Fluorophotometric Determinations of Riboflavin*

By Wm. S. Jones and W. G. Christiansen

A number of methods, microbiological as well as chemical, have been employed to assay products and materials for riboflavin. For several years we have been working with the method of Hodson and Norris (1). It involves sulfuric acid extraction of the riboflavin; removal of foreign pigments by mild reduction, followed by mild oxidation with air; and photoelectric measurement of the fluorescent green light, produced when a solution of riboflavin is excited by a blue light. Our experience with this method, the accumulation of information regarding the factor of color in the solutions and data on substances highly fortified with riboflavin have enabled us to modify and simplify the procedure; phosphoric acid has been adopted instead of sulfuric acid as the extracting agent and the reduction-oxidation steps have been omitted. The phosphoric acid acts not only as the extracting agent but also forms part of the buffer system later on in the process; this was helpful because riboflavin gives maximum fluorescence at pH 7.0.

METHOD

A. Instrument.—We have used the Pfaltz and Bauer Fluorophotometer, Model A[1] with a G. E. Mercury Lamp, Type H3, 85 Watts as the source of light. Filter glass (Jena) combination GG-15 and BG-12, giving a peak at 4500 A., is placed between the solution in the 4-cm. glass cuvette and the source of light. An orange filter glass 315, Corning, is placed between the cuvette and photocell. The iris diaphragm, controlling the amount of light admitted to the solution in the cuvette, is opened about 3 cm.

The intensity of the activating light is regulated to give a definite galvanometer reading. This may be done in two ways: first, by using a standard solution of quinine activated by ultraviolet light at 3650 Å. (this requires switching of filter glasses during testing period), or, second, by direct galvanometric setting for the transmitted light picked up by the rear photocell. Once the resistance has been set for a definite fluorometric reading, the intensity of light is held constant by opening or closing iris diaphragm, with a voltage stabilizer in the line. This change in intensity of light is very small.

B. Reagents.—Standard Riboflavin Solution A: Fifty micrograms of anhydrous riboflavin are dissolved in 500 cc. of 25% aqueous alcohol containing 2.0 cc. of 85% phosphoric acid. The solution is kept in an amber bottle in refrigerator. Each cc. contains 100 micrograms riboflavin.

Standard Riboflavin Solution: Prepared daily by diluting 5 cc. of standard solution and 2.0 cc. of 85% phosphoric acid to 500 cc. with distilled water.

N/10 Sodium hydroxide.

Sodium hydrosulfite solution: One Gm. of sodium hydrosulfite and 1 Gm. of sodium bicarbonate are dissolved in 20 cc. of ice-cold distilled water and kept in an ice bath.

Table I.—Results Obtained with the Hodson-Norris Method

	Bioassay, or Expected Value		Found by Hodson-Norris Method	
Riboflavin concentrate, powder	3.0	mg./Gm.	3.193	mg./Gm.
	4.0	mg./Gm.	4.07	mg./Gm.
Liver concentrate	170	μg./Gm.	90	μg./Gm.
Yeast powder, type A	75	μg./Gm.	64	μg./Gm.
	85	μg./Gm.	61.6	μg./Gm.
	85	μg./Gm.	50.7	μg./Gm.
	75	μg./Gm.	59.2	μg./Gm.
	85	μg./Gm.	54.7	μg./Gm.
Yeast powder, type B	43.5	μg./Gm.	36.6	μg./Gm.
Power containing B complex, type A	350–425	μg./Gm.	117.0	μg./Gm.
Powder containing B complex, type B	65	μg./Gm.	52.0	μg./Gm.
	70	μg./Gm.	51.0	μg./Gm.
	75	μg./Gm.	83.0	μg./Gm.
Powder containing B complex, type C	200	μg./Gm.	193	μg./Gm.
	200	μg./Gm.	226	μg./Gm.
	250	μg./Gm.	216	μg./Gm.
	250	μg./Gm.	198	μg./Gm.
Yeast concentrate, liquid, type A	165–180	μg./cc.	71.6	μg./cc.
Yeast concentrate, liquid, type B	58	μg./cc.	50.0	μg./cc.
	78	μg./cc.	62.0	μg./cc.
Yeast concentrate, liquid, type C	250	μg./cc.	124	μg./cc.
Multiple vitamin capsule, type A	114	μg./cap.	112	μg./cap.
Multiple vitamin capsule, type B	1250	μg./cap.	1200	μg./cap.

C. Procedure.—To the sample (1 to 2 Gm. of powders, 1 to 2 cc. of liquids or 2 capsules) in a 250-cc. Erlenmeyer flask are added 100 cc. of distilled water and 0.4 cc. of 85% phosphoric acid solution. The flask is shielded from light by means of close-fitting blackened tin. The mixture is heated on the steam bath for one hour, cooled and filtered by means of paper into a volumetric flask. Any insoluble matter on the filter is washed with distilled water. The filtrate is made up to such a volume that each cc. will contain about 1 μg. of riboflavin; 0.4 cc. of 85% phosphoric acid is added for each 100 cc. additional volume over and above the original 100 cc. of water added. The measurement

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[1] A newer type is now available.

of fluorescence is carried out as follows having the instrument regulated as described above:

(*a*) To 2 cc. of the solution on test, in a 25-cc. amber cylinder, are added 2 cc. of the *N*/10 sodium hydroxide and distilled water to make 25 cc.; 12.5 cc. of this solution are placed in the 4-cm. cuvette and a galvanometer reading is recorded.

(*b*) Two cc. of the Standard Riboflavin Solution B are treated as in (*a*).

(*c*) To a second 2-cc. portion of the solution on test in a 25-cc. amber cylinder are added 2 cc. of Standard Riboflavin Solution B, 4 cc. *N*/10 sodium hydroxide and sufficient distilled water to make 25 cc. Measurement carried out as under (*a*).

(*d*) Repeat (*c*) except that 2 cc. of the hydrosulfite solution are added prior to making up to 25 cc. with distilled water. This constitutes the blank.

(*e*) To 2 cc. of the Standard Riboflavin Solution B are added 2 cc. of *N*/10 sodium hydroxide solution, 2 cc. of the hydrosulfite solution and sufficient distilled water to make 25 cc. Then 12.5 cc. of this solution are placed in the cuvette and fluorescent reading on the galvanometer is recorded. This constitutes a blank, which varies from 1.5–3.5. The difference between (*b*) and (*e*) equals the reading for 1 μg. of riboflavin.

$$\mu\text{g. riboflavin} = \frac{a-d}{b-e} \times \frac{b}{c-a} \times \text{dilution} \times \frac{1}{\text{am't of sample}}$$

Results obtained by meticulously following the Hodson and Norris method are found in Table I. The agreement with the biological or expected values is excellent with some samples and fair with others but in still others the difference is extremely large. Results obtained by the phosphoric acid method are found in Table II and in most cases the stated riboflavin content and found values are in very good agreement.

Five samples of yeast powder, type A, and one of powder containing B complex, type C, were assayed by both the Hodson and Norris and the simplified phosphoric acid procedures. The results, listed in Table III, obtained by the two methods are in fair agreement although somewhat lower than the biological assay figures.

Table II.—Results Obtained with the Phosphoric Acid Extraction Method

	Bioassay, or Expected Value		Found by Phosphoric Acid Extraction Method	
Riboflavin concentrate, powder	2.0	mg./Gm.	1.985	mg./Gm.
Liver concentrate	170	μg./Gm.	150	μg./Gm.
Yeast powder, type A	75	μg./Gm.	57	μg./Gm.
	85	μg./Gm.	67	μg./Gm.
	85	μg./Gm.	64	μg./Gm.
	75	μg./Gm.	57	μg./Gm.
	85	μg./Gm.	62	μg./Gm.
Powder containing B complex, type C	250	μg./Gm.	202	μg./Gm.
	260	μg./Gm.	194	μg./Gm.
	230	μg./Gm.	205	μg./Gm.
	270	μg./Gm.	180	μg./Gm.
Powder B complex in capsules	575	μg./cap.	530	μg./cap.
	575	μg./cap.	555	μg./cap.
	575	μg./cap.	530	μg./cap.
	575	μg./cap.	550	μg./cap.
Paste in capsules, type I	5.0	mg./cap.	5.0	mg./cap.
	1.0	mg./cap.	0.94	mg./cap.
	1.0	mg./cap.	0.92	mg./cap.
Paste in capsules, type II	5.0	mg./cap.	5.0	mg./cap.
	5.0	mg./cap.	4.9	mg./cap.
	1.0	mg./cap.	1.02	mg./cap.
	1.0	mg./cap.	1.01	mg./cap.
Paste in capsules, type III	1.0	mg./cap.	0.95	mg./cap.
	1.0	mg./cap.	0.98	mg./cap.
	1.0	mg./cap.	1.00	mg./cap.
	1.0	mg./cap.	1.03	mg./cap.
Paste in capsules, type IV	1.25	mg./cap.	1.15	mg./cap.
	1.25	mg./cap.	1.16	mg./cap.
Paste in capsules, type V	2.5	mg./cap.	2.4	mg./cap.
Paste in capsules, type VI	1.0	mg./cap.	1.05	mg./cap.
Paste in capsules, type VII	125	μg./cap.	124	μg./cap.
	125	μg./cap.	122	μg./cap.
	125	μg./cap.	130	μg./cap.
Paste in capsules, type VIII	25	μg./cap.	28	μg./cap.
	25	μg./cap.	26	μg./cap.
Paste in capsules, type IX	125	μg./cap.	130	μg./cap.
	125	μg./cap.	130	μg./cap.
Paste in capsules, type X	312	μg./cap.	330	μg./cap.
Paste in capsules, type XI	125	μg./cap.	115	μg./cap.

The items listed as Paste in capsules are in all instances multivitamin mixtures with suitable diluents and vehicles in soft elastic capsules.

Table III.—Comparison of Hodson and Norris Method and Phosphoric Acid Method

	Bioassay, or Expected Value	Found	
		Hodson-Norris	Phosphoric Acid
Yeast powder, type A	75 μg./Gm.	64 μg./Gm.	57 μg./Gm.
	85 μg./Gm.	62 μg./Gm.	67 μg./Gm.
	85 μg./Gm.	51 μg./Gm.	64 μg./Gm.
	75 μg./Gm.	59 μg./Gm.	57 μg./Gm.
	85 μg./Gm.	55 μg./Gm.	62 μg./Gm.
Powder containing B complex, type C	250 μg./Gm.	216 μg./Gm.	202 μg./Gm.

Table IV.—Samples Prepared by Enzymatic Digestion

	Bioassay, or Expected Value	Found
Liver concentrate	170 μg./Gm.	100 μg./Gm.
Powder containing B complex, type A	350–425 μg./Gm.	118 μg./Gm.
Yeast concentrate, liquid, type A	165–180 μg./Gm.	121 μg./Gm.
Yeast concentrate, liquid, type C	250 μg./Gm.	144 μg./Gm.

In connection with the fluorophotometric assaying of some materials for B_1 by a procedure in which diastatic digestion is employed, portions of the solutions were tested for riboflavin by the Hodson and Norris method. The data are given in Table IV; they compare fairly well with those obtained by direct application of the Hodson-Norris method (Table I) excepting in one case.

Some 25 lots of synthetic riboflavin have been tested. The solutions were made up in the same manner as the standard solution of riboflavin and compared with the standard riboflavin.

4.75%	of the samples assayed	950,000 μg./Gm.
4.75%	of the samples assayed	960,000 μg./Gm.
9.5%	of the samples assayed	970,000 μg./Gm.
28.6%	of the samples assayed	980,000 μg./Gm.
19.5%	of the samples assayed	990,000 μg./Gm.
33.0%	of the samples assayed	1,000,000–1,020,000 ug./Gm.

Of this series 81% showed 980,000 μg. or better per Gm. of material.

SUMMARY

1. The method of Hodson and Norris for the fluorophotometric determination of riboflavin and a modification of it have been applied to both simple preparations and complex mixtures, natural and artificial.

2. Fair agreement between biological and fluorophotometric determination of riboflavin exists.

The authors appreciate the help of Mr. Frank Howland, Development and Control Laboratory, in carrying out the determinations of riboflavin.

REFERENCE

(1) Hodson and Norris, *J. Biol. Chem.*, 131 (1939), 621.

Book Review

Traffic in Opium and Other Dangerous Drugs for the Year Ended December 31, 1940. Bureau of Narcotics, U. S. Treasury Department. 105 pages. 1941. Washington, D. C.: Government Printing Office. Price, paper, 25 cents.

This publication is the annual report of the Commissioner of Narcotics of the United States. It contains detailed information regarding raw materials, manufactured drugs, new legislation, administration of the Uniform Narcotic Drug Act, control of international trade and coöperative efforts of the nations of the world to control illicit traffic. The number of known medical drug addicts is estimated not to exceed 1 in 3000 population, which represents a reduction of at least 66 per cent in the past two decades. The report should make interesting reading for pharmacists and others interested in the problems of drug addiction.—A. G. D.

Photoelectric Determination of Nicotinic Acid*

*By Wm. S. Jones**

Nicotinic acid, nicotinamide or compounds containing the nicotinic acid nucleus play an important nutritional role as the black tongue and pellagra-preventing vitamin. Recognition of this makes it highly desirable that a rapid method be available for the assaying of materials and products containing this factor.

Many investigators have studied the cyanogen-bromide method of determining nicotinic acid. Harris and Raymond (1) and Kodicek (2) considered *p*-amino acetophenone a more satisfactory amine for this purpose. Kodicek studied further the various factors affecting the reactions and developed a satisfactory method for the extraction and determination of nicotinic acid. The sodium hydroxide hydrolysis to which the sample is subjected gives rise to highly colored substances that interfere badly with the subsequent measurement. Melnick and Field (3) overcame this difficulty by preferential adsorption of the colored substances on Darco G-60 also known as Darco, Coleman and Bell. In our hands this procedure for removal of color gave rise to a loss of nicotinic acid and we did not pursue it further. Other investigators, Arnold, Dehreffley and Lipsuirs (4), have followed the practice of extracting the colored cyanogen-amine compound of nicotinic acid with ethyl acetate and measuring the color photoelectrically. However, even under these conditions the blanks show more color than is desirable. Furthermore, the extra operations incident to the ethyl acetate extraction are time consuming. Moreover, we find that the intensity of the color in the ethyl acetate is subdued and is less, *i. e.*, $^1/_2$ to $^1/_3$, than that in aqueous alcoholic solutions and this is a disadvantage.

Giri and Naganna (5) describe a method in which the foodstuff is hydrolyzed with

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