these compounds would seem to compare with, for example, M and B 693. There is just a suggestion that they had some effect because at the 12th hour (see Table 1) the controls showed definite signs of illness, whereas the mice under treatment appeared quite well. By the 24th hour there is no therapeutic effect evident.'

Table 6

Compound	Activity	
	<i>Myco. tuberculosis</i>	<i>Cl. welchii</i>
Formylambamide	<1/4	Inactive
Butyrylambamide	<1/1	"
<i>n</i> -Heptane-1-carbonylambamide	<1/1	"
<i>n</i> -Tridecane-1-carbonylambamide	<1/1	"
<i>n</i> -Heptadecane-1-carbonylambamide	<1/1	"
Formyl V 335	<1/5	"
Acetyl V 335	<1/1	"
Propionyl V 335	<1/3	"
<i>n</i> -Undecane-1-carbonyl V 335	<1/1	"
<i>n</i> -Pentadecane-1-carbonyl V 335	<1/1	"

Table 7. *The effect of formyl, propionyl, and butyryl V 335 and butyrylambamide on an infection with Clostridium septicum in mice*

(Mice. Weight, 18–20 g.; 10 mice in each group. Infection: about 10 minimal infecting doses (50 spores); 0.05 ml. saline spore suspension + 0.05 ml. 5% aqueous CaCl₂ mixed immediately before injection intradermally into shaved abdomen. Drugs: 20 mg. of the drug suspended in 0.5 ml. saline given by mouth at each dose, (i) 1 hr. before infection, (ii) 4 hr. after infection, (iii) 8 hr. after infection and (iv) 12 hr. after infection.)

Drug given	State of animals					
	12 hr. after infection		24 hr. after infection		48 hr. after infection	
	Sick	Dead	Sick	Dead	Sick	Dead
Controls (no drug)	7/10	0/10	2/10	8/10	—	10/10
Formyl V 335	0/10	0/10	3/10	7/10	—	10/10
Propionyl V 335	0/10	0/10	1/10	9/10	—	10/10
Butyryl V 335	0/10	0/10	—	10/10	—	—
Butyrylambamide	0/10	0/10	1/10	9/10	—	10/10

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The Fluorimetric Estimation of Riboflavin in Foodstuffs and other Biological Material

By E. KODICEK AND Y. L. WANG

Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

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Various methods have been published for the estimation of riboflavin (Hodson & Norris, 1939; Conner & Straub, 1941; Andrews, 1943; Barton-Wright & Booth, 1943; Slater & Morell, 1946; Morell & Slater, 1946; Morell, 1947). Some of them employ adsorption of the vitamin on activated surfaces and its subsequent elution. The disadvantages of the adsorption techniques are that they are tedious, and may result in serious losses because of incomplete adsorption or elution of riboflavin (Rubin & DeRitter, 1945). Since 1942 we have used with satisfactory results the method described below, which, by using a sensitive photocell-galvanometer circuit, enabled us to estimate the vitamin

directly without concentrating it by adsorption. A similar technique has been described by Rubin, DeRitter, Schuman & Bauernfeind (1945) and by Scott, Hill, Norris & Heuser (1946).

EXPERIMENTAL

Principle of method

The main features of our technique are:

(1) Extraction on the water bath with 0.1 N-HCl. Precipitation of proteins by metaphosphoric acid, and/or digestion with takadiastase when starchy materials are assayed.

(2) Washing of the extract with chloroform at acid pH, and oxidation with saturated potassium permanganate solution followed by treatment with hydrogen peroxide to decolorize the remaining permanganate.

(3) The extracts are adjusted to pH 6, and methanol added to clarify the extract if necessary.

(4) Fluoriphotometric readings are taken using a blue filter for the incident light and a yellow filter between the solution and photocell. A sensitive photocell-galvanometer circuit is used.

(5) In the blank, the riboflavin is reduced to a non-fluorescent compound with a neutralized solution of sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$).

Reagents

HCl (0.1 N); NaOH (20%, w/v); acetic acid (1%, w/v); metaphosphoric acid (25%, w/v) freshly prepared; KMnO_4 (4%, w/v); H_2O_2 w/v (3%, w/v); chloroform; methanol; light petroleum; acetone-0.2 N- H_2SO_4 solution (1:1); sodium dithionite solution: 0.5 g. $\text{Na}_2\text{S}_2\text{O}_4$, 0.6 g. NaHCO_3 dissolved in 10 ml. water, prepared immediately before use. Takadiastase (Parke, Davis and Co.).

Riboflavin standard. Riboflavin (25 mg.) in 50 ml. distilled water (at 50°) + 1 ml. glacial acetic acid. Mix with a further 500 ml. distilled water (at 50°), stir until completely dissolved, and make up to 1 l. with distilled water and ethanol to a final concentration of 20%. The solution was kept in a dark brown bottle in the refrigerator. A dilute standard solution, containing 5 $\mu\text{g.}/\text{ml.}$, was prepared each week by diluting 10 ml. of the strong riboflavin standard to 50 ml. with 20% ethanol acidified with 1 drop of conc. HCl.

Apparatus

Single photocell fluorimeter (Cohen type), connected with a sensitive galvanometer (Cambridge Instrument Co., short period galvanometer, 460 Ω resistance at 20°, external resistance for critical damping 2800 Ω , 4.4 sec. period, 4600 mm. deflexion for 1 $\mu\text{amp.}$ at 1 m. The deflexion was doubled by placing the scale at a distance of 2 m.). A photocell was chosen with a high sensitivity and a suitable inner resistance to obtain the critical damping of the galvanometer. The light source was a Hewittic Ulviarc Therapeutic Lamp, a.c. burner, 3500 c.p., with a suitable optical arrangement.

The Hilger fluorimeter can be used as a Cohen type instrument by connecting the galvanometer directly with the right-hand cell as described by Bolton (1944).

The following filters were used: Calorex and Wratten no. 47A (blue) between the light source and solution; Flavazin filter Wratten no. 16 between the solution and photocell.

Preparation of extracts

Different extraction procedures have to be used depending on the material examined. The extraction procedures should be carried out in a semi-darkened room.

(a) For foodstuffs rich in proteins

When dried eggs are assayed, a preliminary treatment is necessary. Wash the weighed sample of the egg powder with

light petroleum, centrifuge, repeat washing, centrifuge, evaporate off the remaining light petroleum on a water bath and proceed as below with the HCl extraction.

Heat a weighed ground sample of the foodstuff (containing about 15 $\mu\text{g.}$ of riboflavin) with 50 ml. of 0.1 N-HCl on a boiling water bath for 30 min. Cool and add 5 ml. of 25% metaphosphoric acid. Mix, wait 10 min., then centrifuge. Decant the supernatant fluid. Re-extract the residue with 35 ml. of 1% acetic acid for 10 min. on the water bath. Centrifuge and unite the liquid with the previous extract. Wash residue with 10 ml. of 1% acetic acid (no heating necessary) and centrifuge. Combine the extracts and make up to 100 ml. with water. Wash a sample (30–50 ml.) with an equal volume of chloroform in a separating funnel. Centrifuge to hasten the separation of the layers. Oxidize 25 ml. of the clear aqueous layer by adding a 4% solution of KMnO_4 drop by drop with continuous stirring. Allow the pink colour to disappear completely before adding the next drop. Continue the additions until a faint pink lasts for 30 sec. Decolorize the excess KMnO_4 with 1–2 drops of a 3% H_2O_2 solution. Wait 5 min., then neutralize to pH 5.5–6.0 with 2% NaOH (i.e. yellow to bromothymol blue and blue to bromocresol green, used as external indicators). Make up to 75 ml. with water. Centrifuge if necessary.

(b) For cereals and foodstuffs containing starch

Use 5–10 g. of material for extraction. Add 50 ml. of 0.1 N-HCl and, stirring continuously, bring the mixture to the boil. Continue with the extraction as described above, omitting the metaphosphoric acid. Centrifuge while warm. Bring the combined supernatants to pH 4.5 with 20% NaOH (bromocresol green as external indicator). Digest the solution with 10–20 mg. of takadiastase (Parke, Davis and Co.) for 2 hr. at 37° in an incubator in the presence of a few drops of sulphur-free toluene. Adjust the volume to 100 ml. Centrifuge the digested extract. Add to a sample of the supernatant fluid 4 drops of conc. HCl and wash with an equal volume of chloroform in a separating funnel. Proceed as above by taking 30 ml. of the washed extract for oxidation. After adjusting the pH of the solution to 5.5–6.0, add 10 ml. of methanol and bring the volume to 50 ml. with water. Centrifuge for a short time if a precipitate has formed.

(c) Acetone- H_2SO_4 (1:1) extraction, suitable for cereals and vegetables

An alternative extraction procedure has been devised which gives comparable results with the extraction procedure (b), and can be used in some samples where extraction (b) does not result in clear extracts.

Reflux 5 g. of the sample with 50 ml. of the acetone-0.2 N- H_2SO_4 solution on a water bath for 30 min. Cool and centrifuge. Decant off the liquid and return the residue to the flask with 35 ml. of fresh acetone- H_2SO_4 solution. Reflux for a further 15 min. Centrifuge, and wash the residue with 15 ml. of fresh acetone- H_2SO_4 solution. Combine the supernatant fluids and evaporate off the acetone completely *in vacuo* at about 60°. Dilute the residual aqueous solution to 50 ml., wash with chloroform, and proceed as under (b).

Notes. The takadiastase preparation should be tested for the presence of appreciable amounts of riboflavin, and if necessary the proper correction should be made. It is also advisable to check the reagents, especially methanol, for a

possible blank fluorescence. The final extracts should be water clear even under inspection in ultraviolet light, otherwise quenching will interfere with the accuracy. Auto-claving with 0.05N-HCl is a possible alternative extraction procedure, but extraneous colours were observed with certain foodstuffs which decreased the reliability of this procedure.

Measurement of fluorescence

Add to glass cell A 0.2 ml. water, and to glass cell B 0.2 ml. (1 μ g.) of the dilute riboflavin standard. Measure 17 ml. of the unknown solution into each of the two glass cells A and B. Take at least three alternate readings in quick succession on both solutions by moving the sliding carrier into position. Then add 0.2 ml. of a freshly prepared $\text{Na}_2\text{S}_2\text{O}_4$ solution to glass cell A, stir and take readings again. As the reduction may take 30–60 sec. to complete take readings until repeats agree. This last reading gives the value for the blank.

Calculation

$$\text{Riboflavin} = \frac{Q_1 - B}{Q_2 - Q_1} \times \frac{V_2}{17} \times \frac{V_1}{V_2} \times \frac{1}{S} \mu\text{g./g.},$$

where S = amount of sample taken (g. or ml.); Q_1 = galvanometer reading in mm. of the unknown solution (i.e. glass cell A before reduction); Q_2 = galvanometer reading in mm. of the unknown solution + 1 μ g. of riboflavin (i.e. glass cell B); B = galvanometer reading in mm. of the blank (i.e. glass cell A after reduction); V_1 = vol. of combined aqueous extracts; V_2 = vol. of aqueous extract taken for oxidation; V_3 = vol. of final solution prepared for fluorimetry.

Fig. 1. Standard curve for pure riboflavin.

Sensitivity of method and standard reference curve

We have found that 1 μ g. of riboflavin in 17 ml. of 0.05M-phosphate-acetic buffer of pH 6 gives a maximum galvanometer deflexion of 182 mm.; by closing the adjustable slit which decreases the exciting light, we normally work with a deflexion of 90 mm. for 1 μ g./17 ml. of solution.

Fig. 1 shows the response curve. There is a straight-line relationship between galvanometer readings and concentration of riboflavin up to at least 5 μ g./17 ml. of solution.

Reproducibility

To check the reproducibility of the method, several samples were analyzed in 8–12 replicates (Table 1). The coefficient of variation ranged from 2.2 to 4.6%. The

Table 1. Reproducibility of results

Material	No. of estimations	Riboflavin		
		Mean ($\mu\text{g./g.}$)	Standard error of single determination ($\mu\text{g./g.}$)	Coefficient of variation (%)
Yeast, dried	8	50.2	± 1.082	2.2
Meat, dehydrated	8	6.92	± 0.321	4.6
Soya bean	12	3.72	± 0.171	4.6

Table 2. Riboflavin content of various foods

Material	No. of samples (different sources)	Riboflavin ($\mu\text{g./g.}$)	
		Mean	Range
Beef, fresh	3	1.6	1.5–1.7
Beef, dehydrated	1	6.2	—
Beef, corned	2	2.0	1.8–2.2
Pork, fresh	2	2.4	2.1–2.7
Pork, dehydrated	3	4.5	4.3–4.8
Liver, calf	6	13.5	11.8–14.6
Liver, sheep	10	29.4	21.6–36.0
Liver, guinea pig	1	40.0	—
Eggs, dried	10	13.8	8.2–15.3
Milk, fresh, cow's	3	1.5	1.4–1.8
Milk, human	6	0.6	0.4–0.7
Wheat flour, 100% extraction	5	1.2	1.05–1.35
Rye, flour mixed, whole	1	2.7	—
Oatmeal	1	1.0	—
Sussex oats, ground	1	1.5	—
Maize meal, yellow	1	0.8	—
Soya bean, Manitoba brown	1	3.7	—
Soya flour, low fat	1	7.2	—
Soya flour, high fat	1	12.0	—
Peanuts	1	2.8	—
Potatoes, fresh	4	0.33	0.29–0.39
Potatoes, peeled, dehydrated:			
Doon Star	1	1.3	—
King Edward	1	1.5	—
Majestic	1	0.6	—
Tomatoes	1	0.5	—
Carrots	3	0.55	0.42–0.76
Carrot puree	1	0.27	—
Pea puree	1	1.4	—
Cabbage, inner leaves	2	0.3	0.21–0.45
Grass, vacuum dried	1	4.4	—
Yeast, <i>Torula utilis</i> , dried	3	37.2	28.7–50.2
Yeastrel	1	42.6	—

accuracy of the method decreases with decreasing concentration of riboflavin in the material examined. This low variation was confirmed by Bolton (private communication), who found with our procedure a coefficient of variation of 5.3% when analyzing ground-nut cake in 12 repeats (2.38 μg . riboflavin/g., standard deviation ± 0.127 , or 5.3%).

RESULTS

Various foodstuffs were examined for their content of riboflavin. As may be seen in Table 2 the method is applicable to a wide variety of material. A number of foodstuffs have been assayed both chemically and microbiologically. The latter method is described in a following paper (Kodicek, 1949). Good agreement was found.

The method described above obviates the lengthy process of adsorption. The extraction of riboflavin when combined with enzymic digestion seems to be complete. The resulting extracts are clear and almost colourless.

It is important to point out that when dithionite is used as the reducing agent, blue light is

essential for obtaining the correct blank reading. We have observed that under ultraviolet light $\text{Na}_2\text{S}_2\text{O}_4$ reduces very strongly, or rather extinguishes almost completely, the total fluorescence of the extract. When a cell containing this reducing agent is placed in a beam of ultraviolet light in front of a cell containing riboflavin, the solution does not fluoresce. When blue light is used as the exciting source the fluorescence is almost unaltered.

SUMMARY

1. A modification of a fluorimetric method for the estimation of riboflavin has been described.

2. It estimates directly the fluorescence of riboflavin by using a sensitive photocell-galvanometer circuit without recourse to adsorption.

3. Washing of the extracts with chloroform and oxidation by potassium permanganate and hydrogen peroxide reduces the interference by unspecific fluorescent substances. The reduction of riboflavin by sodium dithionite has been used to obtain the value of the blank.

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The Fluorimetric Estimation of Nicotinamide in Biological Materials

BY D. K. CHAUDHURI AND E. KODICEK

Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

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Scudi (1946) has reported that when a solution of nicotinamide is treated with cyanogen bromide (CNBr) and then extracted with *isobutanol* at an alkaline pH it fluoresces like the F_2 compound formed from N^1 -methylnicotinamide. Pyridoxal, vitamin B_1 , N^1 -methylnicotinamide and certain alkaloids also produce a similar fluorescence under these experimental conditions. Kodicek (1947) has modified the conditions by using methyl ethyl ketone in a single phase at an alkaline pH instead of

extracting with *isobutanol*. Even then vitamin B_1 , pteroylglutamic acid, N^1 -methylnicotinamide, pyridoxal and quinolinic acid interfere.

On re-examination of the reaction we have found that a fluorescent compound can be obtained from nicotinamide on treatment with cyanogen bromide, and on standing at an alkaline pH, without recourse to an organic solvent. By further modifications it was possible to eliminate the interference of other fluorescent substances. A method has thus been