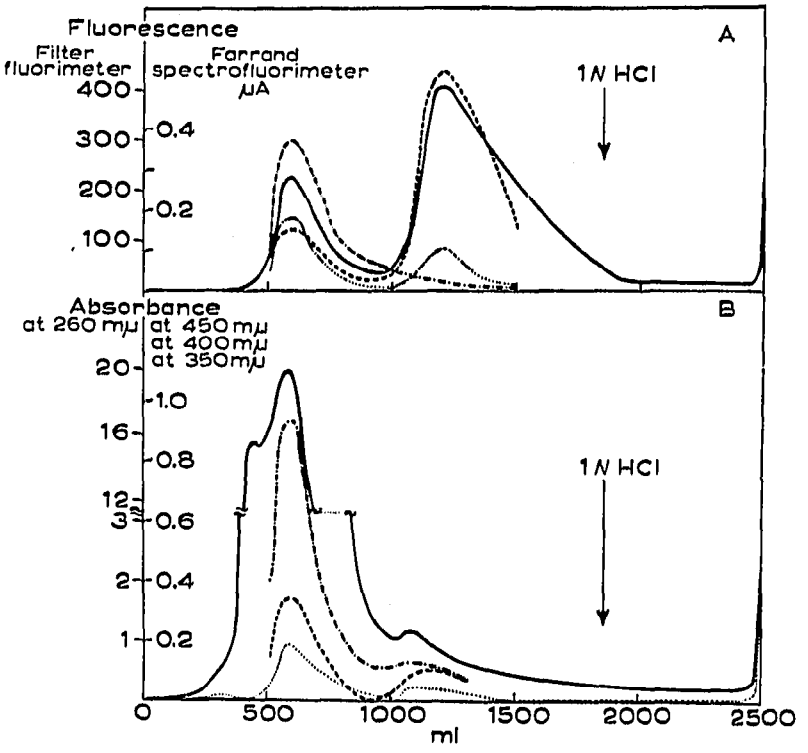

Fig. 2. Elution pattern on a carboxylic ion exchanger of the extract of a flavinogenic strain of *Ashbya gossypii*. 37.8 g of mycelium (wet weight) from a submerged culture at the fourth day of fermentation, corresponding to 1.3 g of proteins, were separated from the broth by filtration, and extracted with 0.01 *M* KCl–(0.03 *M* KH_2PO_4–K_2HPO_4 buffer, pH 6.8) (94:4) at 80°. The extract was applied to a column 100 cm × 75 cm^2, packed with Amberlite IRC 50, H form. Eluant was H_2O, followed by 1 *N* HCl, at a rate of 40 ml/h. 8-ml fractions were collected at 2°. (A) Fluorescence intensity of the eluate, in μA; measured with a spectrofluorimeter: (–·–·–·–) excitation at 340 mμ, emission at 425 mμ; (– – – –) excitation at 415 mμ, emission at 475 mμ; (· · · ·) excitation at 445 mμ, emission at 520 mμ; (————) fluorescence intensity measured with a filter fluorimeter as compared to a standard containing 0.079 μg fluorescein/ml taken as 100. (B) Absorbance of the eluate: (————) at 260 mμ; (–·–·–·–) at 350 mμ; (– – – –) at 400 mμ; (· · · ·) at 450 mμ.

step for the assay of mold extracts. The elution was followed by recording the absorption at 260 mμ. Each peak was then measured at 350 mμ, 400 mμ and 450 mμ. As a first approach fluorescence was measured throughout the elution with a filter fluorimeter. Each peak was then assayed at the excitation and emission wavelengths characteristic for each compound. The elution pattern of the mycelial extract from a flavinogenic strain is shown in Fig. 2. Fig. 3 shows the pattern obtained, under identical conditions with a non-flavinogenic strain. In this case only one peak, containing FMN and FAD, is eluted by water. Regarding the sensitivity of the methods, no 6-methyl-7-hydroxy-8-ribityllumazine was detected and the second peak due to 6,7-dimethyl-ribolumazine, did not appear. Only small amounts of riboflavine were eluted by 1 *N* HCl.

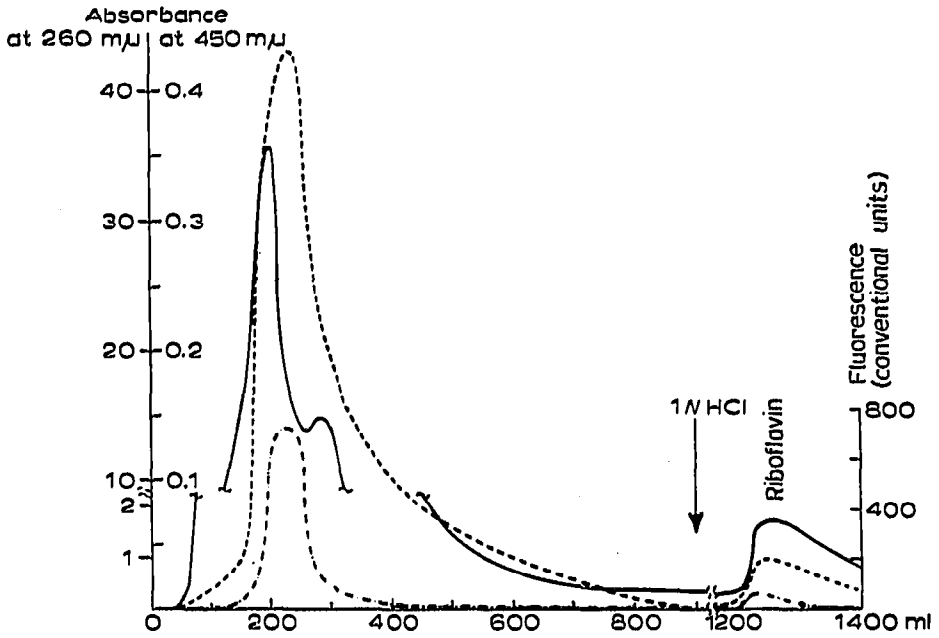

Fig. 3. Elution pattern on a carboxylic ion-exchanger of the extract of a non-flavinogenic strain of *Ashbya gossypii*. 83 g of mycelium (wet weight) from a submerged culture at the fourth day of fermentation, corresponding to 3.44 g of proteins, were separated from the broth by filtration, and extracted with 0.01 *M* KCl–(0.03 *M* KH_2PO_4–K_2HPO_4 buffer, pH 6.8) (94:4) at 80°. The extract was applied to a column, 50 cm × 3 cm^2, packed with Amberlite IRC 50, H form. Eluant was H_2O followed by 1 *N* HCl, at a rate of 30 ml/h. 5-ml fractions were collected at 2°. Ordinates: (————) absorbance at 260 mμ; (– – – –) absorbance at 450 mμ; (–·–·–·–) fluorescence intensity measured with a filter fluorimeter as compared to a standard containing 0.079 μg fluorescein/ml taken as 100.

The first peak eluted by H_2O from the Amberlite column, containing the flavine coenzymes and, in flavinogenic strains 6-methyl-7-hydroxy-8-ribityllumazine, was fractionated by chromatography on a cellulose column. A preliminary treatment was required. Indeed, other non-identified U.V. absorbing substances from the mold contaminate the eluate from the ion exchanger and on the cellulose column interfere with the separation of 6-methyl-7-hydroxy-8-ribityllumazine from FMN. The ribolumazine is therefore extracted quantitatively from the effluent from the ion-exchanger with *n*-butanol followed by ether. Traces of FMN in this extract are readily separated on the cellulose column. FAD and the bulk of FMN remain in the water phase with U.V. absorbing contaminants. Contaminants are satisfactorily separated

on the cellulose column where they move faster than FMN in the alcoholic solvent.

The analytical data are summarized in Table II. FAD and FMN were demonstrated in both types of strains and a relationship was found between accumulation of riboflavine and presence of lumazines.

DISCUSSION

The methods developed in the present work permit the separation both on an analytical and on a preparative scale, and the quantitative estimation of flavines and lumazines. The procedures described in the literature do not generally consider simultaneously both groups of compounds. Those aimed at the determination of flavines in tissues[12,16,17] do not account for a simultaneous estimation of lumazines. Chromatography on a cellulose column yields in one operation a satisfactory separation on a semi-preparative scale of mixtures of all pure lumazines and flavines. If necessary, the treatment described in the text also permits the analysis of extracts contaminated by other material of biological origin.

The combination of methods outlined permits quantitation of the compounds in a mixture containing FAD in amounts about 2,300 times less than riboflavine and 500 times less than 6-methyl-7-hydroxy-8-ribityllumazine. This proved extremely useful in the study of *Ashbya gossypii*, since it facilitated the determination of small amounts of flavine coenzymes in strains which accumulate large quantities of riboflavine.

Flavine coenzymes could be predicted as necessary cofactors for oxidative enzymes in *Ashbya gossypii* since this ascomycetes is a strictly aerobic mold, and flavinogenesis is greatly enhanced by aeration of the culture. So far however FAD and FMN had never been demonstrated, and it is of interest that strains which do or do not accumulate riboflavine synthesize, on a protein basis, similar amounts of flavine coenzymes despite the very different production of riboflavine. It has been shown previously[11] that the low content of FMN and FAD is not to be attributed to breakdown of these compounds by the mold. The ratio of vitamin to coenzymes in non-flavinogenic strains is about the same as that existing in animal tissues[12] and in many microorganisms[18]. Lumazines, if present, are less than $1 \cdot 10^{-3}$ μmoles/g of mycelial protein.

Despite their dramatic accumulation of riboflavine, flavinogenic strains have a growth rate similar to non-flavinogenic ones[11]. Previous data[11] indicate that riboflavine accumulation in flavinogenic mold is not, at least in *Ashbya gossypii*, the consequence of a shift from a cytochrome type of terminal respiration to the flavinoprotein type, as has been suggested for *Eremothecium ashbyii*[19]. Flavine accumulation is more likely a side event in the life of the mold, and is limited to the free vitamin and to its near precursors.

The lack of lumazines, at least in detectable amounts, in non-flavinogenic strains, suggests that flavinogenesis may be differently regulated in flavinogenic and non-flavinogenic strains, at a level involving the precursors of the vitamin.

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SUMMARY

The separation both on an analytical and on a preparative scale of flavines and ribolumazines is described. The quantitation of these compounds in flavinogenic and non-flavinogenic strains of *Ashbya gossypii* is reported. The content of each coenzyme in flavinogenic strains is similar to the corresponding flavine in the non-flavinogenic strain. Flavinogenic strains contain about 2,300 times more riboflavine than FAD. Lumazines were not detected in non-flavinogenic strains.

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