URINARY EXCRETION OF RIBOFLAVIN OF COLLEGE WOMEN ON A SELF-CHOSEN DIET AND THE RESPONSE TO A SATURATION DOSE OF RIBOFLAVIN AND THIAMINE

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Ruth Louise Ingalls

1945

THESIS

This is to certify that the

thesis entitled

Urinary Excretion of Riboflavin of College Women on a Self-Chosen Diet and the Response to a Saturation Dose of Riboflavin and Thiamine.

presented by

Ruth Louise Ingalls

has been accepted towards fulfilment of the requirements for

M. S. degree in Foods and Nutrition

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Date September 1, 1945.

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by

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A THESIS

Submitted to the Graduate School of Michigan State College of Agriculture and Applied Science in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Department of Foods and Nutrition

1945

The writer wishes to express her appreciation to Dr. Margaret A. Ohlson for her interest, supervision and helpful suggestions, to Miss Wilma D. Brewer for her valuable assistance, encouragement and advice throughout the study, to Dr. W. D. Baten for help with the statistical analysis of the data and to all the students for their willing cooperation in the study.

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INTRODUCTION

Riboflavin has been known as an essential dietary factor for a comparatively short time. Though early seen as a fluorescent substance in whey (Blyth, 1879), recognition of this vitamin as part of the B complex came within the last decade (György, '35). Now it is conceded that riboflavin functions in the oxidation and reduction system as a catalyst in the energy transfer of phosphorylated hexoses (Quastrel and Webley, '41).

The importance of the vitamin to man became evident when symptoms were observed which were due directly to a dietary deficiency of riboflavin (Sebrell and Butler, '39). This has led to investigations of man's requirement of riboflavin and to experimental studies attempting to measure deficiency and saturation as evidence of riboflavin status (Axelrod, Spies and Elvehjem, '43). These studies were given new impetus when the National Research Council in 1941 attempted to establish standards of vitamin requirement.

Urinary excretion of riboflavin has been used as an index to riboflavin nutrition. The riboflavin excreted in a 24-hour urine sample has been used generally as the criterion of riboflavin status (Emmerie, '36; Terrebee, '41;

Williams, and others, '43), but the response to a saturation dose of riboflavin has been found more satisfactory in some cases (György, '42). The micrograms of riboflavin excreted per milliliter of urine in the one-hour sample after a night of fasting has been found by one investigator (Feder, Lewis and Alden, '44) to give good agreement with the micrograms excreted per milliliter during 24 hours.

The purpose of this study was to determine the range of daily riboflavin excretion of college girls on a self-chosen diet, the correlation of the 24-hour sample with a one-hour fasting sample, and the response of each girl to a saturation dose of riboflavin. Since thiamine and riboflavin are thought to be closely related in animal metabolism, the effect of a saturation dose given simultaneously as it affects the riboflavin excretion was studied. Another investigator from this laboratory will report on thiamine excretion and the response to a saturation dose of thiamine.

LITERATURE

Riboflavin Excretion Studies

The excretion of riboflavin does not depend directly on intake, but is complicated by many factors. It has been demonstrated that urinary excretion parallels intake (Sebrell, and others, '41; Williams and co-workers, '43; Najjar, Holt and others, '44) and that fecal excretion remains nearly constant at varying intakes (Najjar and others,'44). The total urinary and fecal excretions, however, plus the small amount excreted in perspiration (Tennent and Silber, '43) normally represents only part of the total intake. Sebrell and others (1941) in their studies with humans noticed that with increasing riboflavin intakes there was an increase in the amount of riboflavin not accounted for when the amount excreted was totaled. This was more pronounced at the higher intakes. That is, the riboflavin which could not be measured in the excretions was much more at an intake of 0.11 milligram per kilogram than at 0.085 milligram per kilogram. Each additional dietary increment was accompanied by a greater proportion of unmeasured riboflavin. Even at 0.025 milligram per kilogram intake there was some unaccounted loss.

Sure and Ford (1943) fed rats 1000 micrograms riboflavin and thiamine for 30 days. At the end of that time,

the bodies of the animals were assayed for these vitamins. The entire body of an animal contained only 783 micrograms riboflavin. On the same intake the daily excretion of the animal showed a utilization of 76 to 88 per cent of the amount ingested. In vitro studies made at the same time demonstrated a destruction of riboflavin in tissues (liver, heart, lung, stomach, large and small intestine). When 1000 micrograms riboflavin were incubated with these tissues at 37° centigrade for 24 to 48 hours, three to 28 per cent destruction occurred. These workers concluded that there is destruction of riboflavin by the tissues, perhaps after the tissue has made use of the vitamin.

Another factor to be considered when measuring riboflavin excretion is the possibility of bacterial synthesis. Najjar and others (1944) showed that bacterial synthesis does occur in the large intestine and at times may be enough to lower dietary requirement.

When urinary excretion is used as an index of riboflavin status, the loss of part of the intake through tissue destruction and the augmentation of the vitamin supply through bacterial synthesis should be remembered. Other factors, such as poor absorption, the effect of impaired kidney function, and the influence of the other vitamins or of any of the nutrients also may have a bearing on the problem (Seyle, '43; Unna and others, '44; Mannering, Orsini and Elvehjem, '44).

Thus, the desirability of using urinary excretion

alone to measure the adequacy of riboflavin in the diet of the individual has been questioned. Axelrod, Spies and Elvehjem (1941) found that the blood of twenty normal individuals contained an average of 0.42 micrograms riboflavin per milliliter of blood while the blood of deficient patients contained 0.40 micrograms per milliliter. Strong, Feeney and Moore (1941) found human blood to contain 0.5 micrograms per milliliter. Majjar and others (1944) found that fecal excretion of riboflavin remained at 200 to 600 micrograms per day even when riboflavin was injected. These findings lead to the conclusion that riboflavin status cannot be measured by analysis of fecal excretion of riboflavin or of riboflavin concentration in the blood.

The early investigations of riboflavin status were made using individuals with what appeared to be marked deficiency. Oden, Oden and Sebrell (1939) noted cases described as ariboflavinosis and recorded the symptoms. Axelrod, Spies and Elvehjem (1941) also described riboflavin deficiency in hospital patients. Both investigators cured the syndrome with a therapeutic dose of three to five milligrams of riboflavin. Sydenstricker, Kruse and coworkers (1940) cured certain ocular changes with four to 10 milligrams of riboflavin. Sanstead (1942) failed to effect a cure with the same dosage. Recently, Machella and McDonald (1943) attempted to remove symptoms ascribed to ariboflavinosis by riboflavin therapy and failed. The work with cases of extreme deficiency symptoms probably is not

comparable to what has been described as borderline deficiency.

Since urinary excretion follows intake closely, the individual's daily urinary excretion may be used as a measure of dietary intake (Williams and co-workers, '43). The amount excreted varies from day to day with variations of intake but, in general, remains within a certain range for the individual. This range of excretion should give an indication of the adequacy of riboflavin in that individual's diet. It also has been found (Feder, Lewis and Alden, '44; Oldham and others, '44) that the riboflavin in a one-hour fasting sample and the four-hour and the 24-hour samples after a saturation dose may be used as a measure of nutritional status in regard to riboflavin. Excretion of less than 0.3 microgram of riboflavin in the one-hour sample or of less than 35 per cent of the saturation dose was interpreted to indicate deficiency.

As early as 1936 an attempt was made to find the range of urinary excretion of riboflavin in apparently normal individuals when Emmerie reported the average daily excretion of males to be 819 to 1250 micrograms per 24 hours. Helmer (1937), using the rat-assay method, estimated excretions of 120 to 175 Sherman-Bourquin units or 360 to 875 micrograms per day. The methods used were questionable but the findings are interesting when compared with more recent data. Ferrebee (1940) found the range of excretion to be 700 to 1700 micrograms. Axelrod, Spies and Elvehjem (1941)

found 477 to 835 micrograms or an average of 625 micrograms excreted by two patients on a regular hospital diet. At the same time Sebrell and others (1941) found the range of excretion in eleven young men to be 234 to 1740 micrograms daily. Strong, Feeney, Moore and Parsons (1941) found 500 to 800 micrograms excreted by women. Klopp, Abels and Rhoads (1943) found 210 to 1200 micrograms per 24-hour urine sample. Williams and others (1943) recorded the range of urinary excretion compared directly with intake. When riboflavin intake was one milligram per day or less, 150 to 350 micrograms were excreted. At an intake of 1.6 milligrams per day excretion was raised to 400 to 700 micrograms. When 3.6 milligrams was the total intake (diet plus supplementary administration of the vitamin), the excretion was 1600 to 1800 micrograms. Feder, Lewis and Alden (1944) stated that consideration of these data sets the range of urinary excretion at 500 to 1000 micrograms with an average of 800 micrograms when the riboflavin intake meets the National Research Council standard of two to three milligrams per day. These data are summarized in table 1.

Some investigators have used the one-hour fasting sample rather than the 24-hour sample as a measure of riboflavin status. Feder, Lewis and Alden (1944) found that the micrograms per milliliter in the fasting hour sample agreed well with the per milliliter excretion in the corresponding 24-hour sample. Feder also stated that when

TABLE 1

Range of urinary excretion of riboflavin reported in the literature as normal

Investigator	RANGE OF RIBOFLAVIN EXCRETION		PER CENT RESPONSE TO SATURATION			Comment
	24-Hour	One-Hour fasting	1 hour	4 hours	24 hours	
	µg.	µg.				
Emmerie, '36	819-1250	30-50	---	---	---	One hour excretion was not taken in fasting state
Helmer, '37	360-875	---	---	---	---	---
Najjar, '41	---	6-49	---	28-68	---	Saturation dose of one milligram injected
Ferrebee, '41	700-1700	---	---	---	---	Study on young men
Axelrod, Spies and Elvehjem, '41	477-835 625 average	---	---	---	---	Observation on hospital patients
Sebrell, '41	234-1740	---	---	---	---	Studies on hospital patients
Strong, Feeney, Moore, Parsons,'41	500-800	---	---	---	---	---
Klopp, Abels, Rhoades, '43	210-1200	---	---	---	---	Studies on hospital patients
Williams, '43	150-1800	---	---	---	---	Excretion regulated by controlled intake
Ferrebee and Weismann, '43	---	---	30-35	---	---	Saturation dose of one milligram riboflavin injected
Feder, '44	500-1000 800 average	1.4-114.4 or 0-1.77 per ml.	---	---	35	Saturation dose injected intramuscularly
Oldham, et al.,'44	---	---	---	---	23-32	Study with children

riboflavin excretion falls below 0.3 micrograms per milliliter in the fasting hour, the individual may be considered deficient. The values found in that study for the fasting hour sample range from 0.00 to 1.77 micrograms per milliliter. The range in total micrograms per hour was 1.4 to 114.4 micrograms for four subjects. Najjar and Holt (1941) found six to 49 micrograms excreted during the fasting hour.

The excretion of a saturation dose of riboflavin demonstrates the ability of the body to excrete any excess of the vitamin. It is thought that the amount of the test dose excreted depends on the individual's need for the vitamin. The amount of the test dose has varied as has the method of administration. Klein and Kohn (1941) found 25 per cent of two milligrams of riboflavin excreted. Axelrod, Spies and Elvehjem (1941) injected 200 to 400 micrograms riboflavin per kilogram body weight intravenously and found no correlation between the per cent excreted by normal and by deficient subjects. Najjar and Holt (1941) injected one milligram intravenously. Individuals having an adequate dietary intake retained 32 to 72 per cent of the test dose at the end of the four-hour period, while deficient individuals retained 81 to 93 per cent. Ferrebee and Weisman (1943) injected one milligram riboflavin and one milligram thiamine. They reported 30 to 35 per cent of the test dose of riboflavin excreted in one hour and values for the two, three and 24-hour response in agreement with the

data of Najjar and Holt. Feder and co-workers injected 0.016 milligram per kilogram by the intramuscular route and found that an excretion of less than 35 per cent of the test dose indicated deficiency. Oldham and co-workers (1944) gave a test dose of 75 micrograms per kilogram orally to two normal children and found 23 to 32 per cent respectively excreted in 24 hours.

In summary of these studies, the 24-hour excretion of riboflavin by normal subjects has been found to average 500 to 1000 micrograms. The amount found in the one-hour fasting sample varies widely, but less than 0.3 micrograms per milliliter seems to indicate low storage of the vitamin, and less than 25 to 35 per cent excretion of an injected saturation dose has been interpreted to denote the same thing. However, the application of the last figure to this study is uncertain, since the only response to an oral test dose reported in the above literature was in the study with children.

Very little is known of the relationships existing between the vitamins of the B complex. There has been some study of the interrelationship between thiamine and riboflavin. This work has been chiefly with animals. Sure (1944, 1945) noted that riboflavin excretion in rats is increased two to three times in chronic thiamine deficiency, but found no difference in riboflavin content of tissues or in absorption. Singher, and others (1944) found a 50 per cent increase in livers of thiamine-deficient animals.

Sure (1945) maintained this to be transient riboflavin found during digestion and absorption. Ferrebee and Weissman (1943) could find no difference in the riboflavin excretion of human subjects with thiamine deficiency. Following an injection of one milligram of each vitamin the riboflavin response was similar to the response reported by Najjar and Holt (1941), who injected only riboflavin. Klopp, Abels and Rhoads (1943) found an increased excretion of riboflavin in human subjects after an injection of thiamine. Continued daily injections, however, did not produce a riboflavin deficiency.

At the present time the analysis of riboflavin excretion in the urine appears the most satisfactory method of judging riboflavin status. The range of riboflavin excretion in 24 hours, the amount of riboflavin excreted in a one-hour fasting sample, the response to a saturation dose of the vitamin, the effect of a saturation dose of thiamine on this response, and the existing relationships between these factors have not been established conclusively. This study has attempted to correlate these scattered data by the investigation of all these techniques within one experiment.

Evaluation of Chemical Method

The choice of a method for any routine analysis depends on the accuracy and convenience of administration of

that method. In vitamin assay there are three types of method used, biological, microbiological and chemical. The last two of these have been used successfully in analyses of riboflavin in the urine. The microbiological method of Snell and Strong (1939) has been used by many workers (Axelrod, Spies and Elvehjem, '41; Strong, Feeney, Moore and Parsons, '41; Oldham and co-workers, '44). The results of the fluorometric method of riboflavin determination compared well with the microbiological (Najjar and Holt, '41).

The fluorometric method depends on the fact that pure riboflavin in aqueous solution shows a yellow-green fluorescence which can be measured in a fluorophotometer. It has been used for the estimation of riboflavin in food (Hand, '39; Hodson and Norris, '39; Connor and Straub, '41) and in urine(Najjar and Holt, '41; Ferrebee, '40; Sure and Ford, '42; Keys and co-workers, '44). The accuracy of this method depends on the removal of certain interfering factors, so that the fluorescence measured will be due to riboflavin alone. Some of these interfering factors were found to be the pigments in the urine, turbidity of the solution, the formation of gaseous emulsions during the extraction process and the scattering of light rays through the solution (Najjar and Holt, '41).

The pigments found in normal urine are urochrome, urobilin and uroerythrin. The last of these is non-fluorescent. The turbidity of urine is due to a precipitation of calcium phosphate in urine voided directly after a

meal, also to an alkalinity resulting from the decomposition of urea to form ammonia. Other sedimentary substances also are present in varying amounts (Hawk and Bergeim, 1942). Some of these are easily filtered out of the urine, but some remain in the sample. Various methods have been used to remove these substances. Sure and Ford (1942), using a modification of the method given by Hodson and Morris (1939), oxidized the interfering substances in the filtered urine sample with potassium permanganate and subtracted a blank reading from the sample.

Several workers have used the adsorption technique to remove the pigment and to concentrate the riboflavin in urines of low value. Emmett and McKim (1917) found that fuller's earth adsorbed the vitamin fraction of yeast. Narayanan and Drummond (1930) and Salmon and co-workers (1928) also showed that fuller's earth adsorbed certain of the B vitamins. Supplee, Bender and Jensen (1939) and Conner and Straub (1941) demonstrated the use of adsorption in the estimation of riboflavin in food. Ferrebee (1940), Najjar and Holt (1941), and Keys (1944) have used it in the riboflavin analysis of urine.

Since all interfering substances are not removed by adsorption and by oxidation with potassium permanganate, a blank reading has been found advisable. The blank has been obtained by various methods. Sure and Ford (1942) reduced the riboflavin in the sample with sodium hydrosulfite after the sample reading had been taken. This reduces the ribo-

flavin to a non-fluorescent leuco form which may be re-oxidized by atmospheric oxygen (Hodson and Morris, '42), thus introducing an error in the blank reading (Najjar and Holt, '41). Ferrebee (1940) used a constant blank obtained by passing pyridine-acetic acid solution through the adsorption columns and then treating this solution as a sample. Najjar and Holt (1941) prepared blanks by exposure of the sample or of a duplicate sample to light, using either a mercury vapor lamp or direct sunlight for one to two hours. Keys (1944) irradiated a duplicate of each sample for one to two hours under a mercury vapor lamp at an acid pH. DeMerre and Brown (1944) found complete destruction of riboflavin at all pH values upon exposure of the solution to strong daylight.

The adsorption technique of Ferrebee with the modifications suggested by Keys appear to eliminate most of the interfering substances in urine and to give accurate results as judged by the recovery of added riboflavin.

EXPERIMENTAL PROCEDURE

Subjects

The urinary excretion of riboflavin of 20 junior and senior college women was studied. The subjects ate their customary dict obtained at the college dormitories or at the Home Management Houses. All the subjects were in apparent good physical health at the time of the study. The importance of accuracy in making collections was impressed on the subjects; each kept a record of time of collection, noting any error made in collection of a sample, and reported regularly to the laboratory. Each subject kept a record of her diet during the five-day collection period. This explained any marked deviation in riboflavin excretion and at the same time gave a pattern of the subject's dietary habits. In addition, a history of past illnesses, food likes and dislikes and general information concerning the health and habits of the subject were obtained.

The length of the experimental period was five days for each subject. This was divided into two periods, a pre-saturation period and a period of saturation response. Twenty-four hour urine samples were collected for three days. On the fourth day each subject was instructed to drink a glass (200 cc.) water on arising. At the end of an hour a fasting urine sample was obtained. At this time the subject was given three milligrams riboflavin and three

milligrams thiamine in aqueous solution, orally, as a saturation test dose. Urine was collected for the four hours after the saturation dose and kept separate from the collection for the remaining 19 hours. The urine for the following 24 hours also was colledted in order to observe any delayed response to the saturation dose.

Collections were made directly into amber jars with screw tops containing five milliliters glacial acetic acid as a preservative. The subjects were instructed to keep the containers in the dark and away from radiators. Each sample was returned to the laboratory and refrigerated as soon as possible after each period.

Chemical Method

Each 24-hour collection was measured, made up to a volume of 2000 milliliters (2500 if the original volume was over 2000 milliliters), mixed well and a filtered aliquot of 150 milliliters saved. The three pre-saturation aliquots were combined and analysis of this composite made. Trial analysis showed that urinary riboflavin was not destroyed by this procedure (Table 2). Two types of experiments were tried. In the first, 24-hour urine samples for three successive days were analyzed separately for riboflavin and on the third day a composite of the three days' samples was analyzed. In the second type of experiment a single day's urine sample was analyzed at the end of the 24-hour period and again three days after the collection. There was no

TABLE 2

Stability of urinary riboflavin

Experiment 1. Analyses of three successive 24-hour urine samples and the three-day composite of these samples

Sample	Riboflavin excretion
	µg./24 hrs.
First day	1610.4
Second day	1434.0
Third day	1250.0
Average	1431.5
Three-day composite	1397.0
Per cent difference	-2.4

Experiment 2. Analyses of same urine sample at end of 24-hour period and after three days

Subject	I	II	III
µg. riboflavin at end of 24-hour collection	153.5	832.6	366.6
µg. riboflavin in same sample after 3 days	161.0	812.9	350.0
Per cent difference	+5.2	-2.4	-4.5

loss of riboflavin after storage of the urine sample.

The collections for the other periods were measured and made to volume as follows:

One-hour fasting sample ------ to 100 milliliter

Four-hour saturation period -- to 1000 milliliter

Nineteen-hour period --------- to 2000 milliliter

Twenty-four hour period after saturation --- volume recorded and sample used without dilution to any given volume.

This dilution made the urine concentration of all subjects comparable and facilitated judgment of the size of the sample taken for analysis.

The size of the aliquot taken for analysis depends on the amount of riboflavin expected in the urine sample. Three to 15 milliliters filtered urine were pipetted into a 50-milliliter beaker and made to a volume of 20 milliliters with distilled water. The sample was adjusted to pH6 with sodium hydroxide, using Nitrazine paper as indicator. Adsorption columns were prepared with activated florisil and the urine sample passed through. The beakers were rinsed twice with warm distilled water and these rinsings passed through the columns also. The riboflavin was eluted with 30 to 35 milliliters of an aqueous solution of 20 per cent pyridine and two per cent glacial acetic acid, the eluate collected into 50 milliliter volumetric flasks and made to volume with the pyridine-acetic acid solution. A 15-milliliter aliquot of this eluate was pipetted into a 25-milliliter volumetric flask, one milliliter of four per

cent potassium permanganate solution added to oxidize interfering substances and the flask shaken for three minutes. The solution was decolorized with a three per cent hydrogen peroxide solution, usually one milliliter being needed. The sample was made to volume with the pyridine-acetic acid solution and was ready to be read in the photo-electric fluorescence meter.

Correction for interfering fluorescent substances was made by using a separate blank for each sample and reading this blank against its duplicate sample. A duplicate aliquot of each sample was pipetted into a 250-milliliter beaker, the volume made to 20 milliliters and the beaker placed under a mercury vapor lamp for two hours to destroy the riboflavin (Keys, '44; DeMerre and Brown, '44). The fluorescence which remained was due to the interfering substances. After the two hours any loss in volume due to evaporation was replaced with distilled water. The blank was then made to pH6, passed through the adsorption columns and treated in the same manner as the sample.

A reagent blank was prepared by passing 30 to 35 milliliters of the pyridine-acetic acid solution through an adsorption column, making to a volume of 50 milliliters with the same solution and treating like the samples.

All samples and blanks were read in a photo-electric colorimeter equipped with a mercury vapor lamp (Lumetron). The instrument was standardized at 100 with a solution of pure riboflavin containing 0.1 gamma per milliliter. The

zero setting of the instrument was made against the reagent blank to suppress any incidental fluorescence from the reagents. Figures in the slide dial were in logarithmic relationship so that readings were directly in terms of percentage concentration of the standard solutions. The micrograms riboflavin for the total volume were calculated using this formula:

$$\frac{\text{Final reading} \times 50(\text{volume of eluate}) \times 25(\text{final volume})}{\text{Aliquot of urine sample} \times 15(\text{milliliters of eluate})} \times \text{total volume} = \text{total micrograms riboflavin}$$

RESULTS

Pre-Saturation Period

The range of daily urinary excretion of riboflavin of the twenty subjects during the pre-saturation period of three days was 58.2 to 1030.0 micrograms (Table 3). The average excretion was 446.1 ± 238.7 micrograms (Table 5). Since this range was so wide with a few subjects at the two extremes in excretion, an attempt was made to find a more representative range of excretion for this group of college girls. When the data were grouped and the figures for the highest and the lowest excretions eliminated, the range of riboflavin excreted by 14 subjects was 250 to 750 micrograms per day (Table 6). The range in micrograms of riboflavin per milliliter of urine excreted during this period was 0.078 to 0.757 micrograms (Table 4) with an average excretion of 0.444 ± 0.213 micrograms per milliliter (Table 5).

The range of urinary riboflavin excreted during the fasting hour was 0.4 to 37.8 micrograms (Table 3) with an average of 12.7 ± 10.8 micrograms. The micrograms of riboflavin per milliliter of urine excreted during the fasting hour ranged from 0.023 to 1.686 micrograms (Table 4) with an average excretion of 0.488 ± 0.263 micrograms. The correlation between the average total excretion for this fasting hour and for the previous 24-hour periods was not

TABLE 3

Urinary excretions of riboflavin by college women

Subject	PRE-SATURATION EXCRETION		POST-SATURATION EXCRETION				
	Average daily	One-hour fasting	1-4 hours after saturation	Per cent excreted in 4 hours	4-24 hours after saturation	Per cent excreted in 24 hours	24-48 hours after saturation
	µg.	µg.	µg.		µg.		µg.
HH	1030.0	23.6	1717.0	31.6	2305.2	42.5	564.6
AB	833.3	11.3	1223.2	21.3	1742.9	30.3	629.9
FR	666.6	10.1	335.2	5.6	986.9	10.7	611.0
GH	649.9	no sample	656.5	12.8	1487.6	27.9	474.0
ER	576.3	37.8	877.8	19.0	1638.1	35.4	546.6
LM	563.8	27.9	777.8	20.4	2647.3	69.5	813.6
JC	516.3	8.9	764.6	13.8	1277.6	25.3	279.1
JG	499.9	16.6	912.5	21.2	1641.6	38.1	662.4
JF	472.2	25.2	1568.7	40.6	1917.4	49.6	781.0
MD	468.3	11.2	1098.7	26.7	1730.7	42.0	537.4
MW	466.6	5.8	1162.5	28.5	1618.3	39.1	966.6
BJ	437.9	11.3	489.5	10.2	1167.4	24.3	549.0
RN	415.5	22.2	263.9	4.5	866.2	14.7	333.4
KB	338.2	11.0	1084.0	29.9	1707.4	47.1	260.5
BT	320.8	8.8	538.8	11.9	955.9	21.1	429.6
MP	266.6	4.8	352.7	6.9	721.5	15.2	275.0
MK	209.6	10.6	547.0	14.9	1157.3	31.6	625.0
FF	159.7	0.8	424.0	9.8	524.8	12.1	70.9
LH	95.5	6.5	543.1	16.4	850.6	25.2	274.0
IO	58.2	0.4	459.5	13.1	668.0	19.1	58.4
Average	446.1 ±238.7*	12.7 ±10.8	789.8 ±131.7	17.9	1380.6 ±580.6	31.0	487.1 ±240.0

*Standard deviation of the items from the mean.

TABLE 4

Urinary excretions of riboflavin*

Subject	PRE-SATURATION EXCRETION		POST-SATURATION EXCRETION		
	Daily average	One-hour fasting	1-4 hours after saturation	4-24 hours after saturation	24-48 hours after saturation
	μg./ml.	μg./ml.	μg./ml.	μg./ml.	μg./ml.
HH.	0.756	1.686	2.516	0.849	0.482
AB	0.737	0.753	2.005	0.847	0.632
FR	0.591	0.055	0.972	0.980	0.568
GH	0.670	no sample	1.026	0.902	0.389
ER	0.356	0.266	1.015	0.556	0.309
LM	0.560	0.821	1.353	4.332	0.768
JC	0.757	0.746	4.749	0.528	0.396
JG	0.600	0.722	1.383	0.843	1.019
JF	0.310	0.927	1.318	0.434	0.514
MD	0.423	0.509	1.066	0.906	0.625
MW	0.453	0.242	2.801	0.667	1.080
BJ	0.502	1.410	0.906	0.595	0.409
RN	0.436	0.067	0.497	0.482	0.351
KB	0.330	0.314	2.331	0.957	0.431
BT	0.390	0.213	0.547	0.454	0.367
MP	0.503	0.098	1.764	1.156	0.618
MK	0.194	0.707	1.609	0.833	0.601
FF	0.142	0.023	0.689	0.194	0.078
LH	0.083	0.158	0.735	0.303	0.188
IO	0.078	0.040	2.137	0.221	0.068
Average	0.444	0.488	1.571	0.852	0.495

*Micrograms of riboflavin excreted per milliliter of urine.

TABLE 5

Summary of average riboflavin excretions

Period	Micrograms excreted during period		Micrograms excreted per milliliter during period	
	Average	S.D.	Average	S.D.
	µg./24 hrs.		µg./ml.	
24-hour excretion before saturation	446.1	±238.7	0.444	±0.213
One-hour fasting excretion	12.7	±10.8	0.488	±0.263
Four-hour excretion after saturation	789.8	±131.7	1.571	±1.003
24-hour excretion after saturation	1380.6	±560.6	0.852	±0.86
48-hour excretion after saturation	487.1	±240.0	0.495	±0.477

TABLE 6

Range of riboflavin excretion found by grouping the data

Range in 24-hour period before saturation

Group	Range	Frequency	Average within group
	µg.		µg.
I	750-999	2	931.6
II	500-749	6	578.0
III	250-499	8	398.0
IV	0-249	4	137.7
		Average	449.0 ±215.0 µg.

Range in 24-hour period of fifth day

Group	Range	Frequency	Average within group
	µg.		µg.
I	750-999	3	853.7
II	500-749	8	595.7
III	250-499	7	332.1
IV	0-249	2	64.2
		Average	524.0 ±166.5 µg.

significant (r = 0.34) for the number of samples studied. The lowest correlation at which significance can be assumed for this size of sample (19) is 0.37. The correlation of 0.34 found in the comparison of the one-hour fasting sample and the preliminary 24-hour urine sample is very near to a significant figure. When the average micrograms of riboflavin per milliliter excreted during the fasting hour and the pre-saturation 24-hour periods were compared, there was good correlation (r = 0.53). There was further confirmation of this relationship when no significant difference (t = 0.99, D.F. = 18) was found between the two averages, the average micrograms of riboflavin excreted per milliliter of urine during the one-hour and during the 24-hour periods. There was no correlation between the volume of urine and the micrograms of riboflavin excreted during the one-hour fasting period.

Saturation Period

During the four hours after the three milligram test dose of riboflavin was given, the range of excretion was 263.9 to 1717.0 micrograms of riboflavin with an average of 789.8 ± 131.7 micrograms. This represented 3.6 to 40.6 per cent, or an average of 17.9 per cent, of the three milligram test dose excreted in the four hours (Table 3).

At the end of the first 24 hours after the test dose the total riboflavin excretion ranged from 524.6 to 2647.3 micrograms, an average of 1380.6 ± 580.6 micrograms. The fraction of the test dose that had been excreted by the end

of 24 hours was 10.7 to 69.5 per cent or an average of 31 per cent (Table 3). The second 24-hour excretion immediately after the test dose gave a high correlation ($r = 0.68$) with the 24-hour average of the presaturation period suggesting that the test dose was excreted within the first 24 hours.

Thus, during the fifth day, which was the second 24 hours after the administration of the test dose, the urinary excretion of riboflavin appeared to have returned to the range found during the 24-hour period before the test dose was given. The range of riboflavin excretion of the fifth day of the experimental period was 58.4 to 966.6 micrograms (Table 3) with an average of 487.1 ± 240.0 micrograms (Table 5). This average was not significantly different ($t = 0.55$, D.F. = 19) from the average excretion of the pre-saturation period. Fifteen of the subjects excreted between 250 to 750 micrograms, or an average of 524.0 ± 166.5 micrograms of riboflavin during this period (Table 6). The micrograms of riboflavin per milliliter of urine excreted during this day ranged from 0.068 to 1.080 micrograms with an average of 0.495 ± 0.477 micrograms. This average was not significantly different ($t = 0.55$, D.F. = 19) from the average micrograms per milliliter of the pre-saturation period, though there was some individual variation.

Dietary Study

The dietary intakes of protein, thiamine and riboflavin were calculated from food consumption records (Short method, Reynolds,'44). An average of the dietary intake of each subject for one day was obtained from the intake of the five-day experimental period. This average was used as an indication of the subject's dietary habits. The average intake of protein was 58.2 grams and for thiamine was 1.056 milligrams. Riboflavin intake varied from 1.03 to 2.2 milligrams, or an average of 1.55 ± 0.339 milligrams per day (Table 7). The protein and thiamine in the individual diet appeared to have no relationship to the amount of riboflavin. The correlation between the number of glasses of milk in the diet and the riboflavin excreted in the preliminary period was high ($r = 0.668$). This seemed to indicate that milk was an important constituent of the diet in determining the amount of riboflavin excretion. There was good correlation ($r = 0.525$) between dietary intake of riboflavin and the micrograms of riboflavin excreted in the 24-hour period before the test dose (Figure 1). There was possibly some relationship between dietary intake of riboflavin and the percentage of the test dose excreted during the first 24 hours after the administration of the test dose (Figure 2).

Relationship between Riboflavin and Thiamine

No effect on riboflavin excretion due to the administration of three milligrams of thiamine could be observed.

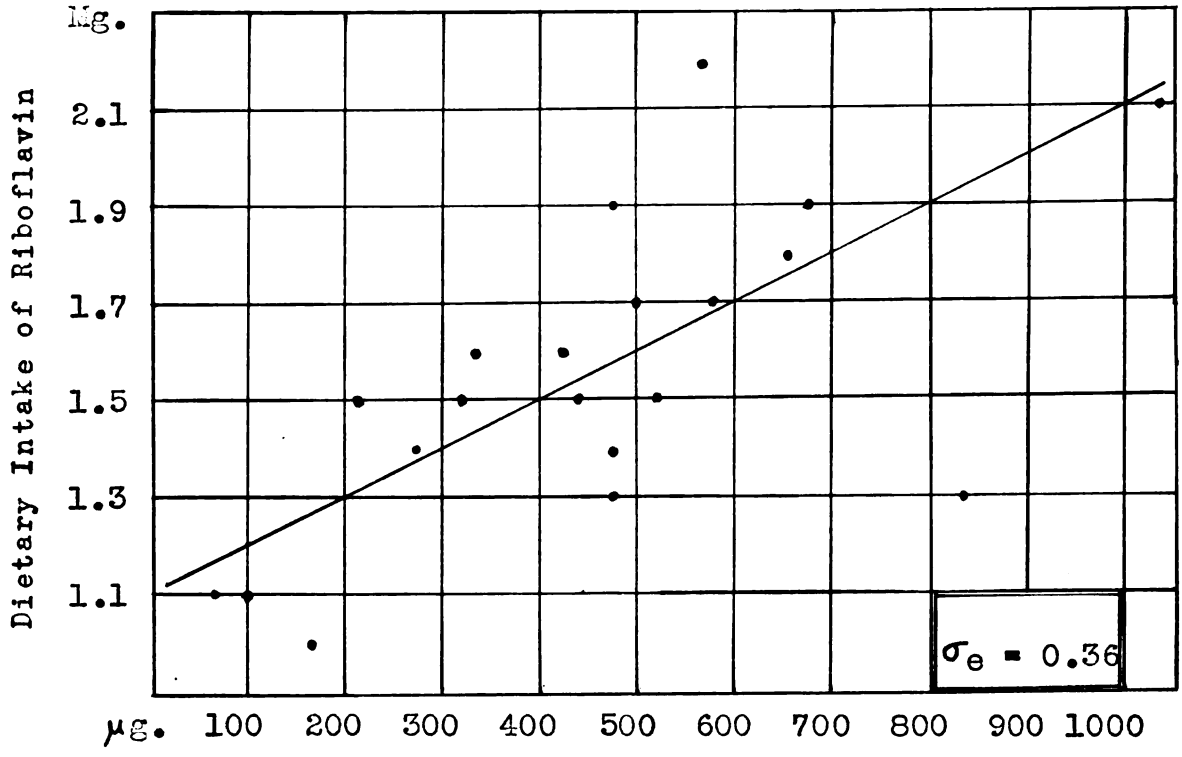
TABLE 7

Average daily dietary intakes of protein, thiamine and riboflavin

Subject	Protein	Thiamine	Riboflavin
	gms.	mgs.	mgs.
HH	62.6	1.65	2.113
AB	51.5	1.011	1.269
FR	57.0	1.0	1.865
GH	71.8	1.221	1.888
ER	67.0	1.086	1.715
LM	71.8	1.294	2.203*
JC	46.0	0.916	1.458
JG	57.1	0.861	1.707
JF	71.1	1.479	1.846
MD	55.5	1.128	1.267
MW	72.6	0.946	1.419
BJ	58.7	0.926	1.456
RN	61.5	0.847	1.614
KB	70.2	1.170	1.623
BT	42.7	0.932	1.477
MP	44.9	0.783	1.401
MK	51.9	0.868	1.451
FF	55.3	0.910	1.030
LH	48.9	1.041	1.045
IO	46.4	1.052	1.123
Averages	58.2	1.056	1.584

*Liver eaten on day of saturation; value for pre-saturation days = 1.71

DAILY DIETARY INTAKE AND 24-HOUR
URINARY EXCRETION OF RIBOFLAVIN



Riboflavin Excreted in 24-Hour Urine Samples
of Pre-Saturation Period

Figure I

PERCENTAGE OF SATURATION DOSE
EXCRETED IN 24 HOURS AND
DAILY DIETARY INTAKE OF RIBOFLAVIN

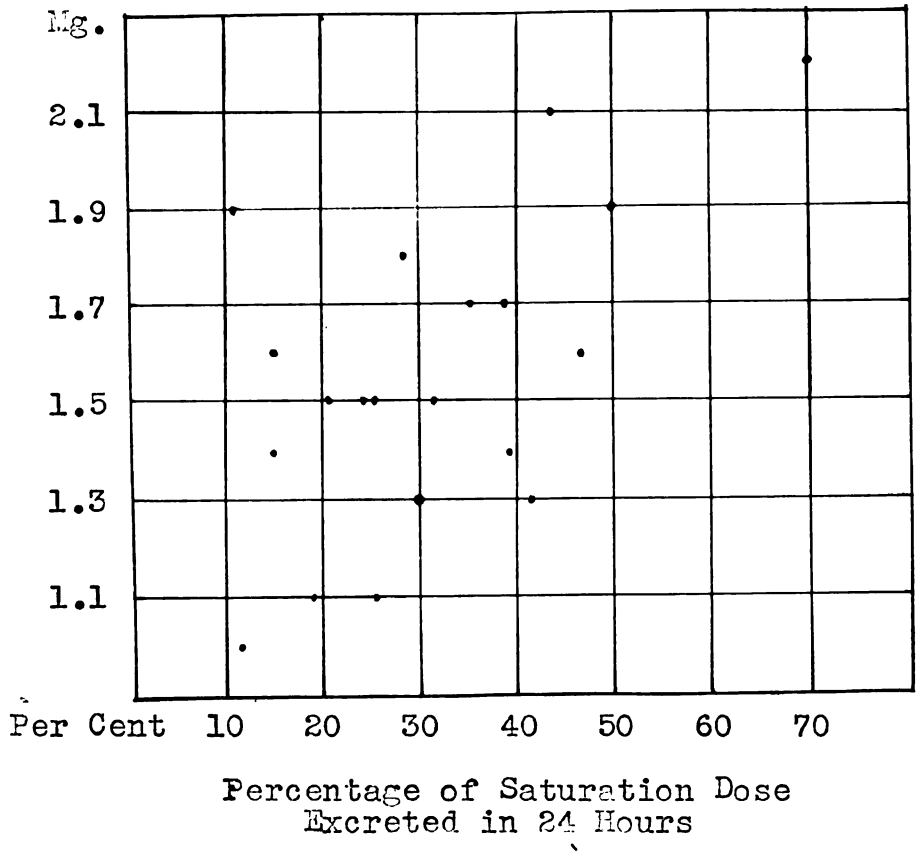


Figure II

Also, the dietary intake of thiamine seemed to have no relationship to the dietary intake of riboflavin. The thiamine excretions will be reported in detail by another investigator from this laboratory.

DISCUSSION

The range of urinary excretion of riboflavin found in this study was lower than that previously reported (Table 1). This may be due to the fact that the findings in the literature have been reported on studies made almost entirely with men (Sebrell, '41; Williams, '43). The lower values previously reported were from the studies with hospital patients (Axelrod, Spies, and Elvehjem, '41). In an attempt to compare the figures obtained in this study with the data reported in the literature, the riboflavin excretion of each subject was recalculated on the basis of 70 kilograms of weight (Table 8). The adjusted range of excretion found was 65 to 1273.7 micrograms or an average of 539.3 micrograms. These values compared with the lower range of presumably normal data reported in the literature.

The fact that the 24-hour urine samples were held for three days before analyses did not explain the lowered excretion values. Hagedorn and co-workers (1945) stored urine samples for three weeks with no significant variation in riboflavin content. Trial analyses in this laboratory indicated that there was little variation between the average riboflavin content for three 24-hour urine samples analyzed immediately at the end of each 24-hour period and the riboflavin content of a composite of three days analyzed at the end of a three-day period.

TABLE 8

Urinary excretions of riboflavin calculated to 70 kilograms weight

Subject	Height Inches	Weight Kilograms	Riboflavin excretion	Per 70 kilograms weight
			µg./24 hrs.	µg./24 hrs.
HH	66	55.9	1030.0	1273.7
AB	64	63.6	833.3	917.2
FR	61	63.6	666.6	733.7
GH	66	52.3	649.9	869.9
ER	60	54.5	576.3	740.2
LM	61	51.8	563.8	761.9
JC	65	61.8	516.3	584.8
JG	67	65.9	499.9	530.2
JF	66	67.3	472.2	491.1
MD	64	50.0	468.3	655.6
MW	65	59.1	466.6	552.1
BJ	64	55.0	437.9	557.3
RN	66	75.0	415.5	387.8
KB	63	54.6	338.2	433.6
BT	64	62.7	320.8	358.1
MP	66	58.1	266.6	321.5
MK	66	59.1	209.6	245.3
FF	64	54.6	159.7	204.7
LH	67	64.6	95.5	103.5
IO	62	62.7	58.2	65.0
Average			446.1	539.3

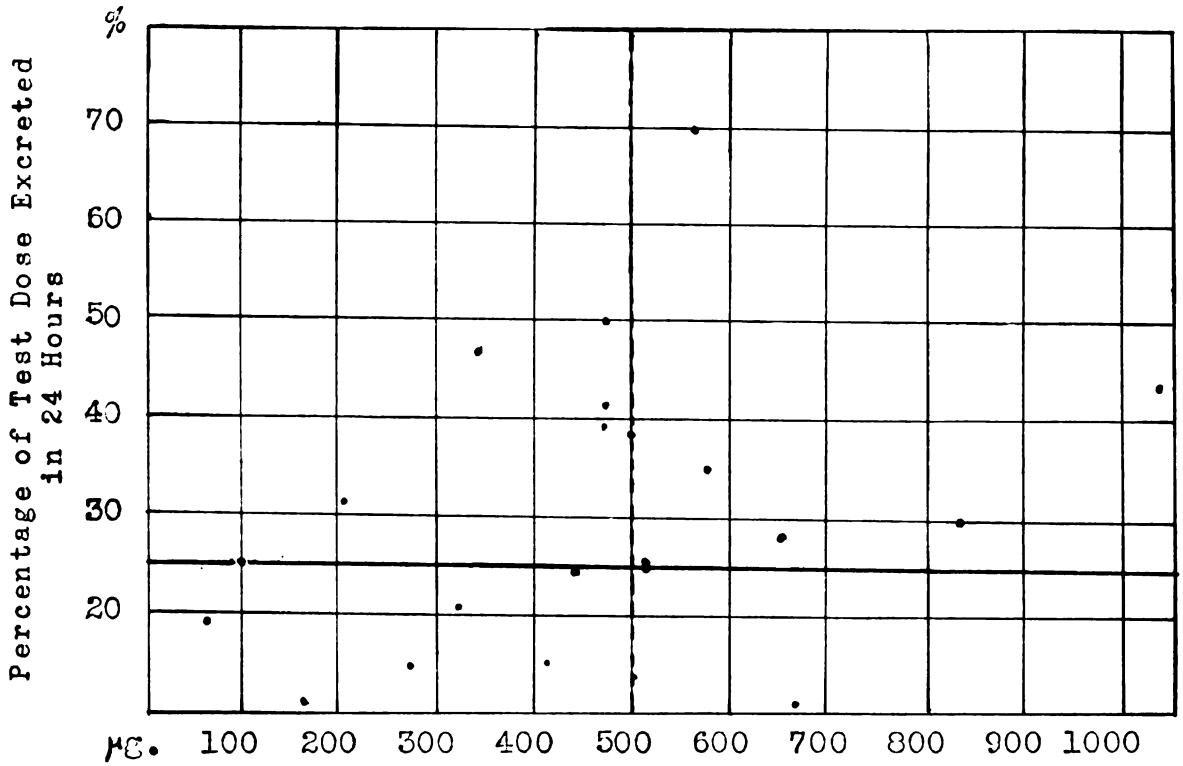
Feder and co-workers (1944) reported that the micrograms of riboflavin excreted per milliliter in the fasting hour sample gave good agreement with the micrograms of riboflavin excreted per milliliter in the 24-hour sample. Hagedorn, and others (1945) found fair agreement in the two measurements but stated that the low concentration of the one-hour sample led to errors in analyses. In this study there was no significant difference between the average micrograms of riboflavin excreted per milliliter of urine in the two periods. The individual variation in riboflavin excretion per milliliter during the two periods appeared greater than statistical analysis would indicate (Table 4). This one-hour fasting excretion seemed, therefore, to be an adequate measurement of riboflavin excretion when a group was studied but inadequate for individual comparisons.

The three-milligram test dose of riboflavin has been referred to in this paper as a "saturation" dose. Keys, and others (1944) found that the 24-hour urinary excretion of riboflavin averaged 12 per cent of the dietary intake when the subject was on a low riboflavin diet. The percentage recovery of a one-milligram test dose was similar. For 26 days the subjects in Key's study were given 11.2 milligrams of riboflavin per man per day. The response to the test dose was 19.7 per cent six days after the excess intake was stopped. When Hagedorn, and others (1945) used a two-milligram test dose given orally, there was 64 per cent excretion of the test dose in 24 hours compared to a

20 per cent excretion when one milligram of riboflavin was used as the test dose. Others (Ferrebee, '41; Williams and others, '43) have injected one milligram of riboflavin as the test dose. In this study three milligrams of riboflavin were given orally as a test dose. In 24 hours after administration, an average of 31 per cent of the test dose had been excreted, a figure which would not suggest marked depletion of body stores of the vitamin.

The excretion of riboflavin in the four hours after the test dose was lower than that reported in the literature (Ferrebee and Weissman, '43; Najjar and Holt, '41; Feder and others, '44). These investigators injected the test dose. A delay in excretion of the oral test dose was to be expected, since the time needed for absorption could not be determined. In this study 24 hours after administration of the test dose an average of 31 per cent was excreted which is comparable to the excretions reported in the literature (Table 1). However, only seven of the subjects responded to the test dose by an excretion of 25 per cent or more and also excreted 500 micrograms of riboflavin or over per 24 hours during the preliminary period. These limits, 500 micrograms riboflavin excreted per 24 hours and 25 per cent excretion of a test dose in 24 hours, are the lower limits of riboflavin excretion proposed in the literature as indicating good riboflavin nutrition. Figure 3 illustrates this. This would mean, if former investigations are used as the criteria, only seven of the twenty subjects studied were in good riboflavin nutrition. Six of the

URINARY EXCRETION OF RIBOFLAVIN
DURING 24-HOUR PERIODS BEFORE TEST DOSE AND
PERCENTAGE EXCRETION OF TEST DOSE IN 24 HOURS



—— Cited in literature as lower limit of percentage response to test dose.
--- Cited in literature as lower limit of micrograms riboflavin excreted in 24 hours.

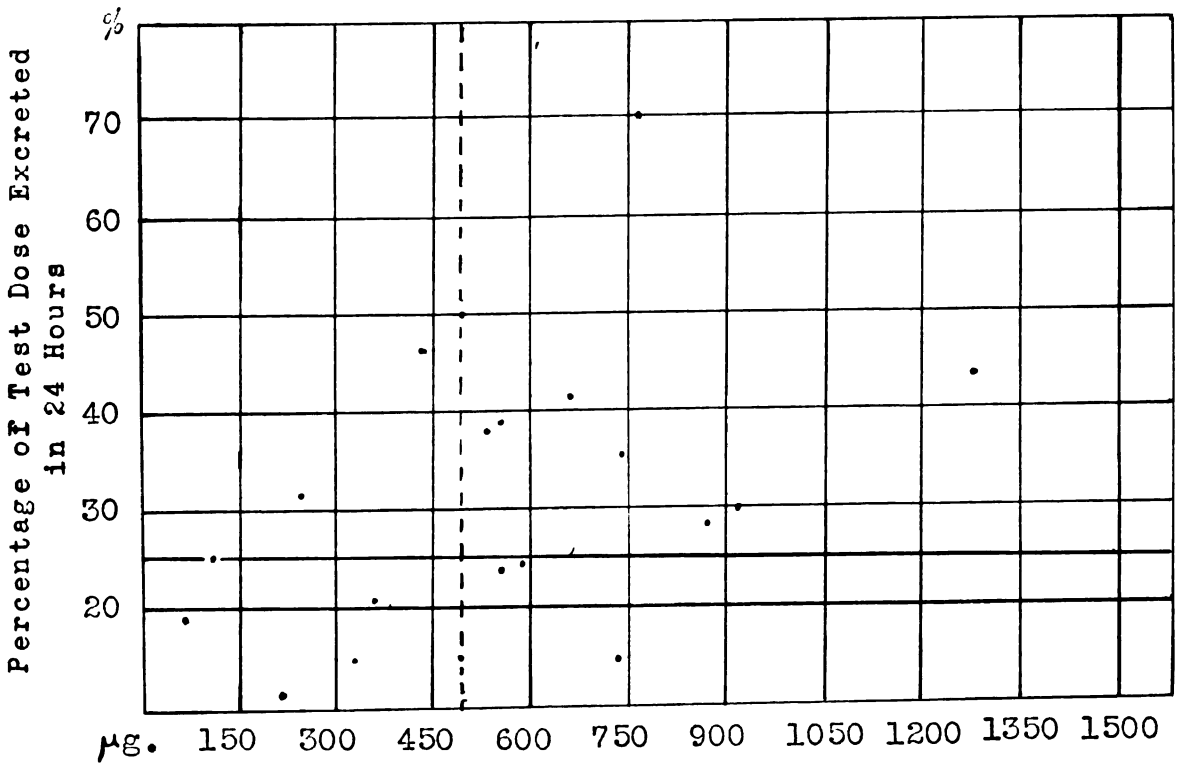
FIGURE III

subjects excreted less than the minimum 500 micrograms in the 24 hours but showed a 25 per cent or more response to the test dose.

When the 24-hour urinary excretion of riboflavin was recalculated on the basis of 70 kilograms weight, all but four subjects (Figure 4) fell within the standards proposed by the literature. Three of these four subjects excreted less than 500 micrograms per 24 hours even with the new calculations, but the response of these three subjects to the test dose was above 25 per cent. This may have been due to a lower kidney threshold for riboflavin in these subjects than in the other subjects, if riboflavin can be considered a "threshold" substance. The response of the one subject who excreted 666.6 micrograms of riboflavin in 24 hours during the preliminary period and only 10.7 per cent of the test dose could not be explained. The recalculation of the riboflavin excretion values on the basis of size was done arbitrarily. The effect of size on the urinary excretion of riboflavin is not known, but the results as expressed in Figure 4 seem to indicate that there may be some relationship, particularly since no complaints attributable to riboflavin deficiency were made by the subjects.

There appeared to be some relationship between dietary intake of riboflavin and the percentage of the three-milligram test dose excreted in 24 hours after administration (Figure 2). This relationship might have been more pronounced if there had been more predise dietary data.

URINARY EXCRETION OF RIBOFLAVIN
DURING 24-HOUR PERIODS BEFORE TEST DOSE
RECALCULATED FOR 70 KILOGRAMS WEIGHT AND
PERCENTAGE EXCRETION OF TEST DOSE IN 24 HOURS



Riboflavin Excreted in 24-Hour Period
before Test Dose Recalculated
for 70 Kilograms Weight

—— Cited in literature as lower limit of percentage response to test dose.
--- Cited in literature as lower limit of micrograms riboflavin excreted in 24 hours.

FIGURE IV

The diets for the five-day experimental period were calculated. The calculations were made on the basis of records kept by the subjects and all measurements of food intake were estimated. This method gave an indication of the subject's dietary habits, but did not furnish absolute values of intake.

The urinary excretion of riboflavin on the fifth day of the experimental period showed that there was very little delay in the excretion of the test dose (Table 3). At the end of the first 24 hours after administration of the test dose, the riboflavin excretion returned to the average excretion found during the 24-hour periods before the test dose. This suggested that the excretion on the fifth day might be eliminated from a similar experimental study without sacrificing useful information. The fact that there is no significant difference between the average micrograms of riboflavin excreted per milliliter of urine during these two periods emphasizes this relationship.

In this study it was found that the daily variation in urinary excretion of riboflavin between 20 college women was over 900 micrograms. There was also individual variation in the amount of riboflavin excreted in one period in relation to the amount excreted in another period. One subject excreted a little over 300 micrograms riboflavin daily and excreted 47.1 per cent of the test dose in 24 hours. Another subject excreted over 600 micrograms of riboflavin daily, but excreted only 27.9 per cent of the

test dose in 24 hours. Still, the riboflavin excretion of the group of subjects as a whole fell into a definite pattern. This is shown in Figure 3, but is more apparent after the figures were recalculated to a uniform weight basis as is shown in Figure 4. The subjects who excreted the most riboflavin daily were, in general, the same ones who excreted the highest percentage of the three milligrams of riboflavin in the first 24 hours after the test dose was given. The six subjects with low daily excretion were the same subjects excreting below 25 per cent of the test dose in 24 hours. Figure 4 shows only four subjects of the 20 who did not follow this pattern.

There were five subjects (Figure 4) who might be said to have poor riboflavin nutrition according to previously reported data (Table 1). All of the subjects were selected for this study because of their apparent good health. No indication of riboflavin deficiency could be noted. Each of the subjects had a record of previous good health. Is the degree of saturation a measure of riboflavin deficiency when no deficiency can be detected in subjects who, by laboratory measurements, are desaturated? Others (Hagedorn and co-workers, '45) have questioned the validity of a saturation test when the effects of tissue desaturation cannot be measured. There is a definite need for further investigation of methods of measuring deficiency and the effects of riboflavin deficiency on the human subject before any conclusions concerning the riboflavin status of the individual may be made.

SUMMARY

The usual range of urinary excretion of riboflavin in twenty college women on a self-chosen diet and the response to a test dose of three milligrams of riboflavin and three milligrams of thiamine were studied. The range of urinary riboflavin excreted in 24 hours was found to be 58.2 to 1030.0 micrograms or an average of 446.1 micrograms. Fourteen of these twenty subjects excreted between 250 and 750 micrograms for the 24 hours. The fasting hour excretion ranged from 0.4 to 37.8 micrograms, an average of 12.7 micrograms. Four hours after a test dose of three milligrams of riboflavin was given, 17.9 per cent of the amount had been excreted. The average excretion of the test dose of riboflavin in 24 hours after administration was 31 per cent which compared with the 25 to 35 per cent output reported in the literature. The range of urinary excretion of riboflavin during the second 24 hours after administration of the test dose returned to the range found during the 24-hour period previous to the test dose.

There was no significant difference between the average micrograms of riboflavin excreted per milliliter of urine during the 24-hour periods before the test dose, the one-hour fasting period and the second 24-hour period after the test dose.

The dietary intake of riboflavin gave a high correla-

tion with the daily 24-hour excretion and was reflected in the response to the saturation dose.

No effect on the urinary excretion of riboflavin due to the administration of a three-milligram test dose of thiamine could be observed.

A wide variation was found in the range of riboflavin excretion of the group and in individual excretions after the test dose. However, a certain pattern of excretion was shown, the subjects with the higher daily excretions having the greater percentage excretion of the test dose. No sign of riboflavin deficiency could be detected in the subjects who had maintained a low riboflavin excretion in all of the periods. The reliability of using the degree of saturation as an index to riboflavin status of human subjects should not be accepted without further study.

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