DETERMINATION OF RIBOFLAVIN-5'-MONOSULFATE

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As reported previously, FMS* has been synthesized as a coenzyme analogue and has been found to inhibit D-amino acid oxidase in competition with FAD. On the other hand, it has been observed that FMS has an inhibitory action on the growth of riboflavin requiring bacteria.

From these results, it was supposed that FMS, as an antivitamin B₂, disturbs the metabolic pattern in higher animals. To study this antivitamin action of FMS, the effect of its administration to animals may be observed by physiological and nutritional experiments. In this connection, we tried to establish a determination method of FMS in animal tissues. This paper reports a method suitable for the separating micro-determination of flavins including FMS. The principle of the present work is to develop the previously reported method for separating determination of flavins so as to be adopted for the samples containing FMS as well as physiologically existing flavins, FAD, FMN and FR.

EXPERIMENTAL

To adopt the above-mentioned procedure as a method for separating determination of flavins including FMS, the following requirements must be satisfied: (1) The absolute concentration of FMS should be determined by lumiflavin fluorescence method. (2) FMS should be separated from the other flavins by paper chromatography and the recovery from the paper including the separation procedure must be same as other flavins. (3) FMS in the tissue must be extracted as the other flavins. These requirements were examined in detail.

1) Photodecomposition of FMS

To know the rate of formation of lumiflavin, the following experiments were carried out. Each 2 ml of aqueous solution of various concentration (0.01 μg/ml–0.5 μg/ml) of FMS was mixed with an equal volume of 1 N NaOH, irradiated at 20° for 30 minutes in the photo-decomposition apparatus, acidified with 0.2 ml of acetic acid and then lumiflavin formed from FMS was extracted with 6.0 ml of chloroform. The intensity of the fluorescence of chloroform layer was measured by the micro-photofluorometer designed by Yagi et al. and the results are shown in Fig. 1.

The results indicate that the intensity of fluorescence is proportional to

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* Following abbreviations are used: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FR, riboflavin; FMS, riboflavin-5'-monosulfate.
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the quantity of FMS over the range of the concentrations mentioned above.

Under the same condition, FR, FMN and FAD produced fluorescence at the same rate.

2) Addition test

Each 1.0 ml of water extract of rat liver (0.1 g/20 ml) was mixed with FMS and made up to 2.0 ml so as to contain graduated quantity of FMS. When these were treated in the same way as described above, the fluorometer reading exactly showed the FMS quantity added.

3) Separation of FMS by paper chromatography

Various solvent systems for the paper chromatography to separate FMS, FAD, FMN and FR were examined and pure samples of such flavin compounds were separated each other by any of the following solvent systems: n butanol/methanol/5% sodium phosphate dibasic/acetic acid (60/15/30/10, v/v/v/v), n-butanol/acetone/saturated boric acid/acetic acid (5/2/3/1, v/v/v/v), and n-butanol/acetone/acetic acid/water (5/2/1/3, v/v/v/v).

For the separation of flavins in the extract of animal tissue, however, the system of n-butanol/acetone/acetic acid/water was most convenient. Using this solvent system and Whatman No. 1 filter paper, the Rf values of FAD, FMN, FMS and FR were 0.08, 0.15, 0.25 and 0.42 respectively.

4) Recovery of flavins in their paper chromatographic separation

The mixture of equimoles of FMS, FAD, FMN and FR was saturated with ammonium sulfate and extracted with liquid phenol twice and the phenol was extracted again with a small quantity of water after an addition of ether.

The aqueous extract of flavin mixture was applied to the filter paper and the paper was developed with the solvent. After development, each spot area was cut out and the quantity of each flavin was determined by lumiflavin fluorescence method.

Each flavin was recovered at the same rate.

5) Extraction of flavins from tissues

The warm-water-extraction method was again examined. The liver of rat which was injected with 500 µg of FMS, was extracted either with warm water or with sulfuric acid. The total flavin contents in the two samples were measured by lumiflavin fluorescence method. The value obtained by the warm-water-extraction was higher than that by sulfuric acid extraction.
FMS was not destroyed during the incubation at 80° for 20 minutes.

6) Recovery of FMS through the whole procedure

Flavin content in rat liver and that in rat liver added with FMS were analysed by the method mentioned above. As shown in Table 1, the added FMS was exactly determined.

<table>
<thead>
<tr>
<th>TABLE 1. Addition Test of FMS</th>
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<tbody>
<tr>
<td>Liver added with FMS</td>
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<tr>
<td>Rat liver (g)</td>
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<tr>
<td>Added FMS (µg)*</td>
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<tr>
<td>Homogenate (ml)</td>
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<tr>
<td>Total flavin content by lumiflavin method (µg/25 ml)*</td>
</tr>
<tr>
<td>FAD (µg/25 ml)*</td>
</tr>
<tr>
<td>FMN (µg/25 ml)*</td>
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<tr>
<td>FMS (µg/25 ml)*</td>
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<td>FR (µg/25 ml)*</td>
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* Calculated in terms of FR.

STANDARD PROCEDURE

The above-mentioned results showed that the previously reported method could be adopted for the separating determination of FMS, FAD, FMN and FR by changing the solvent system for paper chromatography.

Thus the following standard procedure was obtained.

1) Determination of total flavin content

Tissue is excised from animals, immediately weighed (a g), cut into small pieces and put into 5 ml of water previously warmed at 80°C and then kept for 5 minutes. Then, the tissue is homogenized in a glass homogenizer, diluted with water and warmed again at 80°C for 15 minutes. The total volume of the extract is made up to b ml exactly with water and an aliquot of the supernatant is used as test solution for lumiflavin fluorescence method to obtain the total flavin content (µg/g) of the tissue.

2) Ratio of each flavin

The remaining part of the warm water extract of the tissue is saturated with ammonium sulfate and centrifuged. The supernatant fluid is extracted with each 2 ml of liquid phenol twice.

The flavins in the combined phenol layer are transferred to few drops of water by addition of 20-30 ml of ether and the water extract of flavins is placed on a filter paper. After development with n-butanol/acetone/acetic acid/water (5/2/1/3, v/v/v/v), each spot area are clipped, cut into small pieces, suspended in 2.0 ml of water, added with 2.0 ml of 1 N NaOH, followed by the irradiation and successive procedures of lumiflavin fluorescence method.
3) Calculation

The amount of each flavin is calculated by the following formulas, \( a, b, c \) and \( d \) being the reading of fluorometer for FMS-, FAD-, FMN-, and FR-sample.

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\begin{align*}
\text{FMS} &= f \times \frac{a}{a+b+c+d} \quad (\mu g/g) \\
\text{FAD} &= f \times \frac{b}{a+b+c+d} \quad (\mu g/g) \\
\text{FMN} &= f \times \frac{c}{a+b+c+d} \quad (\mu g/g) \\
\text{FR} &= f \times \frac{d}{a+b+c+d} \quad (\mu g/g)
\end{align*}
\]

These values are expressed in terms of FR. When absolute amount is required, these values, should be multiplied by 1.21, 2.09, 1.21 and 1.0 respectively.

4) Example

The rat liver, excised 50 minutes after the injection of 500 \( \mu g \) of FMS, contained 33.2 \( \mu g/g \) of total flavin, 23.2 \( \mu g/g \) of FAD, 6.1 \( \mu g/g \) of FMN, 3.1 \( \mu g/g \) of FMS and 0.8 \( \mu g/g \) of FR.

REFERENCES