SIMPLIFIED LUMIFLAVIN METHOD FOR THE MICRO-DETERMINATION OF FLAVIN COMPOUNDS IN ANIMAL TISSUES

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For the micro-determination of flavin compounds in animal tissues, Bessey et al. (1) had already reported a method suitable for the separative determination of flavin compounds. In determination of the total quantity of flavin compounds by this method, however, it requires 24 hours for the hydrolysis of flavin-adenine dinucleotide (FAD) to flavin mononucleotide (FMN) by trichloroacetic acid.

To estimate the total quantity of flavin compound in short time, the lumiflavin fluorescence method is more convenient, for this method gives the result in 1 or 2 hours. When the respective quantities of flavin compounds are to be determined, the ratio of each other obtained from the warm-water extract (2) by using filter paper chromatography (3) or paper electrophoresis (4), or by separating each other by ion-exchange resins (5) can be adopted.

Accordingly, the lumiflavin fluorescence method, if simple and exact, is considered to be excellent one for the micro-determination of flavin compounds. Though the lumiflavin method is not quantitative for the flavin solution of relatively high concentration (6), it was proved to be practiced quantitatively in dilute flavin solutions extracted from bacteria (7).

Using a micro-photofluorometer, the micro-determination procedure was examined in detail. The results and the standard procedure are described in this paper.

MATERIALS AND APPARATUS

Flavins—Free riboflavin (FR) and FMN were furnished from Hoffman-La Roche Co. FMN was purified by electrophoresis on a thick filter paper to remove a small amount of FR and other derivatives (5).

FAD was the powder extracted from Eremothecium Ashbyii (8). The purity of
it was over 85 per cent, and it contained neither nucleic acid nor other flavin compounds.

Glass-stoppered Centrifuge Tube—For the irradiation of these flavins to convert to lumiflavin, glass-stoppered centrifuge tubes, as shown in Fig. 1, were used. Using these tubes, the procedures following irradiation can be conducted without changing tubes.

Micro-photofluorometer—To estimate the fluorescence of lumiflavin, a micro-photofluorometer designed by Yagi and Arakawa (9, 10) was employed. Using this apparatus, instructions of meter were parallel with the concentrations of lumiflavin in chloroform solution within the range of $1.0 \times 10^{-4} - 2.5 \gamma/ml$. (10).

EXPERIMENTAL

Condition of Photodecomposition

As the photodecomposition of flavins to lumiflavin is the chief procedure of the method, the conditions were examined in detail.

Light Source and Irradiation Time—It was already demonstrated that visual light of shorter wave length than 530 mμ, other than ultraviolet ray, is also effective for the photodecomposition of flavins (11), and that fluorescent lamp (Mazda FL 20 D) is more excellent than ordinary tungsten lamp or high pressure mercury lamp as a practical light source for the photodecomposition of flavins (12). So, an apparatus shown in Fig. 2, was used for the photodecomposition.

To know suitable time for irradiation by this apparatus, the following experiments were carried out. Each 2.0 ml. of the aqueous solution of 0.5 γ/ml. ($1.33 \times 10^{-9}$ mol./ml.) of riboflavin or equimolar concentration of FMN or FAD was mixed with equal volumes of aqueous $N$ NaOH, irradiated for varying periods, acidified with 0.2 ml. of acetic acid, and then lumiflavin produced from flavins was extracted with 6.0 ml. of chloroform. The intensity of the fluorescence of chloroform layer was measured by the micro-photofluorometer, and the results are shown in Fig. 3. It can be seen that the suitable time of irradiation under these conditions lies in 30–60 minutes.

Temperature of Solution—Each 2.0 ml. of the above-mentioned aqueous flavin solution was mixed with equal volume of $N$ NaOH, irradiated
for 30 minutes at different temperatures of the solution. The results are shown in Fig. 4, which indicates that 20° is the most suitable temperature for each flavin solutions, and that within 10–30° the rate of lumiflavin production is almost same in three flavin derivatives.

**pH of Solution**—Each 2.0 ml. of the above-mentioned aqueous flavin solution of was mixed with equal volume of $M/10$ glycine-NaOH buffer. In each tube, final concentration of glycine was adjusted to $M/20$, and pHs values of the solutions were graduated from 7 to 14. After irradiating at 20° for 30 minutes, lumiflavin produced was extracted with chloroform.
and the intensity of the fluorescence of it was measured. As shown in Fig. 5, it indicates that pH of the solution must be strongly alkaline above pH 13 to obtain the same rate of lumiflavin production in each of the three flavins.
Relation between Flavin Quantity and Fluorometer Reading

In the Case of Flavin Aqueous Solution—Two ml. of the above-mentioned aqueous flavin solution was mixed with equal volume of \( N \) NaOH, irradiated under the best condition obtained from the above experiments, and was extracted three times with each 5 ml. of chloroform. The chloroform layers were combined and the intensity of fluorescence was estimated. By comparing with the intensity of fluorescence of standard lumiflavin in chloroform solution, the production of lumiflavin was calculated as 95 per cent.

As a practical method to estimate flavin quantity, a single chloroform extraction for once will be sufficient to evaluate the initial flavin quantity, unless the volumes of the irradiated solution and chloroform layer are not changed in each tube. Using this procedure, the relation between flavin quantity and fluorometer reading was examined by the following experiments. Each 2.0 ml. of graduated concentration of flavin aqueous solution was mixed with equal volume of \( N \) NaOH, irradiated for 30 minutes at 20°, acidified with acetic acid, and then lumiflavin was extracted with 6.0 ml. of chloroform. The intensity of the fluorescence of chloroform layer was estimated. The results are shown in Fig. 6.

![Fig. 6. Relation between initial quantity of flavin and the intensity of fluorescence of chloroform layer.](image)

The quantities of FMN and FAD were calculated as FR. The results with FR, FMN and FAD were agreed on the same points of the figure.
From the results, it is considered that intensity of fluorescence exactly shows the initial quantity of flavin.

In the Case of Flavins in Animal Tissues—The warm-water extract (2) of the liver of hen (0.5 g./20 ml.) was used as a sample. Each graduated volume of the solution was made up to 2.0 ml. with water and operated in the same way as described above.

As shown in Fig. 7, fluorometer reading exactly shows the quantity taken. Using the extract of the kidney or the intestine, the same results were obtained.

![Graph](image)

**Fig. 7.** Relation between the volume of the liver extract taken and the intensity of the fluorescence of chloroform layer.

**Addition Test**

Each 0.5 ml. of water extract of hen liver (0.1 g./20 ml.) was put into centrifuge tube. To these tubes, aqueous FR solutions were added and made up to 2.0 ml. so as to contain graduated quantity of flavins. These tubes were operated in the same way as described above, and the results are shown in Fig. 8. From the results, it is clear that fluorometer reading exactly shows the flavin quantity added.

**Standard Procedure**

As a method for extracting flavins from animal tissues, “warm-water-extraction” method (2) was found to be the most convenient one.
FIG. 8. Relation between the quantity of added FR and the intensity of the fluorescence of chloroform layer.

So, after the preparation of the extract by this method, the extract is treated by the procedure examined in the above-mentioned experiments. A standard procedure is given as follows:

*Extraction with warm water*—Tissue is excised fresh from animals, immediately weighed (a g.), and cut into small pieces. (in ordinary cases, 0.1–1.0 g. of tissue is used). A few ml. of water warmed at 80° are added, and warmed at 80° for 3–5 minutes. After grinding it in a glass-homogenizer, the homogenate is transferred into graduated tube, diluted with water up to b ml., and warmed at 80° for 15 minutes. After it is cooled at room temperature, the total volume of the extract is made up to b ml. exactly with water, stirred, centrifuged, and the supernatant is used as test solution.

*Photodecomposition*—One ml. of the test solution, 1.0 ml. of water, and 2.0 ml. of aqueous N NaOH are placed together in a glass-stoppered centrifuge tube A. At the same time, 1.0 ml. of the test solution, 1.0 ml. of 0.1 γ/ml. of FR aqueous solution, and 2.0 ml. of aqueous N NaOH are placed together in the tube B. (The quantity of FR added must be changed so as to be similar quantity with that of test solution. It will be determined by preliminary experiment.)

Then, tubes A and B are set in the light irradiation apparatus for 30–60 minutes at 10–30°.

*Extraction with Chloroform*—To the above two tubes, each 0.2 ml.
of glacial acetic acid and 6.0 ml. of water-saturated chloroform are added, respectively. After they are cooled with cold water, shaken for about 30 seconds, centrifuged, and each 5.0 ml. of chloroform layer of A and B are transferred into cuvettes A' and B', respectively.

On the other hand, 1.0 ml. of test solution, 1.0 ml. of water, 0.2 ml. of glacial acetic acid, and 2.0 ml. of $N$ NaOH are mixed together in the tube C, and extracted with 6.0 ml. of chloroform in the same manner. Five ml. of chloroform layer are put into cuvette C'.

*Estimation of Fluorescence*—The dark electric current of micro-photofluorometer is regulated at 0. After cuvette B' is set in the light path, shutter is opened, and then the slit of micro-photofluorometer is opened gradually until the compass needle notices near 100. The reading is noted as $f_1$, then $A'$ and $C'$ are estimated under the same condition, and the reading are noted as $f_2$ and $f_3$ respectively.

*Calculation*—The difference of $f_2 - f_3$ corresponds to the flavin content of the test solution, and $f_1 - f_2$ to that of FR added.

So, the quantity of flavins calculated as FR in animal tissue, in the case of 0.1 $\gamma$ of FR was added, is

$$0.1 \times \frac{f_2 - f_3}{f_1 - f_2} \times \frac{b}{a} \, \gamma/g.$$

*Example of Measurement*

Using the above-mentioned standard procedure of this method, flavin contents of several animal tissues were estimated, and the results are shown in Table I.

**Table I**

*Amount of Flavin in Animal Tissues*

<table>
<thead>
<tr>
<th>White rat</th>
<th>Liver</th>
<th>33.4 $\gamma$/g.</th>
<th>White rat</th>
<th>Brain</th>
<th>3.2 $\gamma$/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney</td>
<td>33.1</td>
<td></td>
<td>Muscle</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>18.8</td>
<td></td>
<td>Liver</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>6.6</td>
<td></td>
<td>Kidney</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>5.1</td>
<td></td>
<td>Heart</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>5.0</td>
<td></td>
<td>Spleen</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>Testicle</td>
<td>3.7</td>
<td></td>
<td>Lung</td>
<td>3.1</td>
</tr>
</tbody>
</table>

The values are calculated as free riboflavin on the basis of wet tissue weight.
DISCUSSION

For the determination of flavins in living tissues, the fluorometric method is suitable, because flavin content of living body is generally small. To estimate the fluorescence of flavin separately from similar fluorescent substances in living body, the procedure of converting flavins to lumiflavin is of excellent one. In animal tissues, the fluorescent substances soluble in chloroform is in negligible amount. So, the procedure described above is reliable to determine flavins in tissues.

If a sample contains a certain amount of fluorescent substances soluble in chloroform, these substances can be removed by extracting with chloroform before photodecomposition.

When similar samples are determined repeatedly, the procedure can be more simplified by using standard curve of addition test.

By the above mentioned standard procedure, several samples can be estimated in 2 hours.

Further, when separative determination of flavin nucleotides is required, the procedure using filter paper chromatography (3), or filter paper electrophoresis (5) can be used satisfactorily by combining with this method, and also the ion-exchange separation (4) will be applicable.

SUMMARY

To estimate flavin compounds in animal tissues by lumiflavin fluorescence method, the conditions of photodecomposition of flavins to lumiflavin were examined in detail using dilute flavin solutions. The best condition for photodecomposition was 30–60 minutes irradiation by fluorescent lamp at 10–30°C of solution temperature and solution pH higher than 13. Under these conditions, the amount of lumiflavin produced exactly showed the original flavin quantity.

A standard procedure is as follows: Warm-water extract of tissue is mixed with equal volume of $N$ NaOH, irradiated under the above conditions, extracted once with chloroform and intensity of fluorescence of chloroform layer is estimated. At the same time, the addition test is made by the same way, and amount of flavin is calculated.

By this procedure, flavin quantities of several samples will be estimated simply within 2 hours.

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