ISOLATION AND IDENTIFICATION OF FLAVIN ADENINE DINUCLEOTIDE

I STUDIES ON PAPER PARTITION CHROMATOGRAPHY

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The authors previously investigated by paper partition chromatography (PPC) the crude riboflavin obtained by submerged culture of *Eremothecium ashbyii* and found that it contained small quantities of two substances which were supposed, from the report of Crammer (1), to be flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), respectively. Shimizu (2), who also studied the purification of riboflavin by PPC, stated that it was doubtful whether the substance supposed to be FAD by the authors was real FAD.

The present work was undertaken to identify the disputable substance with the samples isolated by column partition chromatography (CPC) and chemical method.

DETECTION BY PPC

Eremothecium ashbyii was subjected to a submerged culture for 30–60 hours and 20 g of wet pale yellow mycelium was obtained from 1.5 l of the broth. The wet mycelium was extracted as such with 10 ml of a mixture of pyridine methanol (1:1) at 80° for 30 minutes, and the yellow extract was subjected to one-dimensional PPC.

The result obtained by observing the chromatogram in the daylight and ultraviolet light is shown in Table I.

ISOLATION OF FAD FRACTION BY CPC

Forty grams of the lower layer of a mixture of acetic acid-*n*-butanol-water (1:4:5) were well mixed with 150 g of purified starch and the mixture was packed in a glass tube with a diameter of 5.5 cm. On this was layered a mixture of 10 g of purified starch and a pyridine methanol extract which was obtained as in the preceding case from the mycelium of 48 hour culture, followed further by 20 g of starch. The packed tower thus prepared was

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Table I
R_F in Various Solvent Systems.

Solvent system	Acetic acid- <i>n</i> -butanol- water (1 : 4 : 5)		Pyridine- <i>n</i> -butanol- water (3 : 4 : 7)	
From the mycelium, cultured for 36 hr	0.03 +	0.30 ++	0.29 +	0.63 ++
" 48 hr	0.03 ++	0.30 +++	0.30 ++	0.62 +++
" 60 hr	0.04 ++	0.30 ++	0.30 +	0.62 ++
From the broth, cultured for 60 hr	—	0.32 +		

+, Fluorescence was observed in ultraviolet light but not in the daylight.

++, Strongly fluorescent and pale yellow in the daylight.

+++, Strongly fluorescent and yellow in the daylight.

irrigated, using the upper layer of the above solvent system as a mobile phase.

About the first 50 ml of the effluent showed no fluorescence, but the next 30 ml exhibited a strong yellow fluorescence, showing only one band for riboflavin by PPC. However, after the flow of further 50 ml the fluorescence was no longer observed.

After the acetic acid-*n*-butanol had fully flowed out, the upper layer of a mixture of pyridine-*n*-butanol-water (3:4:7) was poured on the packed tower and at about the time when 30 ml of the effluent flowed out, the yellow substance began to be eluted. About 50 ml of this colored effluent was shaken with 10 ml of water to transfer the yellow substance into the aqueous layer. The latter was concentrated to about 1 ml *in vacuo*, 5 ml of absolute alcohol was added, and the resulting precipitate was dried to a yellow powder.

An aqueous solution of the yellow powder was subjected to PPC, using acetic acid-*n*-butanol and no band was detected for riboflavin (R_F 0.32), except for one yellow fluorescent band (R_F 0.02—0.03).

ISOLATION OF FAD FRACTION BY CHEMICAL PROCESS.

Twenty milliliters of a pyridine-methanol extract prepared in the same manner as in the preceding case were shaken with 20 ml of water and 50 ml of ether, when all flavin compounds transferred into the aqueous layer. The latter layer was shaken four times with benzyl alcohol to remove riboflavin, then treated with ether to extract the remaining benzyl alcohol, and finally concentrated to about 1 ml. Absolute alcohol, 15 ml, was added to the residue and the resulting precipitate was dried to a powder. The mother liquor from the precipitate was concentrated and absolute alcohol was added to separate a yellow oil. The two products were subjected to PPC with an acetic acid-*n*-butanol solvent system and the chromatograms were observed in ultraviolet light. The result is shown in Table II.

Table II
R_F Values of 2 Samples.

Color of fluoresc.	Yellow	Yellow	Bluish violet
Yellow powder	0.02 +++	0.30 ?	—
Yellow oil	0.02 +++	0.30 ±	0.05 +

?, Band was scarcely detected in ultraviolet light.

±, Fluorescence was dimly observed.

+, †, ‡, Same as in Table I.

HYDROLYSIS OF FAD FRACTION WITH HYDROCHLORIC ACID.

Twenty milligrams of the above-mentioned yellow powder were heated with 5 ml of 10 % hydrochloric acid at 80° for 2–10 hours and the reaction mixture was subjected as such to PPC, using acetic acid-*n*-butanol to give the chromatogram shown in Table III.

Table III
R_F Values of the Hydrolytic Products of FAD Fraction.

Color of fluorescence	Yellow	Yellow	Yellow
After hydrolysis with 10 % HCl at 80°, 2 hr	0.02 +	0.08 ++	0.30 +
" at 80°, 10 hr	—	0.08 ++	0.30 +

PHOTOLYSIS OF RIBOFLAVIN

For comparison with the band of FAD, two solutions of riboflavin, 100 μ /ml and 2 mg/ml, respectively, adjusted to pH 10, were exposed in sealed colorless ampules to sunlight for an hour on a fine day of February and investigated by PPC, using acetic acid-*n*-butanol. The result is shown in Table IV.

Table IV
R_F Values of the Photolytic Products of Riboflavin.

Color of fluorescence	Yellow	Yellow	Yellow	Bluish violet
From neutral solution	0.25 ±	0.32 +++	0.48 ?	0.72 +
From the soln. of pH 10	0.26 ±	0.32 +++	0.49 +	0.72 +

ESTIMATION OF FAD FRACTION BY ENZYMATIC METHOD

According to the methods of Negelein and Brömel (3), as well as Warburg and Christian (4), FAD-free D-amino acid oxidase was extracted from the kidney cortex of the pig at 0° and the extract was evaporated to dryness. To a mixture of 0.5 ml of an aqueous solution containing 1 mg of the dried enzyme preparation, 1.0 ml of *M*/10 phosphate buffer, 0.2 ml of 4.5 % DL-alanine solution, and 0.5 ml of water was added 0.2 ml of an aqueous solu-

tion containing FAD-fraction (yellow powder) as indicated in Table V, the whole (2.4 ml) was shaken in the Warburg manometer in the presence of air at 37°, and the amount of the oxygen absorbed in 30 min was measured.

Table V
Alanine Test of FAD Preparations

Amount of FAD prep.	Oxygen absorbed	Amount of FAD prep.	Oxygen absorbed
γ	μl	γ	μl
0	8.0	5.0	95.7
0.3	15.6	10.0	136.7
0.6	21.6	20.0	188.0
1.3	32.8	50.0	192.0
2.5	40.7		

DISCUSSION

Investigation by PPC of the pyridine-methanol (1:1) extract of the mycelium obtained by submerged culture of *Eremothecium ashbyii* detected four distinct yellow bands with R_F -values of 0.03–0.04, 0.30–0.32 (acetic acid-*n*-butanol) and 0.29–0.30, 0.62–0.63 (pyridine-*n*-butanol), respectively. The bands were observed most distinctly with the sample prepared from the mycelium of 48 hour culture. From the reports hitherto published it is easily assumed that the bands with large R_F 's are for riboflavin and those with small R_F 's are for FAD. On the other hand, the broth filtrate manifested under the same conditions only one band for riboflavin. The amount of FAD in the broth seems therefore to be very small if any. From this fact it is also supposed that in the normal growth of *Eremothecium ashbyii* the bulk of FAD remains in the mycelium despite of its higher solubility than riboflavin.

When the above extract was subjected to CPC, using starch as a supporter, riboflavin, having a large R_F , was easily irrigated and washed out by acetic acid-*n*-butanol-water. The substance with a small R_F remaining in the tower was eluted with pyridine-*n*-butanol-water, and the effluent manifested only one band corresponding to FAD by PPC.

The isolation of a large quantity of the substance by CPC being troublesome, the pyridine methanol extract was shaken with water and ether to transfer all the flavin compounds into the water layer. The latter, after treating with benzyl alcohol to remove riboflavin, was concentrated, absolute alcohol was added, and the resulting yellow precipitate was dried. PPC of the product detected only one band supposed to be for FAD. The mother liquor from the above precipitate was concentrated and absolute alcohol was added to separate an yellow oil, PPC of which detected, besides bands for FAD and riboflavin, some unidentified bands.

The yellow substance was hydrolyzed by heating with 10 % hydrochloric acid for 2–10 hours. PPC of the product of 2 hour hydrolysis showed three bands corresponding to FAD (R_F 0.02), FMN (R_F 0.08) and riboflavin (R_F 0.30),

respectively (Table III), but the product of 10 hour hydrolysis gave no band for FAD.

In order to test, if a photolysis of riboflavin produces a substance mistakable for FAD, the experiment of Hais *et al.* (5) was duplicated. However, under the authors' conditions for the decomposition and PPC only four bands for the undestroyed riboflavin, lumiflavin (R_F 0.48–0.49), lumichrome (R_F 0.72) and an unidentified substance (R_F 0.25–0.26) were detected, and no band for FAD was found in the vicinity of the site corresponding to R_F 0.02.

Although from hitherto published literatures the above-mentioned FAD fraction was assumed to be real FAD, it was thought important to ascertain its co-enzymatic activity.

The enzymatic estimation of the FAD fraction performed by J. Suzuoki according to the method of Warburg and Christian (4), definitely confirmed the activity. Since, however, the purity and properties of the enzyme extracted from the kidney cortex of the pig, as well as the experimental conditions were not necessarily in accord with those of the original report and since moreover no control was employed, it is difficult to decide the purity of the fraction from the result alone.

SUMMARY

The authors investigated by paper partition chromatography the crude riboflavin obtained from the broth of submerged culture of *Eremothecium ashbyii* and found that it contained small quantities of flavin adenine dinucleotide and mononucleotide. The former does not seem to come out into the culture medium until it is decomposed to riboflavin because it is firmly retained in the mycelium despite of its solubility. Therefore, at a proper stage of the culture the mycelium was separated and extracted with pyridine-methanol. Flavin adenine dinucleotide was isolated from the extract and purified by column partition chromatography using starch as a supporter or by a chemical process, riboflavin being removed with benzyl alcohol. The flavin adenine dinucleotide thus obtained was hydrolyzed with hydrochloric acid into flavin mononucleotide and riboflavin, and the activity of the dinucleotide was also confirmed by enzymatic estimation according to the method of Warburg and Christian.

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