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Research Article

THE URINARY EXCRETION OF RIBOFLAVIN
FLUOROMETRIC METHODS FOR ITS ESTIMATION

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Riboflavin, vitamin B_2, serves in its phosphorylated form as the prosthetic group for a number of tissue enzymes important in biological oxidations (1). The involved substrates include glucose, lactic acid, the *d*-amino acids, and other compounds of biological interest (1, 2, 3). Riboflavin deficiency has been described in man (4), and it seems possible that further clinical study of this vitamin may reveal alterations in its metabolism which will increase our knowledge of the rôle played by these enzymatic oxidation systems in health and in disease. To aid in interpreting such a study, a convenient method has been sought for following the urinary excretion of riboflavin.

It is known that riboflavin occurs in urine in the form of uroflavin, a pigment almost identical with it in composition,[1] properties, and vitamin activity (5), but the methods usually recommended for its determination (5, 6, 7, 8, 9) were not found by the writer to be sufficiently convenient or accurate for routine use. More specific and accurate determinations are possible, and smaller and more convenient volumes of urine (1 to 10 cc. instead of 100 to 500 cc.) can be used when the pigment is estimated not by its optical density but by its intense greenish-yellow fluorescence.[2] In order to do this, the effect of interfering substances must be avoided. With urines of normal individuals containing 0.5 gamma or more flavin per cc., this is readily accomplished by diluting down the salts and other materials to the point where they no longer affect fluorescence (10) and by destroying the greater part of other pigments and fluorescent materials in a brief permanganate oxidation (5). With urines of certain patients containing less than 0.5 gamma per cc., some preliminary concentration of the uroflavin is necessary, and can be much more conveniently and effectively carried out by using an adsorption column than by adding large quantities of adsorbing agent directly to urine (11, 12). In measuring the fluorescence of the column eluates or the diluted urine samples, the usual ultra violet radiation is not satisfactory since it excites the fluorescence of too many substances. Measurements of flavin fluorescence are much more specific and much more accurate when carried out with an exciting beam of visual wavelength, Mercury line at 4358A°, and when the fluorometric photoelectric cell is protected by a yellow filter.[3] With such an arrangement, the relationship between fluorescence and riboflavin concentration is linear for column eluates and diluted urine samples (Figures 1 and 2), particularly when the effect of any occasional alteration in optical density is corrected by means of the empirical curve illustrated in Figure 3.

METHODS

I. Adsorption column procedure for measuring the uroflavin content of urines in general

An adsorption column[4] 1 cm. in diameter and 15 cm. in length is set up with granular floridin earth[5] which has been washed free of dust and

[1] The carbon analysis of the purest preparation isolated did not quite agree with that for riboflavin (Koschara (5)).

[2] Pfaltz & Bauer, New York City. Model A is a suitable fluorometer provided with an additional cell for the measurement of optical density.

[3] This optical arrangement is the work of Dr. D. J. Henessey of Fordham University Chemistry Department and is to be described in detail elsewhere by him.

[4] E. Machlett & Sons, New York City. Usually a large number of columns and determinations are run simultaneously.

[5] Pomeroy & Fischer, New York City. (30 mesh.) Recent preliminary experiments have indicated that a heat-activated floridin and a synthetic material, super-

adsorbed gases by treatment with 2 per cent acetic acid and repeated rinsing with water. One to 10 cc. of filtered urine [6] at pH 5 are passed over the column. The total volume of the urine sample should be made up to about 10 cc. so that the time of its passage will be about 5 minutes. The column is then washed several times with distilled water to remove the unadsorbed urinary substances and the uroflavin is eluted by passing over the column 20 cc. of an aqueous solution of 20 per cent pyridine in 2 per cent acetic acid. This eluate is collected and cleared of oxidizable contaminants (5) by the addition of 2 drops of glacial acetic acid and 1 or 2 drops of 4 per cent potassium permanganate. The oxidation is stopped after 2 or 3 minutes by the addition of sufficient 3 per cent hydrogen peroxide to decolorize the solution (usually 1 to 5 drops); the total volume is made up to 25 cc. with water, and the solution is filtered if necessary.

The fluorometer iris is set so that a standard 0.1 gamma per cc. aqueous solution of riboflavin [7] fluoresces with an intensity of 20 galvanometer units while transmitting 50 galvanometer units of light. With this setting of the apparatus the column elution blank is: fluorescence 6, transmission 49. Riboflavin added to this blank solution to give a concentration of 0.1 gamma per cc. has a reading: fluorescence 20, transmission 49. As the fluorescence of flavin in these pyridine solutions is not very stable, they should be protected from light and in taking readings a constant routine should be adopted.

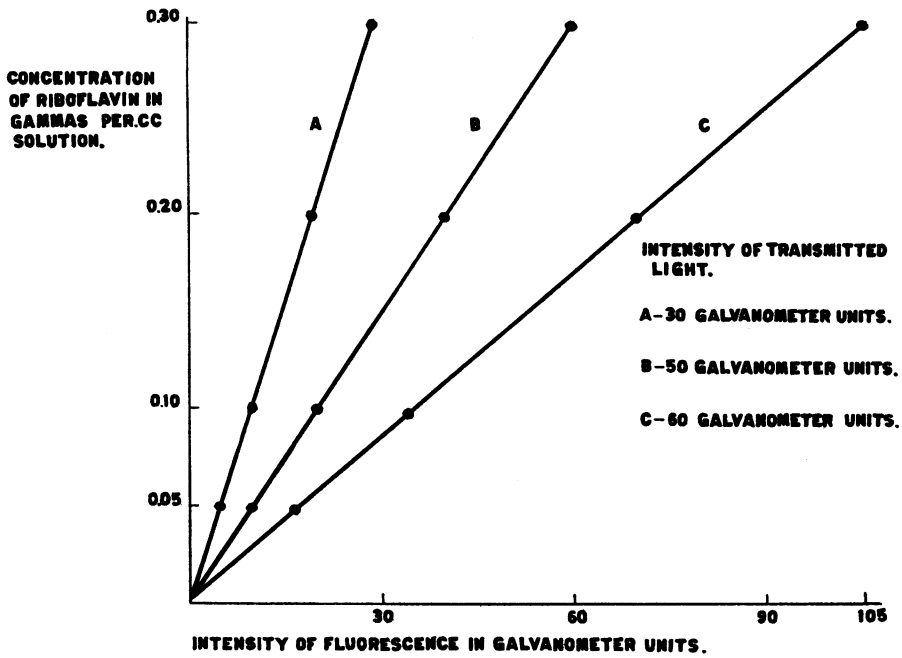

FIG. 1. RELATIONSHIP OF FLUORESCENCE TO RIBOFLAVIN CONCENTRATION AT SEVERAL DIFFERENT INTENSITIES OF ILLUMINATION

The fluorescence and transmission of the eluates are measured and the uroflavin concentration in gammas per cc. urine is calculated.

$$\frac{\text{Fluorescence of unknown}^{8} - 6}{20 - 6} \times 0.1 \times \frac{25}{\text{cc. of urine used}}$$

equals uroflavin in gammas per cc. urine.

sorb, are superior to crude floridin earth. These materials were obtained from Mr. O. Fitzsimons, Floridin Co., Warren, Pa.

[6] Twenty-four hour specimens are collected in dark bottles provided with 3 cc. glacial acetic acid and kept on ice during the period of collection.

[7] Merck & Co., Rahway, N. J. This standard is made up fresh each day from a stock solution of 50 gammas per cc. The stock solution is stable for long periods when preserved with a little acetone and protected from light.

[8] Corrected, if necessary; Figure 3 to compensate for any increase in optical density.

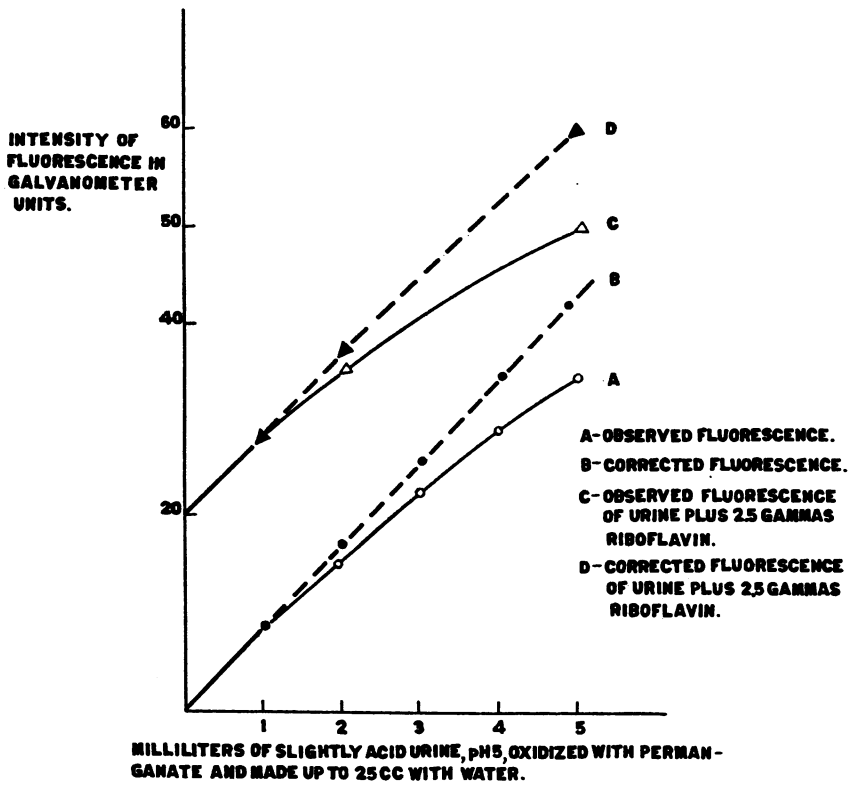

FIG. 2. MEASUREMENTS OF FLUORESCENCE IN SOLUTIONS OF DILUTED URINE, WITH AND WITHOUT CORRECTION FOR OPTICAL DENSITY OF SOLUTIONS

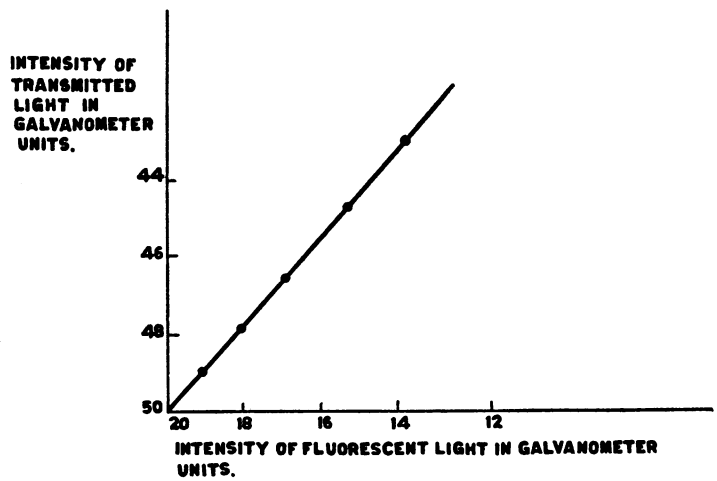

FIG. 3. RELATIONSHIP BETWEEN INTENSITY OF TRANSMITTED LIGHT AND INTENSITY OF FLUORESCENCE FOR A STANDARD RIBOFLAVIN SOLUTION OF 0.1 GAMMA PER CC.

TABLE I

The reliability of flavin determinations using the adsorption column technique

A. Recovery of riboflavin from 10 cc. aqueous solutions of standard riboflavin

Riboflavin in 10 cc. standard *gammas*	Amount recovered in pyridine eluate *per cent*
1.25	107
2.5	100,100,100
5.0	98,100
7.5	99

B. Duplicate determinations of uroflavin

Date	Subject	Uroflavin per cc. of urine *gammas*	Amount urine used *cubic centimeters*
March 25	F	1.1 1.1 1.2	
April 22	F	1.1 1.1	5 10
May 6	F	2.0 1.7	
April 6	McD	0.25 0.25	
May 6	D	0.45 0.43	5 10
May 7	D	0.33 0.33	5 10
May 9	D	0.53 0.56	5 10
May 12	D	0.53 ± .05 (4 determinations)	

C. Recovery of riboflavin added to urine

Uroflavin in specimen of urine *gammas*	Riboflavin added *gammas*	Recovery of added riboflavin *per cent*
2.3	2.5	87
0.63	2.5	100
1.6	2.2	87
2.7	2.5	107
5.6	2.5	104
3.0	2.5	106
1.8	2.5	85
5.4	2.5	100
17.0	2.5	96
16.0	2.5	100

Table I summarizes a study of the reliability of the method. Recovery of riboflavin from aqueous solutions of standard is good over a considerable range (section *A*). Duplicate determinations of uroflavin (section *B*) indicate a high degree of reproducibility which is not affected by altering the amount of urine used. This latter point is important because it demonstrates the method's independence of considerable variation in the concentrations of organic and inorganic matter present during the adsorptions. Column filtrates and washings do not contain significant flavin [9] and a second eluate with 20 cc. of pyridine reagent gives a fluorescence reading equal to that of the column blank. The recovery of riboflavin added to urine (section *C*) is as good as can be expected of an adsorption-elution procedure carried out in the presence of impurities.

II. Direct method for estimating the uroflavin content of urines of high or moderate flavin concentration (0.5 gamma or more per cc.)

One to 3 cc. of filtered urine at pH 5 are diluted to 25 cc. with water; the urinary pigments are destroyed by oxidation with permanganate in the manner described for the pyridine eluates; and the fluorescence of the solution is measured directly in the fluorometer. The value of the blank is given by the reading taken after reduction of the flavin to its non-fluorescent leuko form by the addition of a few particles of sodium hydrosulfite (5). When more than 2 or 3 cc. of urine are used in the dilutions, considerable permanganate, 5 to 10 drops, may be needed for its oxidation and, with highly pigmented urines of low flavin content, this direct method is probably not very accurate (urine M table III). Whether it will prove to be sufficient for routine clinical use is not at present known. The agreement between these direct measurements and the values obtained by the floridin column method, Table III, is evidence for the completeness of the column procedure and indicates the combined effectiveness of the instrument's filter system and the permanganate oxidation in preventing other urinary substances from interfering with the determination of uroflavin fluorescence.

TABLE II

Control conversions of riboflavin to lumiflavin

Amount of riboflavin in original solution *gammas*	Amount recovered as lumiflavin *per cent*
2.5	44
1.25	49
1.25	53
1.25	48

[9] The part played here by aquoflavin (Koschara) is not clear to the writer.

As thus far discussed, the uroflavin method has relied for its specificity upon the assumption that no material in urine other than uroflavin is capable of floridin earth adsorption, of elution by aqueous solutions of pyridine and acetic acid, resistance to permanganate oxidation in acid solution, and greenish-yellow fluorescence when illuminated by blue light. The method may be checked by determining uroflavin in the form of lumi-uroflavin (5). As in the case of riboflavin (13, 14), this chloroform-soluble pigment with intense green fluorescence can be formed from uroflavin by photolysis of strongly alkaline solutions in the cold. Unfortunately, the procedure is at present of little practical use because of the difficulty experienced in obtaining quantitatively consistent conversions and the resulting necessity of control determinations (15, 16).

TABLE III

Agreement of uroflavin determinations by different techniques

Patient	Direct measurement of fluorescence of diluted urines treated with acid permanganate		Measurement by adsorption column technique		Measurement by lumiflavin technique	
	Uroflavin per cc. urine	†Recovery of 2.5 gamma of added riboflavin	Riboflavin per cc. urine	Recovery of 2.5 gamma of added riboflavin	Riboflavin per cc. urine	Recovery of 2.5 gamma of added riboflavin
	gamma	*per cent*	*gamma*	*per cent*	*gamma*	*per cent*
McK.	0.25		0.22		0.27	
M*...	0.37	100	0.18	85	0.28	96
Wh...	0.40	100	0.30	106	0.35	
S.....	0.70	100	0.54	100		
Fx...	1.6		1.6	100	1.8	90
F....	2.2	100	1.7	96	2.1	

* Concentrated, highly pigmented urine.
† "Recovery" in this particular case means that added riboflavin produces the expected increment in fluorescence.

III. Method for determining uroflavin as lumi-uroflavin

A preliminary alkalinization of 5 cc. of urine is carried out in the dark and followed by acidification and extraction with chloroform so that the final chloroform extracts will contain only materials that have been rendered chloroform-soluble by exposure to light. The extracted urine is brought into the light, made up to half normal with concentrated sodium hydroxide, and exposed in an ice bath to a 150 Watt lamp for 1½ to 2 hours. The pH is then brought to 3 with strong acid and the lumiflavin is extracted from an aliquot of the urine by shaking with 20 cc. of chloroform. Control and recovery tubes are treated in a similar manner.

No data could be found in the literature relating the gram molecular fluorescence of riboflavin in water and the gram molecular fluorescence of lumiflavin in chloroform. This relationship was calculated by measuring the concentrations of the 2 pigments with a Koenig-Martens spectrophotometer at 4700A° (17), and comparing their fluorescence in the Pfaltz-Bauer fluorometer. For equal molecular numbers, lumiflavin in chloroform was found to have a fluorescence 2.25 times as great as that of riboflavin in water. It should be noted that this relationship may apply only to measurements made by using the filter system described with this fluorometer. Calculating from this factor of 2.25 and the results of the control and recovery experiments, Tables II and III, one finds that the determinations of uroflavin by this method are in reasonable agreement with those of the other two methods.

In determining the 24-hour uroflavin excretion of various patients, some of whom may excrete very little flavin, Table IV, it is preferable to use the adsorption column procedure in general, and to restrict the use of the direct method to normal urines or to urines containing large amounts of flavin such as those obtained during riboflavin tolerance tests. The lumiflavin method with its necessary controls is at present too time-consuming for routine use.

From the experiences of Table IV, it can be concluded that there is a fairly general dependence of uroflavin excretion upon riboflavin intake (18). This is particularly well illustrated by the patients D, McK, and Fx, and an examination of the actual dietary intakes of the other patients and subjects confirmed this impression; for example, patients G and W, though on regular diets, were both sick and not eating well; patient Kz on a high carbohydrate, high vitamin diet was jaundiced and sick. The normal subjects F and Km excreted considerably more uroflavin when liver or synthetic riboflavin was added to their diets. Normal rats on a riboflavin-deficient diet excreted very little uroflavin at a time when the riboflavin content of their livers had fallen to 40 per cent of normal. When placed on a complete diet their

TABLE IV

Twenty-four hour excretion of uroflavin

A. Normal subjects eating their usual diets

Subject	Uroflavin excretion
	gamma
C	850
G	1100
H	700
F	1700
K	1400

B. Normal subjects on regular hospital diets of 2400 calories

Subject	Uroflavin excretion Daily determinations for 5 days
	gamma
F	1200±200
Km	1000±100

C. Patients on various hospital diets

Subject	Type of diet	Uroflavin excretion
		gamma
G	Regular	450
W	Regular	430
Ch	Regular	560
Kz	High carbohydrate, high vitamin	450
McD	Low potassium	170
McD	Low potassium	220
D	Low potassium	260
D	Low potassium	200
D	Low potassium	250
D	Regular, eating heavily	1200
McK	Low residue, high caloric	200
McK	Low residue, high caloric, plus 1 mgm. Riboflavin	500
McK	Low residue, high caloric, plus 2 mgm. Riboflavin	800
Fx	1 liter 10 per cent cream every day for one week *	1300

* Contained 1.5 milligrams lactoflavin by direct measurement of acetone filtrate.

uroflavin excretion increased ten-fold. No observations were made of the bearing which phosphorylation (19, 20) may have upon riboflavin utilization and uroflavin excretion, but some evaluation of this factor and that of intestinal adsorption is being attempted by means of flavin tolerance studies now in progress.

SUMMARY

The importance of studying the metabolism of riboflavin has been pointed out.

Fluorometric methods for estimating the urinary excretion of riboflavin have been described.

Studies have been presented to show that these methods are convenient and accurate enough for clinical use.

The relationship of riboflavin intake to uroflavin excretion has been discussed.

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