FLUOROMETRIC MEASUREMENTS OF RIBOFLAVIN AND ITS NATURAL DERIVATIVES IN SMALL QUANTITIES OF BLOOD SERUM AND CELLS*

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The concentrations of many of the vitamins or their natural derivatives in blood serum and cells reflect the relatively recent intake of the particular dietary essential (*e.g.*, ascorbic acid). Such a relationship between blood level and intake has led to the usefulness of chemical analytical methods for the evaluation of nutritional status. However, there is, at present, insufficient information available to indicate whether or not measurements of the blood levels of riboflavin in any of its forms would be helpful in this way. This paucity of data on riboflavin blood levels is primarily attributable to the lack of satisfactory analytical methods. Results with microbiological methods (1–3) have been somewhat discordant, apparently because of the difficulty of measuring the low concentrations of the vitamin in the presence of persistent substances in blood extracts which may enhance or inhibit bacterial growth. The microbiological methods also fail to differentiate between riboflavin and its derivatives. By a manometric technique (4, 5), the flavin-adenine-dinucleotide has been measured in red cells and plasma. This procedure requires so much material and labor as to appear impracticable for wide usage.

The recent development of a very sensitive fluorometer (6) now makes possible methods which are simple, rapid, and reproducible to 3 to 5 per cent, and by which riboflavin can be differentiated from its natural derivatives. With this instrument the fluorescence of as little as 0.2 mγ of riboflavin in volumes of 0.5 ml. is readily measurable.

Methods will be described for determining (1) both free and total riboflavin in 50 c.mm. of blood serum (25 c.mm. for total alone), (2) the free riboflavin, flavin-mono- and dinucleotide as separate fractions in 0.2 ml. of serum, (3) the total riboflavin in 20 c.mm. of red cells or whole blood, and (4) the total riboflavin in white cells and platelets from 0.1 ml.

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of blood. Illustrative data include the influence of riboflavin intake on blood levels in the rat, some values for riboflavin concentration in supposedly normal human serum, red blood cells, and white blood cells, and a preliminary study of the effects of large doses and short periods of deprivation of riboflavin on the serum riboflavin of a few human subjects.

EXPERIMENTAL

Principles of Methods—The differentiation of the flavin compounds depends upon the following properties which will be fully discussed elsewhere:[1] (1) At neutrality, on a molar basis, riboflavin and riboflavin monophosphate (flavin-mononucleotide (FMN)) exhibit the same fluorescence, whereas flavin-adenine-dinucleotide (FAD) is only about 14 per cent as fluorescent under the conditions of analysis. (2) Riboflavin is much more readily extracted by benzyl alcohol from aqueous solution than is either FMN or FAD. (3) FAD is completely hydrolyzed to FMN in 5 per cent trichloroacetic acid in 20 hours at 37°.

Materials—

1. Microphotofluorometer fitted with special adapter and tubes as described elsewhere (6) (Farrand Optical Company, Inc., Bronx Boulevard and East 238th Street, New York 66). The commercial instrument requires slightly larger volumes than those used in the present work. The minimum fluid volume required for the particular instrument and cuvettes used should be determined and the corresponding changes made in specimen and reagent volumes.

2. Serological tubes, 6 × 50 mm. (Kimble, No. 45060).

3. Pyrex test-tubes, 3 and 8 ml. capacity.

4. Constriction pipettes 5, 20, 25, 50, 100, 110, 200, 400, and 500 c.mm. (7).

5. Syringe pipette set for 1 ml. volume (Mr. Herman Ruf, 5023 192nd Street, Flushing, Long Island, New York).

6. Pipettes and other equipment described for phosphorus analysis of white blood cells (8).

7. Wide tipped constriction pipette calibrated to contain 20 c.mm. of red blood cells.

8. Trichloroacetic acid, 100 gm. of redistilled acid diluted to 100 ml. From this, 5, 10, and 13 per cent solutions are prepared.

9. 0.16, 2.4, and 4 M K_2HPO_4 solutions.

10. Benzyl alcohol, C.P., redistilled, and saturated with water.

11. Chloroform, C.P., redistilled, and saturated with water.

12. Riboflavin standard solutions, 0.2 to 1.6 γ per ml. in 0.01 N HCl.

[1] Lowry, O. H., Bessey, O. A., and Love, R. H., in preparation.

Although these dilute solutions appear to keep well protected by black cloth in the cold, it is perhaps advisable to prepare them frequently from a stronger stock solution, *i.e.*, 20 γ per ml. in 0.01 N HCl.

13. Sodium hydrosulfite ($Na_2S_2O_4$). 0.5 gm. dissolved within 30 minutes of use in 5 ml. of 5 per cent sodium bicarbonate. This reagent is preferably kept in a tube in ice water to delay oxidation by the air.

14. Fluorescein secondary standards approximately equivalent in fluorescence to 1, 2, and 4 mγ of riboflavin per ml.

15. Reagents as needed for phosphorus determination in white blood cells (8).

16. Potassium oxalate, 1.6 per cent freshly diluted from 8 per cent solution kept in the refrigerator.

17. NaCl, 1 per cent solution.

Because of the small fluorescence measured, reagents must be sufficiently pure, and equipment sufficiently clean so as not to contribute more than a trace of fluorescence to the sample. Glass-redistilled H_2O is used in making all reagents. All tubes are cleaned by boiling first in half concentrated HNO_3 and then, after thorough rinsing, in distilled H_2O.

Since the riboflavin in the neutralized extract is quite sensitive to blue light, it is desirable to work with such extracts in a darkened room equipped with red lamps. Tubes are kept covered whenever possible to prevent contamination.

Some of the reagents listed are not required for certain of the determinations.

Procedure

Determination of Free Riboflavin and Total Riboflavin in Serum—50 c.mm. of serum are well mixed with 1.0 ml. of 5 per cent trichloroacetic acid at 0° in a 3 ml. tube, allowed to stand at 0–5° for 15 minutes, and centrifuged in cold cups at 0–5° for 10 minutes. 0.4 ml. of the supernatant is transferred to each of two fluorometer tubes, the first of which contains 0.1 ml. of 2.4 M K_2HPO_4. The remaining sample is reserved for the determination of total riboflavin as described below. The apparent riboflavin content (A) of the tube containing the neutralized extract is measured in the microphotofluorometer within 1 to 2 hours of neutralization. Meanwhile, care is taken to keep the tube in the dark. The tube is carefully wiped with a slightly damp cloth and three readings are made with the same instrument setting against fluorescein: an initial reading, R_1, a second reading, R_2, after the addition of an internal riboflavin standard (5 c.mm. equivalent to 1 mγ of riboflavin), and a reduced reading, R_3, after the addition of 5 c.mm. of sodium hydrosulfite solution ("Materials," item (6)). Complete blank determinations are also made on reagents, usually in triplicate. A reading

on a tube containing redistilled water is made as a measure of the contribution from scattered light and possible fluorescence of the tube itself.

For measuring the total riboflavin of the filtrate, the fluorometer tube containing the second sample is stoppered with a clean paraffined cork or very clean dust-free rubber stopper and is allowed to hydrolyze in the dark at 37° for 20 hours, usually overnight. It is then neutralized with 0.1 ml. of 2.4 M K_2HPO_4 and its apparent riboflavin content (B) is measured as above. Great care is taken to mix all solutions thoroughly and to keep the lower portion of the tube free from finger-marks and dust. If carefully calibrated fluorometer tubes are used, it is unnecessary to use an internal standard with more than a few samples, since little or no quenching has been observed with these serum filtrates.

Calculation—The readings R_2 and R_3 are corrected for the dilution resulting from the addition of internal standard and reducing agent, and may be designated R'_2 and R'_3. (The reading for redistilled water is subtracted from the sample readings before correcting for dilution, since the contribution from scattered light would not be affected by dilution.)

$$\frac{\text{Micrograms riboflavin added}}{\text{Ml. serum in aliquot}} \times \frac{R_1 - R'_3}{R'_2 - R_1} \times 100 = \text{micrograms \% riboflavin}$$

Appropriate correction is made in the above figure for blanks which have been treated and calculated in the same manner as the samples.

Since under the conditions of measurement FAD, before hydrolysis, is 14 per cent as fluorescent as riboflavin, then

$$\text{FAD} = \frac{B - A}{0.86}$$

and

$$\text{Free riboflavin (+FMN)} = A - 0.14\text{FAD} = 1.163A - 0.163B$$

(Ordinarily, very little FMN has been found present in serum.)

If the determination of total riboflavin only is desired, 25 c.mm. of serum are sufficient. The serum is precipitated with 0.5 ml. of 5 per cent trichloroacetic acid at room temperature and may be allowed to stand 30 minutes to an hour before centrifuging. A 0.4 ml. aliquot is hydrolyzed and measured as above. Prolonged contact of the protein precipitate with the trichloroacetic acid should be avoided as being likely to increase the blank reading.

Determination of Free Riboflavin, FMN, and FAD—A serum filtrate is prepared at 0–5°, as described above, from 0.2 ml. of serum and 2.0 ml. of 5 per cent trichloroacetic acid in an 8 ml. tube. After centrifuging, 1.0 ml. of the supernatant is transferred into a 3 ml. tube containing 0.25 ml. of 2.4 M K_2HPO_4 and mixed at once. The free riboflavin plus FMN is meas-

ured on a 0.5 ml. aliquot of this sample (A). The total riboflavin is measured on a 0.4 ml. aliquot of the remaining supernatant acid extract (B) as described above. To 0.75 ml. of the remaining neutral extract, 2 ml. of benzyl alcohol saturated with water are added. Thorough extraction of free riboflavin is accomplished by vigorously agitating the covered tube with a device such as that previously described (9) or by very thorough tapping. After centrifuging 5 minutes at 3000 R.P.M., the benzyl alcohol layer is drawn off as completely as possible by suction. The aqueous layer is then extracted with 1 ml. of chloroform saturated with water and centrifuged. (The chloroform extraction removes the last of the benzyl alcohol and clarifies the aqueous phase.) A 0.5 ml. aliquot of the (upper) aqueous layer is transferred to a fluorometer tube and the apparent riboflavin (C) is measured as before.

Calculation—The distribution coefficients for riboflavin, FMN, and FAD between benzyl alcohol and the neutralized trichloroacetic acid extract are 3.8, 0.02, and 0.01 respectively.[1] Thus, when 0.75 ml. of the sample is extracted with 2 ml. of benzyl alcohol, the free riboflavin extracted $= 100 \times (3.8 \times 2)/(0.75 + (3.8 \times 2)) = 91$ per cent, leaving 9 per cent in the aqueous phase. Similarly, it may be calculated that 96 per cent FMN and 98 per cent FAD will be left behind after the benzyl alcohol extraction. The FAD left will, as before, show only 14 per cent as much fluorescence as an equivalent amount of riboflavin. Therefore, if A, B, and C are equal to the apparent riboflavin in the three samples above, A = free riboflavin + FMN + 0.14FAD, B = total riboflavin, and C = 0.09 free riboflavin + 0.96FMN + 0.14FAD.

From these equations it may be readily calculated that

$$\text{FAD} = \frac{B - A}{0.86} \tag{1}$$

$$\text{Free riboflavin} = 1.10A - 1.15C + 0.007B \tag{2}$$

For most purposes the extracted FMN is negligible, in which case free riboflavin $= 1.10(A - C)$.

$$\text{FMN} = B - \text{FAD} - \text{free riboflavin} \tag{3}$$

Determination of Total Riboflavin in Red and White Blood Cells

Isolation of Blood Cells—The method of isolation of white blood cells and platelets by differential centrifugation from 0.1 ml. of blood has been described (8). The red blood cells which remain after removal of the white cell suspension are sufficient for red cell analysis. The supernatant solution remaining after the white cells are drawn off, together with the upper portion of the red cell layer, is discarded. The remaining red cells are packed by centrifuging at 4° for an hour. Any supernatant liquid is re-

moved and an aliquot of red cells is taken with a dry, rather wide tipped constriction pipette, calibrated to contain 20 c.mm. This is rinsed into 1 ml. of 1 per cent NaCl. The suspension may be stored frozen if immediate analysis is inconvenient.

Analysis of White Blood Cells and Platelets. Riboflavin Measurement—The white blood cells from 0.1 ml. of blood are distributed evenly by vigorous agitation of the small tube and deproteinized by mixing thoroughly with 0.11 ml. of 5 per cent trichloroacetic acid. The sample is allowed to stand at room temperature for 30 minutes to 1 hour and mixed again. After centrifuging at 3000 R.P.M. for 10 minutes, 0.1 ml. of the supernatant fluid is transferred to a fluorometer tube and allowed to hydrolyze at 37°. The residue and remaining supernatant fluid are reserved for phosphorus determination, on which the weight of white cells and platelets is based.

The hydrolyzed filtrate is mixed with 0.4 ml. of 0.16 M K_2HPO_4 and the riboflavin is measured as described for serum. Blanks are provided by treating 0.1 ml. aliquots of 5 per cent trichloroacetic acid in the same manner as the white cell filtrate.

Measurement of Acid-Insoluble Phosphorus—The acid-insoluble phosphorus is measured as previously described (8), except for a simplification made possible because of the more dilute filtrate. Instead of washing the precipitate to remove acid-soluble phosphorus, the 10 c.mm. of acid solution which remain with the precipitate are included in the phosphorus analysis. To reduce the volume of fluid that needs to be evaporated, 20 c.mm. of 7 N H_2SO_4 may be conveniently substituted for the larger volume of 4.5 N acid previously recommended for digestion. Digestion of the sample, color development, and measurement of the absorption at 690 mμ are carried out as described (8).

Calculation—The sample analyzed for phosphorus contains all of the acid-insoluble P and, because of the 10 c.mm. of acid extract not removed, 9 per cent of the acid-soluble P. Since white cells contain an average of 33 micromoles of acid-insoluble P and 28 micromoles of acid-soluble P per gm. (8), the sample contains $33 + 0.09 \times 28 = 35.5$ micromoles of P per gm. of white cells in the sample. Therefore, the micrograms of riboflavin per 35.5 micromoles of P found are numerically equal to the micrograms of riboflavin per gm. of white cells, or

$$(1)\quad \text{Micrograms \% riboflavin} = \frac{\text{micrograms riboflavin in entire sample}}{\text{micromoles P found}} \times 100 \times 35.5$$

Since the riboflavin in the entire sample = riboflavin found × 110/100

$$(2)\quad \text{Micrograms \% riboflavin} = \frac{\text{micrograms riboflavin found}}{\text{micromoles P found}} \times 100 \times 35.5 \times \frac{110}{100}$$

$$(3)\quad \text{Micrograms \% riboflavin} = \frac{\text{millimicrograms riboflavin found}}{\text{micromoles P found}} \times 3.9$$

Analysis of Red Blood Cells—The red blood cell suspension (20 c.mm. in 1 ml. of saline) is well mixed with 3 ml. of 13 per cent trichloroacetic acid and allowed to stand 30 to 60 minutes, mixed again, and centrifuged 10 minutes at 3000 R.P.M. The supernatant liquid, which must be clear, is allowed to hydrolyze at 37°. Aliquots of 0.4 ml. are pipetted into fluorometer tubes containing 0.1 ml. of 4 M K_2HPO_4. The reducible fluorescence is measured as described above. The reduced readings must be made promptly after the addition of hydrosulfite, as there is a tendency for partial reoxidation of the sample.

Determination of Total Riboflavin in Whole Blood—Total riboflavin may be determined in whole blood in the same manner as in red cells, except that a less dilute extract is prepared. 20 c.mm. of blood are delivered into 2.0 ml. of 10 per cent trichloroacetic acid. (For accuracy the pipette used is calibrated with blood. It is rinsed with dilute ammonia and dried with acetone between samples.) The mixture is shaken promptly to prevent clumping of the precipitate. The rest of the analysis is performed exactly as with red cells. In both cases, it is important that the acid extract does not remain in contact with the protein precipitate for much over an hour at room temperature, or 2 to 3 hours at 4°.

Recovery of Riboflavin and Flavin-Adenine-Dinucleotide—Riboflavin and FAD were added to human whole blood, serum, and red and white blood cells prior to trichloroacetic acid precipitation. Filtrates were prepared, hydrolyzed, and analyzed, at least in triplicate, by the proposed procedures (Table I). Quantitative recovery was obtained. Similar recoveries have been obtained from rat blood, serum, and cells. Recovery, however, was not complete with lower dilutions than those recommended; *viz.*, 1:100 for whole blood, 1:20 for serum, and 1:200 for red cells. In fact, even at 1:200, recovery was found to be incomplete for red cells if 5 per cent trichloroacetic acid was substituted for 10 per cent.

Blood Level and Reproducibility of Analyses—The riboflavin content of serum, white blood cells plus platelets, and red blood cells from small groups of adult human subjects thought to be well nourished is summarized in Tables II and III. The average free riboflavin plus FMN in serum is 0.8 γ per cent, and total riboflavin is 3.2 γ per cent, with ranges of 0.3 to 1.3 and 2.6 to 3.7 γ per cent, respectively. A difference in individuals either as to nutritional level or other factors seems primarily responsible for this rather wide variation, since the methods of analysis are quite reproducible as shown by an estimated standard deviation for the series of 0.07 γ per cent for both the (free + FMN) and FAD. (Estimated standard deviation = $\sqrt{\Sigma \Delta^2/(2 \times N)}$, where Δ = the difference between duplicate analyses and N = the number of sera analyzed. This statistic is equivalent to the true standard deviation that should result from N replicate analyses of one serum.)

In a series of twenty-six duplicate determinations of total riboflavin in serum, the estimated standard deviation was found to be 0.08 γ per cent.

TABLE I

Recovery of Riboflavin and FAD from Human Serum, Whole Blood, and Red and White Blood Cells

Sample	Total riboflavin (initial)	Substance added	Amount added*	Total riboflavin found†	Recovery
	γ per cent		*γ per cent*	*γ per cent*	*per cent*
Serum	3.9	FAD	13.7	17.6	100
"	4.2	Riboflavin	20.3	24.1	98
White blood cells	262	FAD	381	649	101
" " "	262	Riboflavin	546	812	102
Red blood cells	24.3	FAD	94.8	119.4	101
Whole blood	12.2	"	40.6	53.4	102

* Calculated as riboflavin equivalent.
† Average of triplicate analyses.

TABLE II

*Riboflavin Content of Serum from Well Nourished Adults**

The values are recorded as micrograms per cent of riboflavin.

Test subject No.	Free + FMN†	FAD†	Total
1	0.7	3.0	3.7
2	0.9	2.5	3.4
3	1.2	2.5	3.7
4	0.7	2.1	2.8
5	0.9	2.4	3.3
6	0.8	2.1	2.9
7	0.9	2.6	3.5
8	0.5	2.8	3.3
9	0.3	2.4	2.7
10	0.8	1.8	2.6
11	0.4	2.3	2.7
12	1.3	2.2	3.5
13	0.8	1.8	2.6
Average.....	0.8	2.4	3.2
Range.....	0.3–1.3	1.8–3.0	2.6–3.7

* Riboflavin intake thought to be liberal by present nutritional standards.
† Average of duplicate analyses.

The average total riboflavin content of white blood cells plus platelets, and of red blood cells from twelve adults was found to be 252 and 22.4 γ per cent respectively, with ranges of 227 to 293 and 18.0 to 26.2 γ per cent

(Table III). The mean of the twelve standard deviations calculated on three to five samples from each individual was 11 γ per cent for white cells and 1.3 γ per cent for red cells.

Comparison of the analyses of the upper buffy layers of several specimens containing platelets predominantly with the corresponding layers containing leucocytes predominantly has indicated no difference in the riboflavin content of these fractions of the white cell layers.

Stability of Samples—Whole blood samples may be kept as collected at 4° for 48 hours in the dark without measurable change in the concentration

TABLE III

Riboflavin Content of White Blood Cells and Platelets, and of Red Blood Cells from Well Nourished Adults*

The values are recorded as micrograms per cent of riboflavin.

Test subject No.	White blood cells†	Red blood cells†
1	227	18.8
2	243	18.0
3	236	26.2
4	261	21.3
5	245	22.8
6	264	25.0
7	247	25.2
8	293	19.8
9	253	20.8
10	246	21.3
11	242	26.0
12	262	24.3
Average	252	22.4
Range	227–293	18.0–26.2

* Riboflavin intake thought to be liberal by present nutritional standards.

† Averages of three to five replicate analyses.

of riboflavin compounds in the serum when it is finally separated. Serum and white and red cells may be stored at −40° for periods of several months without change in the riboflavin content, and, in the case of serum, without change in the proportions of riboflavin, FMN, and FAD.

When FAD is added to serum at room temperature, about 80 per cent of it is hydrolyzed in 1 hour. However, under similar conditions, hydrolysis of the FAD originally present in serum is not detectable after many hours. This would indicate the presence of an enzyme in serum which is capable of splitting uncombined FAD, but which is inactive toward FAD combined with protein. FAD is hydrolyzed in 5 per cent trichloroacetic

acid at the rate of about 5 per cent per hour at 0°, 12 per cent per hour at 15°, and 52 per cent at 38°, which indicates the need for prompt neutralization of extracts to be used for FAD analysis.

Preliminary experiments on the hydrolysis of riboflavin derivatives (FAD) extracted by trichloroacetic acid from serum indicate the possibility of replacing the long acid hydrolysis by enzymatic hydrolysis as an analytical convenience. An enzyme concentrate prepared from potato was used.[2] Similar rates of hydrolysis were obtained with a purified yeast FAD and with a serum extract. Approximately the same FAD values were obtained

TABLE IV

Riboflavin in Blood Serum, Red Cells, and White Cells of Rats on Various Daily Intakes of Riboflavin

Riboflavin in diet	No. of rats	Growth rate	Serum				White blood cells	Red blood cells	Urine
			Free riboflavin	FMN riboflavin	FAD riboflavin	Total riboflavin	Total riboflavin	Total riboflavin	Riboflavin
γ per gm.		*gm. per day*	*γ per cent*	*γ per cent*	*γ per cent*	*γ per cent*	*γ per cent*	*γ per cent*	*γ per mg. creatinine**
0.5	6	1.88	0.14	0.23	1.18	1.55	107	9.6	0.18 (2)
		±0.22†	±0.11	±0.07	±0.13	±0.09	±7	±0.4	
1.0	7	2.67	0.28	0.19	1.32	1.78	125	10.8	0.14 (5)
		±0.14	±0.07	±0.05	±0.05	±0.05	±4	±0.8	
1.5	9	3.53	0.54	0.22	1.36	2.12	142	12.1	0.20 (5)
		±0.14	±0.05	±0.03	±0.07	±0.10	±6	±0.9	
2.0	7	3.69	0.82	0.38	1.38	2.58	134	12.5	0.16 (5)
		±0.27	±0.22	±0.06	±0.06	±0.26	±3	±0.8	
3.0	7	4.53	1.59	0.23	1.44	3.27	162	13.6	0.75 (4)
		±0.35	±0.08	±0.05	±0.09	±0.18	±8	±0.9	
10.0	8	4.29	3.79	0.36	1.66	5.82	164	15.6	13.8 (4)
		±0.30	±0.22	±0.05	±0.16	±0.23	±2	±0.8	

* The creatinine output was approximately 0.8 mg. per day per rat, or 4.0 mg. per kilo of rat. The figures in parentheses denote the number of rats on which urine analyses were performed.

† Standard error of the mean.

by both acid and enzymatic hydrolysis, which increases confidence in the specificity of the proposed procedure.

Illustrative Data

Relation between Riboflavin Intake and Blood Levels in Rats—The blood of rats receiving diets containing 0.5, 1.0, 1.5, 2.0, 3.0, and 10.0 γ of riboflavin per gm. of diet was analyzed for riboflavin (Table IV). The growth of these rats was improved by successive addition of riboflavin in the diet

[2] Lowry, O. H., Bessey, O. A., and Love, R. H., in preparation.

up to the 3.0 γ per gm. level. Determinations of free riboflavin, FMN, and FAD were made in duplicate on serum samples. Total riboflavin was measured in white and red blood cells.

The free riboflavin in the serum of rats on the lowest intake level was negligible and increased progressively with each successive increase in the riboflavin of the diet. A marked increase was observed at the highest two levels of intake. In all groups the serum FMN found was very low and was not demonstrably affected by the diet. The FAD in the serum in-

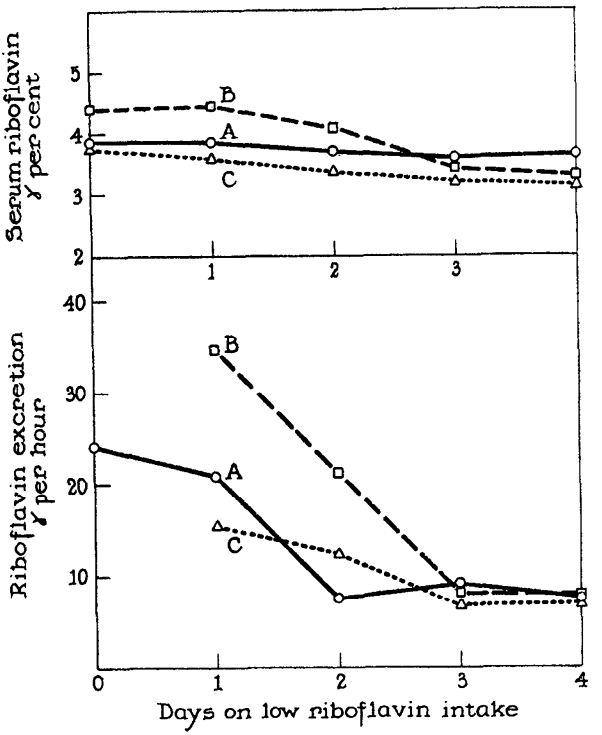

FIG. 1. Serum concentration and urinary excretion of riboflavin of three well nourished human subjects maintained on a low riboflavin diet for 4 days.

creased about 40 per cent from the lowest to the highest intake; *i.e.*, much less than the free riboflavin. Therefore, the changes in the total riboflavin values reflect primarily the changes in the free riboflavin. The group receiving 10.0 γ of riboflavin per gm. of diet had nearly 4 times the total serum riboflavin content of the most deficient group.

The total riboflavin of the white and red cells increased 50 and 60 per cent respectively as the amount of the vitamin in the diet was increased. A comparison of the growth rate in these rats with the blood riboflavin

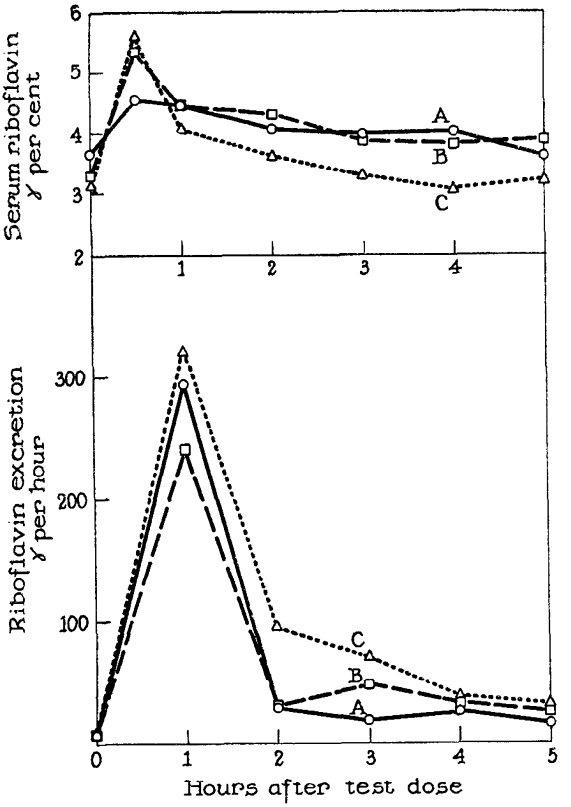

FIG. 2. Serum concentration and urinary excretion of riboflavin of three human subjects following ingestion of 2 mg. of riboflavin.

values (Table IV) suggests possible diagnostic value for the riboflavin measurements. The relation between the serum values and the urinary excretion is of interest.[3]

[3] The rat urine was analyzed as follows: 50 c.mm. of urine buffered with 5 c.mm. of 3.25 M sodium acetate-acetic acid (pH 4.6) were oxidized with 10 c.mm. of 4 per cent $KMnO_4$ for 1 minute. Oxidation was stopped by the addition of 2 c.mm. of 30 per cent H_2O_2. 50 mg. of $(NH_4)_2SO_4$ (previously washed with 95 per cent alcohol and ether to remove fluorescent substances) and 40 c.mm. of benzyl alcohol saturated with water were added. Extraction of the riboflavin was accomplished by vigorous shaking of the tube (9). After centrifuging, 20 c.mm. of the benzyl alcohol layer were pipetted into 0.5 ml. of 45 per cent ethyl alcohol which was 0.1 N in acetic acid and 0.1 N in sodium acetate. The riboflavin was measured as reducible fluorescence compared with an appropriate internal standard in the microfluorometer. The urine from human subjects was similarly analyzed on a macro scale with 4 ml. of urine plus 0.4 ml. of 3.25 M buffer, 1 ml. of $KMnO_4$, 0.1 ml. of H_2O_2, 4 gm. of $(NH_4)_2SO_4$, and 3 ml. of benzyl alcohol, of which 1 or 2 ml. were pipetted into 7 ml.

Comparison of Serum and Urinary Levels of Riboflavin in Man—Three human subjects restricted their riboflavin intake for 4 days to a level of about 0.4 mg. per day. The total riboflavin in the serum dropped only 8, 16, and 25 per cent from the initial levels for the three subjects, whereas the urinary excretion (5 hour collection period) decreased to a fourth to a half of the initial rate[3] (Fig. 1).

The morning of the 5th day, each person received 2 mg. of riboflavin with 600 ml. of water and a few crackers. Serum samples were collected from the finger after 30 minutes and both serum and urine samples were taken at hourly intervals thereafter. The greatest increase in serum riboflavin occurred at 30 minutes (24 to 75 per cent, Fig. 2). An abrupt decrease had already occurred within an hour following the large dose. Thereafter, the serum levels promptly returned to approximately initial values. The increase in urinary riboflavin output was relatively much greater (30- to 45-fold during the 1st hour) and more prolonged than the changes in the serum concentrations. This greater stability of the serum riboflavin would, however, be of diagnostic value only if the levels in the serum reflect the nutritional status as they seem to in the rat. Unpublished data on a few deficient subjects studied with Dr. L. Emmett Holt, Jr., suggest that the total riboflavin in man may be too stable to be of value, but that the free serum riboflavin may prove useful as a measure of nutritional status.

SUMMARY

Methods are described for the measurement of (1) both free and total riboflavin in 50 c.mm. of blood serum (25 c.mm. for total alone), (2) free riboflavin and flavin-mono- and dinucleotide as separate fractions in 0.2 ml. of serum, (3) total riboflavin in 20 c.mm. of red cells or whole blood, and (4) total riboflavin in white cells and platelets from 0.1 ml. of blood. Determinations show a standard deviation of 0.08 γ per cent on serum, 11 γ per cent on white cells, and 1.3 γ per cent on red cells. Two analysts can perform 75 to 100 riboflavin analyses on serum in 2 days, or twenty-five to 50 on red and white cells.

In thirteen adult human sera, from presumably adequately nourished subjects, the average free riboflavin plus FMN was 0.8 and the FAD 2.4 γ per cent; in the white cells and platelets and in the red cells of twelve of these subjects, the average riboflavin content was 252 and 22.4 γ per cent respectively.

Data for the rat are reported on the relationship between the riboflavin in the diet and the riboflavin of the serum, red cells, white cells, and urine.

of the 45 per cent alcohol buffer for measurement of the reducible fluorescence. The oxidation and extraction were designed to avoid interference from reducible fluorescent substances which are oxidized or which are more water-soluble than riboflavin.

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