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A Modification of the Fluorimetric Method of Determining Riboflavin in Biological Materials

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It has been known for some time that solutions of riboflavin exhibit a strong yellow-green fluorescence when irradiated with blue or ultra-violet light. Euler & Adler (1934) were the first to use this property of riboflavin as the basis of a method of determining the vitamin in biological materials and, since then, many workers have proposed various modifications.

Najjar (1941) introduced a new principle by extracting the riboflavin into a non-aqueous phase. This promising method suffers in our opinion from a number of disadvantages, the most serious of which is the possibility that fluorescent compounds other than riboflavin are destroyed during exposure to sunlight. This paper deals with a new modification which overcomes these disadvantages.

The chief differences from Najjar's method are:

- (1) the use of a much larger amount of KMnO_4 (about 10 times as much);
- (2) the introduction of the 'internal standard' procedure to correct for quenching of the fluorescence and to allow for
- (3) a controlled incomplete destruction of the riboflavin in the solutions. A series of short exposures to sunlight enables a check to be made on the specificity of the method.

The following minor modifications of the Najjar procedure have been adopted for convenience:

- (1) the pyridine and butanol are added together;
- (2) 3 g. anhydrous Na_2SO_4 are used instead of 5 g.;
- (3) the solutions are heated above the transition temperature of the reaction

to avoid separation of the decahydrate (cf. Barton-Wright & Booth, 1943);

(4) ethanol is added to the pyridine-butanol layer to avoid subsequent separation of water.

METHOD

If the riboflavin content of a solid material is to be determined, it is first necessary to prepare an aqueous extract of the material containing all the riboflavin. No general procedure for preparing this extract, suitable for all biological materials, can be recommended. Each material must be investigated separately before the most satisfactory method of extraction can be determined. Procedures which have been found suitable for some foodstuffs are given later in the paper. In this section the method of measuring the riboflavin in the aqueous extracts or in protein-free biological fluids such as normal urine will be considered.

Reagents

Acetic acid—glacial, A.R.

Riboflavin solution. A stock solution is prepared every few weeks by dissolving 20 mg. of pure riboflavin (dried over conc. H_2SO_4 —see Note 1 on p. 645) in 1 l. water containing a few drops of acetic acid. Prolonged shaking is necessary for complete solution. The solution is stored in the dark under toluene. From it a solution containing 1.2 $\mu\text{g./ml.}$ riboflavin is prepared on the day of use.

KMnO_4 solution. A 6% aqueous solution is prepared on the day of use.

H_2O_2 . A 3% solution is used.

Na_2SO_4 —anhydrous, A.R.

Working directions

Butanol-pyridine. To 8 vol. redistilled pyridine are added 92 vol. redistilled *n*-butanol. A butanol-pyridine solution can be recovered from residues from previous determinations as follows. The residues are made alkaline to phenolphthalein with 30% NaOH, then filtered free from a brown precipitate and distilled. The distillate with a boiling range 93–118° is collected and its pyridine content determined by titration with 0.1N-HCl, using bromophenol blue as indicator. Enough pyridine is then added to bring the concentration to 8% by vol.

Fluorimeter

The fluorimeter used in this investigation was the Klett-Summerson photoelectric instrument manufactured by Klett Manufacturing Co., New York. The method could, however, be adapted for other fluorimeters. The Klett-Summerson instrument has been described by Kavanagh (1941) and we (Slater & Morell, 1946) have discussed the procedure necessary for accurate results. All measurements have been made with a Corning 554 filter between the light source and the cuvette containing the solution, and Corning 038 and 351 filters between the cuvette and photoelectric cell. The 'standard' solution was 0.25 µg./ml. quinine sulphate in 0.1N-H₂SO₄. On the 'standard' side of the instrument, a Corning 597 filter was placed in the incident beam and a Corning 038 filter in the fluorescent beam. As riboflavin is rapidly destroyed by blue light (especially in butanol-pyridine solution), it is necessary to take a series of readings at various times after placing the cuvette in the light beam and to extrapolate to zero time (see Slater & Morell, 1946).

Procedure

The entire operation must be carried out in weak artificial light, and, if solutions have to stand for any length of time, they should be kept in darkness (see Note 2 on this page).

Into three 50 ml. glass-stoppered measuring cylinders A, B and C (all solutions should be prepared in duplicate, i.e. a total of 6 cylinders is taken), 1 ml. acetic acid is pipetted. Into cylinders A and B, *v* ml. of the extract are pipetted (*v* depends upon the concentration of riboflavin and of interfering substances in the extract; it should not contain more than 1.2 µg. riboflavin). Water (1 ml.) is pipetted into cylinder A, 1 ml. riboflavin solution into cylinder B and (*v*+1) ml. water into cylinder C. To each cylinder 1 ml. KMnO₄ solution is added and the contents are mixed and allowed to stand for exactly 1 min. After addition of 1 ml. H₂O₂ the cylinders are agitated gently to expel gases and 3 g. anhydrous Na₂SO₄ are added through a long-stem filter funnel (so that Na₂SO₄ does not come into contact with the upper part of the measuring cylinder), followed by 25 ml. butanol-pyridine. The cylinders are immersed in warm water (at 45–60°) for a few min. to bring the contents to a temperature of at least 40°. They are then removed from the bath and shaken vigorously so as to dislodge the Na₂SO₄ cake at the bottom. After cooling to room temperature, they are again shaken vigorously for 2 min., the contents poured into a 50 ml. centrifuge tube and centrifuged at 2000 r.p.m. for 4 min. From the clear upper layer 20 ml. are pipetted into thin-walled soda glass test-tubes (see Note 3 on this page), containing 3 ml. ethanol and stoppered with corks covered with tin-foil. After mixing

20 ml. are pipetted into the cuvette of the fluorimeter and the fluorescence read, keeping the solution in the light beam for exactly 30 sec. The solution in the cuvette is returned to the test-tube. When the readings on the three solutions A, B and C have been taken, the test-tubes are placed in sunlight for a time sufficient to destroy between 80 and 95% of the riboflavin (see Note 4 below) and the fluorescence is redetermined in the fluorimeter. With each set of readings the standardization of the instrument should be checked, and if any change has occurred the readings are corrected by the method described by Slater & Morell (1946). Solution C should not change by more than 1 fluorimeter scale reading during exposure to the sun.

Note 1. Our sample of riboflavin contained 1.8% water.

Note 2. DeMerre & Brown (1944) have recently reported on the conditions of laboratory illumination necessary to prevent photochemical decomposition of riboflavin.

Note 3. The test-tubes should be of the same wall thickness and of approx. equal bore. Soda glass test-tubes of 0.5 mm. wall thickness, 15 cm. long and 1.85 cm. external diameter, have been found suitable.

Note 4. Artificial light could be used instead of sunlight, but it would be necessary to have a box so designed that all tubes receive the same intensity of illumination.

Quenching of fluorescence

If desired, the quenching of the fluorescence by foreign substances in the final butanol-pyridine-acetic acid solution may be determined by adding to a measuring cylinder D the same solutions as are added to cylinder C, with the exception that *v* ml. only of water are used and 1 ml. riboflavin solution is included. The method of calculation of the percentage quenching is shown below. The riboflavin content of the extract can be determined without measuring the degree of quenching.

Specimen calculation

$$v = 1 \text{ ml.}$$

Fluorimeter readings (corrected for any change in the instrument):

	A	B	C	D
Before exposure—means	45.8	101.4	6.0	67.0
After exposure to sunlight—means	15.8	22.7	5.8	13.1
Reduction	30.0	78.7	0.2	53.9
Reduction, corrected for change in blank	29.8	78.5	0	53.7

The reduction is due to destruction of riboflavin. The difference, 78.5 – 29.8 = 48.7, is due to the destruction of the added riboflavin, i.e.

$$48.7 \equiv 1.2 \mu\text{g. riboflavin,}$$

$$\therefore 29.8 \equiv \frac{29.8}{48.7} \times 1.2 = 0.74 \mu\text{g.,}$$

i.e. riboflavin content of extract = 0.74 µg./ml.

The percentage destruction may be calculated as follows:

Difference between A and B initial readings = 55.6.

$$\% \text{ destruction} = \frac{48.7}{55.6} \times 100 = 87.5.$$

The residual blank fluorescence (corrected for reagent blank) may be calculated as follows:

$$\text{Initial A} - \text{Initial C} = 39.8$$

$$\text{Final A} - \text{Final C} = 10.0$$

$$\text{Difference} = 29.8$$

$$\text{Residual blank} = 39.8 - \frac{100}{87.5} \times 29.8$$

$$= 39.8 - 34.0$$

$$= 5.8,$$

which is equivalent to

$$\frac{5.8}{55.6} \times 1.2 \mu\text{g./ml. riboflavin} = 0.12 \mu\text{g./ml.}$$

The percentage quenching is calculated thus:

In pure solution, 1.2 $\mu\text{g.}$ riboflavin gives a fluorimeter reading of 53.7.

In the extract, 1.2 $\mu\text{g.}$ riboflavin gives a fluorimeter reading of 48.7.

$$\% \text{ quenching} = \frac{53.7 - 48.7}{53.7} \times 100 = \frac{5.0}{53.7} \times 100 = 9.3 \%$$

It is clear that these calculations are valid only if the following assumptions are justified:

(1) the fluorimeter reading is proportional to the concentration of riboflavin in the original extract;

(2) the reduction of fluorescence is due entirely to destruction of riboflavin;

(3) the percentage destruction of riboflavin is independent of the initial concentration of riboflavin.

The validity of these assumptions is examined in the following section.

EXAMINATION OF METHOD

(1) Relation between fluorimeter reading and the amount of riboflavin in the extract

This relation is shown in Fig. 1. The curves show that the fluorimeter reading is proportional to the amount of riboflavin in the extract up to at least 2.4 $\mu\text{g.}$ riboflavin. We have arbitrarily fixed this as the maximum amount of riboflavin which can be in the solution (1.2 $\mu\text{g.}$ from the extract and 1.2 $\mu\text{g.}$ from the added riboflavin). It is possible that the linear relationship holds for higher concentrations of riboflavin, but we have not found it necessary to use such concentrated solutions.

(2) Photochemical destruction of riboflavin

The decomposition curve of riboflavin, as measured in the fluorimeter, using a Corning 554 filter in the incident beam, is shown in Fig. 2 (curves of similar shape are obtained by exposure to sunlight). The curve shows that destruction of riboflavin by light is not a simple exponential function of time, as would be expected if the destruction was a simple reaction involving only one molecule of riboflavin. It follows from the curve that the proportional destruction in any unit of time is not constant. This could be interpreted in two ways:

(1) that the reaction is not a unimolecular reaction with respect to riboflavin but is of a higher order (if this were so, the proportional destruction would

Fig. 1. Relationship between fluorimeter reading and amount of riboflavin in sample. $\times$ — $\times$ Initial fluorimeter reading minus reagent blank. $\circ$ — $\circ$ Initial fluorimeter reading minus reading after irradiation (90% destruction of riboflavin).

Fig. 2. Photochemical destruction of riboflavin in fluorimeter (Corning 554 filter). Readings are corrected for reagent blank.

be a function of the concentration and would decrease as the concentration of the riboflavin decreased);

(2) that the proportional rate of destruction is dependent on the concentration of decomposition products, i.e. a reversible reaction is involved.

The first possibility was excluded by simultaneously subjecting solutions containing different amounts of riboflavin to sunlight for various intervals of time. The results in Table 1 show that the percentage destruction of riboflavin is independent of the initial concentration of riboflavin.

Table 1. *Effect of concentration of riboflavin on percentage destruction in sunlight*

Time of exposure (min.)*	Destruction (%)			
	0.3 μ g. riboflavin	0.6 μ g. riboflavin	1.2 μ g. riboflavin	2.4 μ g. riboflavin
3	61	59	64	66
8	80	79	78	79
15	89	85	84	85
34	91	89	88	89

* Before the first exposure and between each exposure, the solutions were in the light beam of the fluorimeter for 30 sec.

That the second possibility is the true explanation of the shape of the decomposition curve is illustrated by the following readings obtained in the fluorimeter. A solution (containing 1.2 μ g. riboflavin), which had not been exposed to sunlight, gave an initial reading of 105.2, and there was a destruction of 23% in 1 min. in the light beam of the fluorimeter. Another solution (containing 2.4 μ g. riboflavin), after 5 min. exposure to weak sunlight, gave a fluorimeter reading of the same order of magnitude (95.7) but, in this case, there was only 7.3% destruction in 1 min. in the light beam.

The shape of the decomposition curve suggests that at least two consecutive reactions are involved in the decomposition of riboflavin:

Riboflavin $\rightleftharpoons$ Intermediate decomposition products
(Fast reaction.)

Intermediate decomposition products
 $\rightarrow$ Further decomposition products
(Slow reaction.)

The unusual shape of the decomposition curve of riboflavin allows an easy check of the specificity of the method, that is, of the assumption that the reduction of the fluorescence is entirely due to destruction of riboflavin and does not include any destruction of other fluorescent compounds. This can be accomplished by carrying out a series of exposures to sunlight; after the readings have been taken at the end of the first exposure, the solutions are returned to the test-tubes and again placed in sunlight. The riboflavin content of the extract can be calculated from each series of readings. If the

figures thus obtained are the same (or show small variations without a definite trend), it can be assumed that no other fluorescent compounds unstable to light are present. Examples of the use of this procedure are given in Tables 2 and 3.

Table 2. *Riboflavin content of an extract, calculated from a series of exposures to sunlight. Example 1*

Time of exposure (min.)*	Destruction of riboflavin (%)	Riboflavin content of extract (μ g./ml.)
2	48.6	0.100
7	74.3	0.100
17	87.6	0.100
42	91.7	0.100

* Before the first exposure and between each exposure, the solutions were in the light beam of the fluorimeter for 30 sec.

In the example shown in Table 2, the same result is obtained whether the readings obtained after 2 or 42 min. are used. If a fluorescent compound in the solution decomposed at a rate faster than riboflavin, the results calculated from short exposure times would be higher, and if it decomposed at a slower rate than riboflavin, the results obtained from the long exposure times would be higher. Even if the initial rate of decomposition of this compound was the same as riboflavin, its presence would be revealed unless the shape of its decomposition curve was similar to that of riboflavin.

Table 3. *Riboflavin content of an extract, calculated from a series of exposures to sunlight. Example 2*

(Treatment with KMnO_4 omitted.)

Time of exposure (min.)*	Destruction of riboflavin (%)	Riboflavin content of urine (μ g./ml.)
5	75.6	0.09
15	91.7	0.19
30	94.4	0.42

* Before the first exposure and between each exposure, the solutions were in the light beam of the fluorimeter for 30 sec.

The results in Table 3 were obtained by omitting the KMnO_4 . In this case it is obvious that a fluorescent substance other than riboflavin was present and that it was decomposed more slowly than riboflavin. When sufficient KMnO_4 was used (see below), this urine was found to contain 0.04 μ g./ml. riboflavin.

For the sake of convenience, and also to avoid errors due to the presence of small amounts of slowly decomposing fluorescent substances (and these are more likely to be present than substances which are rapidly decomposed), it is desirable to avoid prolonged exposure to light. On the other

hand, if the exposure time is too short, errors due to slight differences in intensity of illumination of the solutions in the different tubes become more important. The sensitivity also suffers. As a compromise, exposures sufficient to destroy between 80 and 95 % of the riboflavin are considered most satisfactory. However, it is possible to obtain reliable results with most materials, even if the percentage destruction is outside this range. A new type of material should always be subjected to a series of exposures (as in Tables 2 and 3) in order to test the specificity, but only one exposure is necessary as a routine procedure.

The times of exposure necessary for the degrees of destruction referred to above depend upon such factors as locality, weather, season and time of day. The following are examples of exposure times necessary in the mid-afternoon in Canberra (latitude 35° S., altitude approx. 2000 ft. above sea-level):

	Exposure (min.)	Destruction (%)
March, clear	5	69.7
	15	83.8
	30	90.0
August, overcast	60	50.0
July, raining	120	52.8
February, 'weak' sunlight	5	55.9

It should be noted that the percentage destruction refers to the total destruction effected by the 30 sec. exposure to the beam in the fluorimeter (this causes an average destruction of about 14 %), followed by the exposure to sunlight. Test-tubes referred to in Note 3 (p. 645) were used and the tubes were placed vertically in a test-tube rack.

On very overcast or rainy days it is often not possible to obtain 80 % destruction of the riboflavin. However, even under these circumstances, 50 % destruction can be obtained, and it is possible to determine the riboflavin content of the extract from such figures, with a little loss of accuracy.

(3) *Effect of quantity of KMnO_4 used*

The KMnO_4 destroys a large proportion of the non-riboflavin fluorescent compounds, so reducing the residual blank. Moreover, a sufficient excess of KMnO_4 completely destroys light-labile fluorescent compounds, other than riboflavin. This is true for all extracts of biological materials studied by the authors. Sometimes a larger excess of KMnO_4 than usual has been necessary; but, whenever a trial of the type exemplified in Table 3 has shown that light-labile fluorescent compounds, other than riboflavin, are present, a further determination, in which the proportion of the volume of KMnO_4 to the volume of the extract was greatly increased, has given satisfactory results. It was sometimes necessary to use 2 ml. 6 % KMnO_4 to 1 ml. extract.

Table 4 shows that these large amounts of KMnO_4 cause some destruction of riboflavin, but the internal standard procedure avoids any error due to this destruction, provided that the time of reaction is carefully controlled.

Table 4. *Destruction of riboflavin by KMnO_4*

	Vol. of KMnO_4	Mean fluorimeter reading* (corrected for reagent blank)	Destruction of riboflavin (%)
I	0	90.2 ± 1.5†	—
II	1	85.3 ± 1.5†	5.4
III	2	81.5 ± 1.5†	9.6

* These readings were obtained as follows:

I. Taken, 1 ml. acetic acid, 1 ml. riboflavin solution (1.2 µg./ml.), 4 ml. water, then proceeded according to the usual method.

II. Taken, 1 ml. acetic acid, 1 ml. riboflavin solution, 2 ml. water, 1 ml. KMnO_4 , after 1 min. 1 ml. H_2O_2 , then as usual.

III. Taken, 1 ml. acetic acid, 1 ml. riboflavin solution, 2 ml. KMnO_4 , after 1 min. 2 ml. H_2O_2 , then as usual.

† Standard error of mean.

(4) *Effect of volume of sample taken*

This was investigated by adding various volumes of water to cylinders containing 1 ml. acetic acid and 1 ml. riboflavin solution, and then following the usual procedure; 3 g. Na_2SO_4 were sufficient for up to 7 ml. water added, 7 g. were necessary when 15 ml. water were added. The results are given in Table 5.

Table 5. *Effect of volume of sample on fluorimeter reading*

Water added (ml.)	Fluorimeter units (initial reading – reading after irradiation)
0	82.6 ± 1.3*
7	84.5 ± 1.3*
10	87.2 ± 1.3*
15	82.5 ± 1.3*

* Standard error of mean.

They show that the readings actually increase with increasing volume of sample until about 10 ml. are added, thereafter the readings decrease. The increase is probably due to reduction of the volume of the butanol-pyridine-acetic acid layer, without concomitant loss of riboflavin into the aqueous phase. However, when the volume of aqueous phase increases beyond a certain point, the amount of riboflavin, which is not extracted from the aqueous phase, becomes the more important factor. The results indicate that, when small volumes of the extract are used, the extraction of riboflavin into the butanol-pyridine-acetic acid phase is probably complete. They also illustrate the necessity for keeping the volume of the aqueous phase in all the cylinders constant.

The volume of extract which can be taken for the determination is limited by the following considerations:

(1) it should contain not more than 1.2 μg . riboflavin;

(2) there must be a sufficient concentration of KMnO_4 .

With materials containing small amounts of riboflavin, it is desirable that a volume of extract as large as practicable should be taken. This is limited by the necessity for a sufficient concentration of KMnO_4 .

(5) Order of addition of reagents

In the procedure of Najjar (1941) the pyridine is added before the KMnO_4 . We obtain considerably lower reagent blanks, which are, moreover, considerably more stable to sunlight, if the pyridine is not added until the KMnO_4 has been destroyed by the H_2O_2 . This phenomenon may be due to an impurity in our pyridine (redistilled 'Baker's Analyzed' C.P.). The pyridine is most conveniently added in solution with the butanol.

(6) Amount of Na_2SO_4

Najjar (1941) and Barton-Wright & Booth (1943) used 5 g. Na_2SO_4 . However, it is only necessary to saturate the aqueous phase with Na_2SO_4 ; we have found 3 g. to be sufficient for this purpose, even when 7 ml. of extract is taken, and the smaller amount causes less trouble. In our experience, it is important to keep the Na_2SO_4 in the anhydrous state throughout the process. If $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ separates, it occupies a large volume and all of the aqueous layer and much of the organic layer are trapped in the crystals. This causes difficulty in the extraction of the riboflavin from the aqueous to the organic layer. The separation of the decahydrate is prevented by heating above 32.4° , the transition point between the anhydrous salt and the decahydrate. Above this temperature, anhydrous Na_2SO_4 always crystallizes from water (Ephraim, 1934). When this solution is cooled below 32.4° , it crystallizes if nuclei of $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ are present, but if these are excluded the solution becomes more and more unsaturated, since the solubility of anhydrous Na_2SO_4 rises with falling temperature. It is therefore necessary to exclude decahydrate nuclei and this can be most easily achieved by keeping the entire contents of the cylinder above the transition temperature for a short period, since, in this way, any decahydrate present will be converted to the anhydrous form. Barton-Wright & Booth (1943) heated to $35\text{--}40^\circ$. We prefer to heat the cylinders to above 45° , then to mix quickly so that portions of the cylinder above the water-bath will also be above the transition temperature. In this way, any decahydrate on the upper portions of the

cylinder is converted to the anhydrous salt. If this is not done, crystallization of the decahydrate often occurs during the final shaking after the contents have cooled to room temperature. This, too, is the reason for the long-stem filter funnel. Even with all these precautions, decahydrate occasionally separates (especially in cold weather) and the cylinder must be reheated.

(7) Quenching of fluorescence

Pigments reduce the intensity of, or quench, the fluorescence by absorbing part of either the incident light or the fluorescent light, or both. Any error due to quenching is eliminated by the use of the 'internal' standard, since the degree of quenching of the fluorescence of solution A will be the same as that of solution B. This is illustrated by the following example. Sulphaguanidine (0.2%) was added to a portion of a sample of urine in order to test the applicability of the method to urines containing this drug. The riboflavin contents of the untreated and treated urines were determined with the results shown in Table 6.

Table 6. *Quenching of fluorescence by sulphaguanidine*

(Procedure and calculation as described on p. 645.)

	Urine- untreated		Urine- sulphaguanidine added	
	A	B	A	B
Fluorimeter readings:				
Initial	68.0	162.7	45.5	103.5
After irradiation	22.3	38.1	14.9	23.4
	45.7	124.6	30.6	80.1
		45.7		30.6
		78.9		49.5
$\mu\text{g. riboflavin in}$	45.7		30.6	
sample	$78.9 \times 1.2 = 0.69$		$49.5 \times 1.2 = 0.74$	
Quenching (%)		6		41

Thus, although the sulphaguanidine caused a considerable increase in the quenching of the fluorescence, that is, a reduction of all fluorimeter readings, the reduction was in about the same proportion in every case, so that the final calculations for the amount of riboflavin in the sample showed a difference of only about 7%. If the 'internal' standard procedure had not been adopted, it would have appeared that urines containing sulphaguanidine contained 33% less riboflavin than urines not containing the drug.

(8) Reproducibility of the method

The reproducibility of the method is illustrated by the following example. A sample of urine was

analyzed on twelve separate occasions with the following results:

0.547, 0.578, 0.550, 0.584, 0.537, 0.483, 0.516, 0.586, 0.544, 0.532, 0.548, 0.545, mean 0.546 $\mu\text{g./ml.}$; standard deviation 0.0290; coefficient of variation 5.3%.

The percentage error of the method (if one defines the error as a quantity x such that 95% of results will lie within mean $\pm x$) is about $2 \times 5.3 = 10.6\%$.

(9) Use of an adsorbent

Some urines were analyzed by the method proposed in this paper and also by a method involving a preliminary adsorption on Florisil (Floridin Co., U.S.A.). The procedure followed in the latter method was essentially that of Barton-Wright & Booth (1943) up to the elution stage. The sample of Florisil used adsorbed the riboflavin readily, but elution was incomplete. This fact did not, however, introduce any systematic error into the method, as the 'internal' standard procedure was followed.

Experimental procedure. Urine (5 ml.), undiluted in the case of urine 1, diluted 1 to 5 in the case of urines 2-5, was adjusted to pH 5.0 and diluted to 100 ml. Amounts of 20 ml. were transferred to four 50 ml. centrifuge tubes A, B, C and D. Riboflavin solution (1 ml., 1.2 $\mu\text{g./ml.}$) was added to B and D and 1 ml. water to A and C. To each tube was added 0.5 g. Florisil (Floridin Co., U.S.A.) and the mixture stirred mechanically for 5 min. After centrifuging and pouring off the centrifugate, 20 ml. water were added, the mixture stirred by hand for 2 min. and centrifuged. The centrifugate was discarded and as much water as possible drained off. The eluting solution (6 ml. of a mixture of water 9 vol., pyridine 5 vol. and acetic acid 2 vol.) was added, and the contents stirred mechanically for 5 min. and centrifuged. The centrifugate was added to a 50 ml. glass-stoppered measured cylinder and a further 2 ml. eluting solution added to the centrifuge tube. The contents of the tube were stirred by hand for 2 min., centrifuged and the centrifugate added to the same cylinders as previously. The method proposed in this paper was then followed. A fifth cylinder, containing 8 ml. eluting solution, was treated as the reagent blank.

The results are shown in Table 7.

Table 7. Comparison between proposed method and method using Florisil

Urine	Riboflavin content			Residual blank*	
	Proposed method	Method using Florisil	$\frac{\text{II}}{\text{I}} \times 100$	Proposed method	Method using Florisil
	($\mu\text{g./ml.}$) I	($\mu\text{g./ml.}$) II			
1†	0.02	0.01	—	0.14	0.01
2	0.48	0.44	90	0.08	0.01
3	0.35	0.40	115	0.22	0.01
4	1.15	1.03	90	0.16	0.03
5	0.41	0.36	89	0.24	0.06
	Mean		96		

* Equivalent to $\mu\text{g./ml.}$ riboflavin in urine.

† From a riboflavin-deficient rat.

The reproducibility of the method using Florisil was not very satisfactory and this probably accounts for the differences between the riboflavin contents of the individual urines as determined by the two methods. However, there was no significant systematic difference between the results obtained by the different methods.

As would be expected, the method using Florisil gave a lower residual blank, but this appeared to be its only advantage. It was extremely tedious compared with the proposed method.

(10) Comparison of fluorimetric and microbiological methods

In Table 8 some comparisons of the results obtained by the fluorimetric and microbiological methods are given. The microbiological determinations were carried out by Dr C. White, who has a paper on the method in preparation. The micro-organism used was *Lactobacillus casei*. The figures in Table 8 show that there is no systematic difference between the results obtained by the two methods. The results for the urine from the riboflavin-deficient rat show that the fluorimetric method is at least as specific as the microbiological method.

Table 8. Comparison of the fluorimetric with the microbiological method

Sample	Riboflavin content ($\mu\text{g./ml.}$)		M/F $\times 100$
	By fluorimetric method (F)	By microbiological method (M)	
Urine 1*	0.02	0.05	—
Urine 2	0.45	0.43	96
Urine 3	1.15	0.97	84
Urine 4	0.35	0.40	114
Urine 5	0.34	0.31	89
		Mean	96

* From a riboflavin-deficient rat.

APPLICATIONS OF THE METHOD

The method has been applied successfully to the measurement of riboflavin in rat and human urine and in many food materials. Its application to urine and a comparison with other methods which have been used for urine are discussed in another paper (Morell & Slater, 1946).

The results of determinations on miscellaneous foods are given in Table 9. A number of different methods of extraction has been used. It is impossible to specify a single method for all foods; the best method of extraction must be determined by trial with each type of food material. Morell (1946) has compared a number of different methods of

extracting riboflavin from wheat and wheat products. Protein-bound riboflavin is present in some foods and, in addition, riboflavin appears to be readily adsorbed on the protein of eggs and milk. Unless the protein is largely broken up during the preparation of the extract, low figures are obtained. This is revealed by carrying out a 'recovery' test, i.e. a known amount of riboflavin is added to a second sample of food and the same treatment is subsequently applied to both. If any material adsorbs some riboflavin, about the same proportion of the added riboflavin will also be adsorbed and a low recovery will be obtained. The recovery test also reveals whether riboflavin has been lost in any other way (e.g. by exposure to light), but it does not give any information on the completeness of the extraction.

Table 9. *Riboflavin content of some foodstuffs*

Foodstuff	Method of extraction*	Riboflavin content ($\mu\text{g./g.}$)	Recovery of added riboflavin (%)
Tinned meat and vegetable stew	A	1.00	93
Tinned meat and vegetable hash	A	0.85	96
Tinned corned beef hash	A	0.64	97
Tinned meat and beans	A	0.76	95
Tinned black-currant spread	A	0.22	104
Wheatmeal biscuit	A	0.93	105
Fruit and cereal block	A	0.69	98
Tea infusion	—	0.05†	—
Caramel confection	B	5.0	93
Cheese—Kraft	B	4.0	107
Chocolate	B	3.4	95
Wheat lunch‡	B	2.9	—
Peanut butter	B	0.76	93
		Mean	98

* See this page. † $\mu\text{g./ml.}$

‡ A preparation containing 35% wheat germ.

Method of extraction A

The sample was minced or ground and 10–25 g. taken. Acetate buffer (150 ml., pH 4.5), 5 ml. takadiastase suspension (0.2 g. takadiastase suspended in 10 ml. water), 5 ml. papain suspension (0.2 g. papain made into a paste with a little glycerol and dispersed in 10 ml. water), 1 ml. 3.2% KCN and 1 ml. toluene were added. The KCN was added to activate the papain. The recovery test was carried out by adding 5 ml. riboflavin solution (20 $\mu\text{g./ml.}$) to another weighed sample. A blank determination (to allow for riboflavin in the enzymes) was made by mixing 5 ml. takadiastase suspension, 5 ml. papain suspension, 1 ml. KCN, 30 ml. acetate buffer and 1 ml. toluene. All flasks were incubated at 37° overnight and made up to 200 ml., 500 ml. (recovery) and 50 ml. (blank) respectively. The solutions were filtered and the riboflavin determined on 4 ml. of the clear extract. With the black-currant spread, it was necessary to use 2 ml. KMnO_4 and 2 ml. extract prepared from a 25 g. sample.

Method of extraction B

A sample, 2–10 g., was autoclaved with about 80 ml. 0.25N- H_2SO_4 for 30 min. at a pressure of 15 lb./sq. in. The mixture was made up to 100 ml., filtered and the riboflavin determined on 4 ml. of the filtrate.

DISCUSSION

The method described in this paper differs from the other methods of determining riboflavin which have been described in the literature by including a test for specificity. With methods employing the hydro-sulphite blank, there is no certainty that fluorescent substances other than riboflavin are not reduced—indeed, our experience is that this often occurs. Similarly, when the 'irradiation blank' is used according to the methods of Najjar (1941) and Barton-Wright & Booth (1943), there is no test to determine whether other fluorescent substances are destroyed during the irradiation. The failure of the published chemical methods to measure correctly the riboflavin content of urine is discussed in another paper (Morell & Slater, 1946). The specificity of even the microbiological method cannot be accepted without question. Price (1945) states that 'because of the presence, in certain types of sample, of substances stimulating to *L. casei*, invalid assays are not uncommon'. With our method, only substances which are decomposed at about the same rate as riboflavin (with a similar decomposition curve) can pass the specificity test. Direct evidence of the specificity of the method as applied to rat urine is given by the extremely low figure obtained on a sample of urine from a riboflavin-deficient rat.

Our experience with Florisil has been similar to that of Rubin & DeRitter (1945), who have recently discussed the dangers of relying upon this adsorbent. Our results show that the use of Florisil did not increase the specificity of the method.

The shape of the decomposition curve is interesting. Using artificial light, DeMerre & Brown (1944) have observed a somewhat similar phenomenon. They found that, when a solution of riboflavin in butanol-pyridine-acetic acid was exposed to artificial light, 'the final destruction amounted to slightly less than 60% of the original quantity of riboflavin exposed. After 60 min. radiation, very little additional destruction was observed suggesting that some protective factor has been developed during the radiation or that some factor had been introduced which interferes with the accurate fluorimetric estimation of riboflavin.'

The nature of the compound or compounds formed when riboflavin is decomposed in pyridine-butanol-acetic acid solution is unknown. Karrer, Saloman, Schopp, Schlittler & Fritzsche (1934) showed that, in aqueous neutral or acid solution,

lumichrome (6:7-dimethyl-isoalloxazine) is produced by the irradiation of riboflavin, while Warburg & Christian (1933) showed that lumiflavin (6:7:9-trimethyl-isoalloxazine) is formed in alkaline solution. Both these compounds exhibit a blue fluorescence. Karrer *et al.* (1934) showed that the nature of the solvent had a considerable influence on the yield of the decomposition products, but did not investigate the products of decomposition in butanol-pyridine-acetic acid solution. The decomposition in this solution is so much more rapid than in aqueous solution that it is quite possible that the decomposition takes a different course. We found that the pH of the butanol-pyridine-acetic acid solution, diluted fourfold with water and ethanol, was 4.6.

The method is sufficiently sensitive for most purposes. The smallest amount of riboflavin which can be measured with an accuracy of 10% is about 0.1 μg . The limit of sensitivity of the method, i.e. the smallest amount which can be regarded as significantly greater than zero, is about 0.01 μg . The smallest concentration which can be measured depends upon the size of the aliquot taken, which in turn depends upon the amount of interfering substances present.

SUMMARY

1. A modification of the fluorimetric method of determining riboflavin in biological materials, based on the method of Najjar (1941) but differing in several important respects, is described.
2. The method differs from those previously described by including a test for specificity.
3. The photochemical destruction of riboflavin is not a simple exponential function of time, but is dependent upon the concentration of the decomposition products, i.e. a reversible reaction is involved.
4. High concentrations of KMnO_4 rapidly destroy riboflavin.
5. The introduction of an additional purification step involving adsorption on Florisil did not increase the specificity of the method.
6. In general, figures obtained by the proposed method agreed closely with those obtained by a microbiological method.
7. The accuracy of the method is about 10% and the limit of sensitivity about 0.01 μg .

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The Fluorimetric Determination of Riboflavin in Urine

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In a previous paper (Slater & Morell, 1946*a*), we described a specific method for the determination of riboflavin in biological materials. The development of this method was made necessary by our finding that the methods commonly employed for the measurement of riboflavin in urine were non-specific and seriously over-estimated the riboflavin content of urines containing small amounts of the vitamin.

Since much of our knowledge of the metabolism of riboflavin (especially in man) is based upon determinations of the urinary excretion of riboflavin, it is useful to know the order of magnitude of the errors associated with the various methods of estimation. This is the purpose of the present paper, which presents the results of a systematic comparison of various methods of determining riboflavin in human and rat urine.