ON A NEW FLAVIN MONONUCLEOTIDE PRODUCED BY *RHIZOPUS ORYZAE*

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Flavin 5'-mononucleotide (FMN) is well known to be the only naturally occurring riboflavin monophosphate. However, the flavin mononucleotide-like compound of *Rhizopus oryzae* represented as FMN' because of its some different behaviors in paper chromatography and paper electrophoresis as reported by one of the authors (1), has now been revealed physicochemically and enzymatically to be a new natural flavin monophosphate whose phosphate is attached to the other carbon than 5' of ribityl radical.

EXPERIMENTAL AND RESULTS

1. Culture of *Rhizopus oryzae*

Rhizopus oryzae IFO No. 4698 was grown in a medium containing 30 g glucose, 2 g NH_4NO_3 , 1 g KH_2PO_4 , 0.5 g MgSO_4 , 0.5 g KCl, 10 g CaCO_3 and 200 mg riboflavin in 1-liter Erlenmeyer flask at 30° for about three weeks in the dark.

2. Isolation and Purification

After nearly 50 % conversion of riboflavin to the new flavin compound, FMN', was checked by paper chromatography, the culture was filtered, and the filtrate was concentrated to a small volume *in vacuo* at the temperature lower than 50°. The concentrated filtrate was treated three times according to Crammer (2). The final water layer was washed by ether to remove phenol, followed by Amberlite IR-120 (H) column chromatography. The effluent was washed by benzyl alcohol to remove the residual riboflavin. The flavin in the concentrated effluent was then purified with ethanol and water twice each. After being kept the supernatant obtained by the addition of 85 % ethanol overnight at room temperature, fine crystals of the flavin were collected, which were dried in vacuum desiccator over P_2O_5 for a week and subjected to the following analysis.

3. The R_F Values and the Findings in Paper Electrophoresis.

The R_F values and the distance of mobilization were found to be in good agreement with the details of previous paper as shown in Table 1.

Each of the R_F values of FMN' after development with the upper layer of *n*-butanol-acetic acid-water (4:1:5), water saturated with iso-amyl alcohol, 5 % Na_2HPO_4 solution and the upper layer of *n*-butanol-pyridine-water (6:4:3) is almost the same as

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TABLE I
Values of a New Flavin Compound

Flavin	R_F values using the solvent system					PEP ^a distance from center
	I	II	III	IV	V	
FMN'	0.11	0.85	0.51	0.01	0.27	^{mm} + 47
FMN	0.11	0.85	0.51	0.01	0.18	+ 35

I, *n*-butanol-acetic acid-water (4:1:5); II, water saturated with iso-amyl alcohol; III, 5% Na₂HPO₄ aqueous solution; IV, *n*-butanol-pyridine-water (6:4:3); V, phenol-*n*-butanol-water (16:3:10).

^a Paper electrophoresis.

that of FMN. But the value of R_F 0.27 in the solvent system of the lower layer of phenol-*n*-butanol-water (16:3:10) is markedly higher than that of FMN, 0.18 under the same conditions by the ascending technique. In the case of paper electrophoresis carried out at pH 5.9 in 0.05 *M* phosphate buffer at a constant voltage of 200 V (5 to 14 mA) for 5.5 hours, FMN' and FMN, co-spotted on the center of Toyo filter paper No. 50 (2×40 cm), migrated 45—49 and 32—35 mm respectively toward the anode, so that they were completely separated.

4. Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of FMN' in water was measured with Hitachi Model EPU-2A Spectrophotometer employing the cuvetts with 1-cm light path. The positions of the maxima and minima of the absorption spectrum of FMN' coincided with those of FMN measured simultaneously as a control.

The ratios of the optical densities at different wave lengths, E_{222}/E_{450} , E_{260}/E_{450} , E_{275}/E_{450} and E_{375}/E_{450} of FMN' were 5, 2, 1 and 0% higher than those of FMN respectively.

5. Estimation of Phosphate

The method of Youngburg and Youngburg (3) was employed for phosphate determination. The molar ratio of riboflavin to phosphate bound, based on the optical density at 450 mμ of flavin, was 1:1.04, showing the compound to be a monophosphate ester.

6. Oxidation by NaIO₄

The oxidation of FMN' by NaIO₄ was carried out according to the method of Fleury and Lange (4). Three reaction mixtures (8 ml each) consisted of 2 ml of 0.01 *M* FMN', FMN or water, 4 ml of 0.02 *M* NaIO₄ and 2 ml of pH 3.6, 0.1 *M* acetate buffer were kept in a refrigerator at 6°. After 20 hours of oxidation reaction, to 4 ml aliquot of each flask were added 10 ml of saturated NaHCO₃ solution, 3 ml of 0.02 *M* NaH₂AsO₃, 1 ml of 20% KI solution and 1 ml of 1% starch solution, and then the remaining NaH₂AsO₃ was titrated by 0.02 *M* I₂. The result of calculation based on the titration values with 0.02 *M* I₂ in three mixtures showed that one mole of NaIO₄ per mole of FMN' was consumed, in contrast to two moles of NaIO₄ per FMN.

This finding suggested that the possible configuration of FMN' might be 4', 5'-cyclic, 4-, 2', 5'-cyclic, 2', 3'-cyclic or 3'-monophosphate. Meanwhile, the yellow

oxidation product composed of the isoalloxazine of FMN' was chromatographically different from any one of the oxidation products of FMN which should contain the same one as from 4'- or 4',5'-cyclic monophosphate.

Furthermore, the R_F value of FMN' is not similar to that of 4',5'-cyclic monophosphate. It is conceivable, therefore, that the possibilities of the former two configurations; 4',5'-cyclic and 4'-monophosphate, are very little.

7. Enzymatic Hydrolysis

In order to investigate some of the characteristics of the phosphate linkage of FMN', the method of enzymatic hydrolysis was employed. The enzymes for assays were 5'-nucleotidase from bull semen, 3'-nucleotidase from *Bacillus subtilis* and the cell-free extracts of *Aspergillus oryzae*.

No hydrolysis of FMN' was detected by the former two enzymes under the similar conditions as employed in the hydrolysis of 5'- or 3'-AMP (5, 6) except for using 3-fold concentration of each enzyme. When FMN was substituted for FMN' as the substrate, the liberation of riboflavin by the 5'-nucleotidase was detected in a little extent. The cell-free extracts could not so markedly liberate the orthophosphate from FMN' at any of pH 3.6, 7.6 or 8.5 as from FMN under the same conditions.

Although these evidences were not capable of indicating the precise configuration of the phosphate linkage of FMN', it has been enzymatically significant, anyhow, that FMN' is different from FMN in respect of phosphate linkage.

DISCUSSION

It has been obvious from the above details that the crystalline FMN' prepared under the procedures described above has the different configuration from that of FMN.

The point to be considered is whether FMN' in *Rhizopus oryzae* is an artifact produced from FMN initially present. However, we must conclude, on the basis of the following findings, that FMN' is not an artifact, but a natural form in the mold.

There has been no chromatographic difference between the crystalline FMN' and the crude FMN' in the cultured medium before isolation. The treatment of FMN under the same experimental conditions as employed for the isolation of FMN' from the cultured solution of the mold described above resulted in no change of the configuration both chromatographically and enzymatically.

Moreover, it was observed that FMN', not FMN, was formed by the cell-free extracts of *Rhizopus oryzae* as well as under the cultivation mentioned above.

SUMMARY

1. As reported in the previous paper, the R_F value of a new flavin mononucleotide, FMN', in a solvent system of phenol-*n*-butanol-water (16:3:10) was 0.27, which is higher than that of FMN, 0.18, and lower than that of 4',5'-cyclic monophosphate, 0.50. It was further confirmed that FMN' migrated faster than FMN toward the anode in paper electrophoresis.

2. One mole of NaIO₄ was consumed per mole of FMN'.

3. The yellow oxidation product of FMN' by NaIO₄ was chromatographically different from that of FMN, and more stable than that in the course of oxidation.

4. FMN' was more stable than FMN against the hydrolysis by 5'- or 3'-nucleotidase or by the cell-free extracts of *Aspergillus oryzae*.

5. It was suggested that the possible structure of FMN' might be 3'-, 2',5'-cyclic or 2',3'-cyclic monophosphate.

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