URINARY EXCRETION OF RIBOFLAVIN BY HUMAN SUBJECTS ON CONTROLLED DIETS

by

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She would also like to acknowledge the loyal cooperation and interest of the three experimental subjects.
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HISTORICAL BACKGROUND

Riboflavin was the first vitamin to be recognized as a pro-enzyme, and was the first member of the vitamin B₂ complex to be isolated and identified, both chemically and biologically (15). Riboflavin has been known by various names in its past history—including lactoflavin (the European name), ovoflavin and hepatoflavin, named for the origin of the substance, and vitamin G, a former designation used by Sherman.

A brief review as given by Rosenberg (36) states that some of the early research was concerned with milk, as Elyth in 1879 isolated an impure flavin from whey, and in 1913 Osborne and Mendel recognized a water soluble, growth-promoting substance in milk. It was in 1925 that Bleyer and Kallman obtained a yellow pigment, "lactochrome," from milk. Warburg and Christian isolated the yellow enzyme from yeast in 1932, while Banga and Szent Györgyi recognized a respiration co-ferment in yeast called cytoflav. Pure riboflavin was isolated in 1933 by two separate groups: by Ellinger and Koschera and by Kuhn, Györgyi and Wagner-Jauregg. The latter group recognized
the identity of vitamin B₂ and riboflavin activity. By 1935, both the Kuhn and Karrer groups made known the constitution of riboflavin by total synthesis.

**CHEMICAL NATURE AND PROPERTIES**

Riboflavin occurs in the free form and also in combination with phosphoric acid, adenylic acid, and specific proteins.

**Formula:**

\[
\begin{align*}
\text{CH}_3\text{C} & \quad \text{C} \\
\text{N} & \quad \text{C} = \text{O} \\
\text{CH}_3\text{C} & \quad \text{C} \quad \text{C} \quad \text{C} = \text{O} \\
\text{C} & \quad \text{N} & \quad \text{C} \quad \text{C} & \quad \text{N} \quad \text{NH} \\
\text{CH}_2 & \quad \text{OH} & \quad \text{OH} & \quad \text{OH} & \quad \text{OH}
\end{align*}
\]

d-riboflavin; 6,7, dimethyl-9-(d-1 ribytyl)-isoalloxazine

Riboflavin is odorless, comparatively heat stable—especially in acid medium, and the crystals are an orange-yellow color which in a water solution gives a yellow-green color and shows a characteristic yellow-green fluorescence (1,36). Two hydrogen atoms are readily added to the
riboflavin molecule to form leuco-riboflavin which is colorless and shows no fluorescence. The leuco-riboflavin is readily oxidized to riboflavin by air (36).

Riboflavin is extremely labile to light. Irradiation in an alkaline solution yields lumiflavin, while in neutral or acid solution lumichrome is the resulting product. O'Malley and Sievert (35) found that riboflavin could be protected from light by hydrosulphite reduction, but this is not effective when heat is used because the reducing agent is volatilized leaving the riboflavin to be reoxidized by the air, and again susceptible to destruction by light.

**Physiology and Function of Riboflavin in Metabolism**

Riboflavin is necessary for the promotion of normal growth of the human organism. One of the most important known functions of riboflavin is its participation in enzyme systems which regulate the metabolism of carbohydrates, amino acids, and probably fats. The relative ease with which riboflavin can add on and in turn give up hydrogen makes it especially suitable for this role.

In carbohydrate metabolism it has been found that the substrate is first oxidized by nicotinamide-enzyme systems which are in turn oxidized by the riboflavin containing enzymes (36). The reduced riboflavin enzyme
systems are re-oxidized by other enzymatic reactions. Riboflavin enzyme systems also act in the oxidation of d-amino acids to their corresponding keto acids (56). Studying the protein metabolism of grasses Watson and Wynd (57) think that riboflavin may influence some part of the condensation of amino acids to proteins.

Sciclounoff and Alphonse (38) concluded that phosphorylated riboflavin is essential for the absorption of sugars. They found that when intestinal functioning is impaired, phosphorylation of riboflavin does not occur, and thereby sugar absorption is inhibited. This involves another relationship since the adrenal cortex hormone is necessary for the phosphorylation (56).

Heiman (16) is convinced that riboflavin is concerned in the visual function, possibly in cone vision, similarly as carotene is concerned in rod vision. Factors which Heiman outlines as making possible riboflavin's participation in vision are: 1) its function as an oxidation-reduction enzyme, 2) its property of fluorescence, 3) its power to intensify weak light stimuli, and 4) its protective effect against excessive light.

An anemia has been produced in dogs fed a synthetic diet devoid of riboflavin. Spector and coworkers (43) found that dogs could not recover from the anemia unless riboflavin was fed. The size of new cells was partly
determined by riboflavin. Klein and Kohn (23) found that ingestion of riboflavin raised the level of flavin adenine dinucleotide in the blood. The level returns to the previous level when riboflavin ingestion is decreased again.

An increased excretion of riboflavin by man during the late stages of thiamine deficiency is thought by Ferrebee and Weissman (14) to represent unused products of tissue catabolism rather than indicating a riboflavin deficiency. They say that since catabolism makes riboflavin available from the tissues, the dietary requirement may not be as great as normally considered.

SYMPTOMS OF RIBOFLAVIN DEFICIENCY

As the result of research and study, a characteristic syndrome for riboflavin deficiency has evolved. This syndrome includes cheilosis (40,41,26), a sharkskin appearance of the skin around the mouth and across the tip of the nose (45), a specific glossitis (24) characterized by a definite purplish red or magenta color, and various ocular manifestations (45,52).

Sebrell and Butler (40,41) produced experimental cases of cheilosis in women, and were able to cure the condition with crystalline riboflavin. Treatment with nicotinic acid did not prove beneficial. The idea that cheilosis may not be a manifestation of riboflavin
deficiency alone is brought out by Machella (26) who men-
tions that cases of cheilosis have been known to respond to
pyridoxine, nicotinic acid, and in some cases of hemorrhag-
ic lesions, ascorbic acid brought about improvement.

The common ocular symptoms are bulbar conjunctivitis,
lacrimation, burning of the eyes, roughness of the eyelids,
failing vision, eyestrain, and photophobia. Sydenstricker
et al (52) found photophobia and dimness of vision the
most frequent symptoms of their riboflavin deficient
patients, with the burning of the eyes and visual fatigue
nearly as common. They found the physical changes in the
eye such as congestion of the sclera, vascularization and
opacities of the cornea and abnormal pigmentation of the
iris were improved with the administration of riboflavin.

The appearance of cataracts in riboflavin defi-
cient rats has provoked much disagreement (15). Day,
Darby and Cosgrove (8) produced nutritional cataract in
31 percent of a group of rats after about 52 days on a
riboflavin deficient diet. An average of about 67 days
elapsed before the cataracts became mature. When intra-
muscular injections of riboflavin were given as the early
cataract development was noticed, the cataract was
arrested in 17 out of 19 animals. Baum, Michaelree and
Brown (3) found that rats on a ration devoid of ribo-
flavin and those provided an adequate diet do not develop
cataracts, but small amounts of riboflavin below the adequate level induce cataract formation. The susceptibility of different strains of rats to cataract may explain some of the differences of opinion.

By working with dogs, Street, Cowgill and Zimmerman (46) found a riboflavin deficiency resulting in the scaling of the skin of the abdomen and hind legs, loss of weight, opacities of the eyes, and a demyelination of the spinal cord, eventually leading to collapse and death.

Ariboflavinosis is generally considered rather prevalent, but actual studies have been few. Wiehl and Kruse (58) made a study on school children using as their criterion the characteristic vascular invasion of the cornea. They found this condition in 2.3 percent of a private school group, 75.0 percent of a public school group and 58.4 percent of adults in a W.P.A. group. Spies et al (44) observed 472 children (5 months up to 14 years of age) of parents with deficiency diseases and found 113 cases of cheilosis, 93 of linear lesion and ocular manifestations in 167. Response to synthetic riboflavin or riboflavin-rich foods was very favorable. These workers also found that treatment of the mother with synthetic riboflavin or riboflavin-rich food would cure the lesions of the nursing infant.
ASSAY METHODS FOR RIBOFLAVIN

Various properties of riboflavin have led to varied approaches in the methods of assay for riboflavin in biological tissues. Ferrebee (15), Najjar (23), Conner and Straub (7) and Peterson, Brady and Shaw (34) have used the chemical methods which are based on the measurement of the fluorescence of riboflavin with a photo-fluorometer. Ferrebee (15) and Conner and Straub (7) adsorbed the riboflavin on a column of floridin, and eluted with 20 percent pyridine in 2 percent acetic acid. Both methods used H\textsubscript{2}O\textsubscript{2} oxidation to destroy interfering substances and H\textsubscript{2}O\textsubscript{2} to decolorize the excess permanganate, but Ferrebee (15) used sodium hydrosulphite while Conner and Straub (7) used sunlight or mercury vapor lamp to destroy the riboflavin in order to obtain a blank reading. Najjar’s (23) method involved the reading of the fluorescence in a butyl alcohol-pyridine mixture and then destruction of the riboflavin by mercury vapor lamp or direct sunlight.

Riboflavin has also been assayed by biological methods based on rat or chick growth. Helmar (17) used the rat growth method to determine the riboflavin content of urine. Lloyd’s reagent was used to adsorb the riboflavin and the dried adsorbate was fed to the rats. Biological methods involved a great deal of time and a rather
large amount of the material. To overcome these difficulties the microbiological methods have been developed. *Lactobacillus casei* was used by Snell and Strong (42) in their determination. Growth on a riboflavin-free basal medium plus known quantities of an extract of the material being assayed was compared with a standard curve made from the results obtained when the organism was allowed to grow on the basal medium plus known graduated amounts of pure riboflavin. The turbidity of the solutions was measured after 24 hours or the lactic acid production was measured after 72 hours. Isboll et al (20) found that the urea in the medium when urine was being analyzed by this method had an inhibiting effect on the growth of *Lactobacillus casei* and proposed a calculation to correct the error.

Fairly good correlation between the methods was found on most materials being analyzed, though each method had its advantages and disadvantages. The chemical and microbiological were quicker than the biological, and the microbiological required use of a smaller quantity of material for analysis than the other methods. Extraction of the sample is very important in both the chemical and microbiological methods, because no matter how carefully the rest of the process is carried out, if the extraction is incomplete, the results are inaccurate. Modifications have had to be employed when different types of tissues
were being analyzed.

**FOOD SOURCES OF RIBOFLAVIN**

Riboflavin has been found widely distributed in plant and animal cells, although there are not many rich sources. Fish products such as liver roe and fish meals have been found good sources (30). Lanford et al. (25) found that the citrus fruits, banana and tomato appear richer in riboflavin than the pome fruits (apples and pears). Liver, milk, eggs, and leafy green vegetables are the best sources of riboflavin found in the human dietary. Cheldelin and Williams (5) in their studies of the average American diet found that dairy products contributed nearly one-half of the riboflavin in the diet, while meats contributed about 1/3, cereals 1/6, and fruits and vegetables 1/6. They found milk to be the highest single contributor. Variable figures for the riboflavin content of milk are given, but Holmes and Holmes (19) concluded that a milk of relatively uniform riboflavin content could be produced by standardizing the feed and management of the herd.

Tea and coffee were until recently considered devoid of food value, but Drummond and Moran (9) have studied the "unconsidered trifles" in our diet and found that tea contains 9 micrograms of riboflavin per gram, coffee 1.7 micrograms per gram and cocoa 2.7 micrograms per gram.
From these figures it was calculated that a cup of tea would provide 10 micrograms of riboflavin.

With riboflavin as with the other nutrients, an adequate supply in the diet is dependent upon a wise selection plus proper care in handling. The stability of riboflavin during processing of milk was studied by Zeigler and Keevil (61) who found that 9 to 16 percent was destroyed during pasteurization, 5 to 8 percent during vitamin D enrichment by irradiation and 3 to 5 percent during bottling and the brief storage previous to delivery. Holmes (18) studied the losses of riboflavin brought about by the holding and flash processes of pasteurization and concluded that the losses were negligible since only 2.0 percent was lost by the holding method and no loss occurred with the flash method. The effect of sunlight on milk was studied by Peterson, Haig and Shaw (35) by exposing pint bottles of milk to the direct sunlight on an open porch at temperatures between 60 degrees F. and 72 degrees F. The respective losses observed in 30, 90, 120, and 210 minutes were 28, 50, 66, and 72 percent, showing a very rapid destruction of riboflavin under these conditions.

That the riboflavin content of canned foods varied considerably in different samples was reported by Thompson and coworkers (54), but they could not offer any explanation for the variations. Strong, Barle and Zeman (47) have
found no considerable loss of riboflavin occurred on stor-
age, cooking and preservation of foods. They were sur-
prised that so small a quantity was removed by boiling the
vegetables in water. Losses of riboflavin in cooking were
also studied by Cheldelin, Woods and Williams (6) who
stated that the losses were small in the absence of light,
and the opacity of many foods tended to prevent riboflavin
destruction even in the light.

METHODS OF ASSESSING THE LEVEL OF RIBOFLAVIN NUTRITION

The riboflavin content of blood, muscles and urine
has been studied in the effort to gain a better under-
standing of the nutritional status of individuals with
respect to riboflavin. Emmerie (11) found that the normal
urines of human males contained 819 to 1250 micrograms per
day, and found that the excretion was increased by the in-
gestion of liver which is a rich source of riboflavin.
Strong, Feeney, Moore and Parsons (43) reported a daily
urinary excretion of 500 to 800 micrograms by normal human
adults. They further studied four women and found that on
a dietary intake of 1 to 2 milligrams per day the excretion
decreased to 50 to 150 micrograms. An average of 357 mi-
crograms per day was excreted by seven women on an institu-
tion diet who were studied by Sebrell, Butler, Wooley and
Isbell (39). Hajjar and Holt (30) observed the riboflavin
excretion of one adult living for 30 days on a diet devoid of riboflavin. His excretion dropped from 135 micrograms to 0 in 16 days and remained at 0 from the 16th to 30th day. Axelrod et al (2) reported the average daily excretion of riboflavin by human adults on regular hospital diets to be 236 to 270 micrograms while on deficient diets it was 58 to 91 micrograms. Ferrebee (13) found the daily excretion in five normal subjects varied from 700 to 1700 micrograms per day while they were eating their usual diets.

It has been shown that the daily urinary excretion varies with the daily intake. The variation of excretion among individuals is quite large, but Axelrod et al (2) found the day to day variation for a given individual is very small. Feder, Louis and Alden (12) recommend the measurement of excretion per milliliter as the most constant value for comparison since they found there was better correlation per unit of volume than per unit of time. In the interpretation of their results they placed 0.3 micrograms per milliliter as the arbitrary level below which they would consider the person deficient in riboflavin. They selected this level because their studies showed that a liberal intake resulted in an excretion of 0.9 micrograms or more per milliliter while a low intake resulted in 0.5 micrograms or less per milliliter being
excreted.

The daily urinary excretion reflects the daily intake and is not usually taken as the index of nutritional status. Many of the previously mentioned research workers (12, 30, 2, and 59) have used the saturation test dose recovery as a basis for determining nutritional status. The test dose consists of taking an excess amount of riboflavin orally, intramuscularly or intravenously and measuring the quantity of riboflavin recovered. Foder et al (12) used a test dose of 0.016 milligram per kilogram of body weight and assumed that figures below 35 percent excretion in a fasting sample represented a deficiency. Najjar and Holt (30) thought a good index was the measurement of the excretion following a dose of 1 milligram of riboflavin given intravenously. They found that in the four hours after injection normal individuals excreted from 277 to 685 micrograms while four riboflavin deficient subjects excreted only 74 to 194 micrograms. Axelrod, Spies, Elvehjem, and Axelrod (2) report that too high test doses must be avoided to get the best results from the saturation test. Williams et al (59) used a 2.0 milligram test dose and later suggested that 1.0 milligram of sodium riboflavin given intravenously would be more satisfactory than the 2.0 milligram dose for adults whose requirement was between 1.5 and 3.0 milligrams. Axelrod and coworkers
(2) could not find a correlation between the daily urinary excretion of riboflavin and the degree of retention of a given test dose, but this may have been influenced by the fact that their subjects were suffering from multiple deficiency. Oldham et al (32) considered a 20 percent return of the test dose of riboflavin within four hours an indication of a satisfactory nutritional status. They thought the one-hour fasting excretion could be used as well as the return of the test dose in determining the nutritional status.

**HUMAN REQUIREMENT FOR RIBOFLAVIN**

More specific knowledge of the riboflavin requirement of human subjects has been the aim of much research. Oldham et al (32) studied two five-year-old boys and concluded that 0.50 milligrams per 1000 calories seemed to be an adequate supply for these boys. An intake of 0.50 milligrams per 1000 calories seemed to be approximately the minimal requirement of riboflavin for the human adults studied by Williams, Mason, Cusick, and Wilder (59). They found an intake as low as 0.35 milligrams per 1000 calories to be associated with tissue depletion while at 0.8 milligrams per 1000 calories tissue depletion did not occur. Strong et al (48) considered an intake of 1 to 2 milligrams per day for the young woman they studied not more
than marginal and perhaps even insufficient to furnish the
daily requirement. Sebrell and coworkers (59) found that
an intake of 0.985 milligrams per kilogram of body weight
was probably a greater amount than that needed for ribo-
flavin saturation. These workers concluded that 0.035
milligrams per kilogram of body weight is not enough to
meet the adult requirement while 0.06 milligrams per kilo-
gram of body weight is slightly above the required amount.

The recommendations of the National Research Council,
Committee on Food and Nutrition (31), published in 1941,
are as follows:

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<tr>
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<th>Man (70 kg)</th>
<th>Woman (56 kg)</th>
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<tr>
<td>Moderately active</td>
<td>2.7 mgs</td>
<td>2.2</td>
</tr>
<tr>
<td>Very active</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Sedentary</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregnancy 2.5</td>
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<td></td>
<td></td>
<td>Lactation 3.0</td>
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Children up to 12 years:

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<td>Under 1 year</td>
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</tr>
<tr>
<td>1 to 3 years</td>
<td>0.9</td>
</tr>
<tr>
<td>4 to 6 years</td>
<td>1.2</td>
</tr>
<tr>
<td>7 to 9 years</td>
<td>1.5</td>
</tr>
<tr>
<td>10 to 12 years</td>
<td>1.8</td>
</tr>
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Children over 12 years:

<table>
<thead>
<tr>
<th>Age</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Girls 13 to 15 years</td>
<td>2.0 mgs</td>
</tr>
<tr>
<td>16 to 20 years</td>
<td>1.8</td>
</tr>
<tr>
<td>Boys 13 to 15 years</td>
<td>2.4</td>
</tr>
<tr>
<td>16 to 20 years</td>
<td>3.0</td>
</tr>
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</table>
FACTORS INFLUENCING RIBOFLAVIN REQUIREMENT

That the body could accumulate a reserve of riboflavin was shown by Keys et al (21), but this reserve was small and rapidly lost in a few weeks. Van Duyne and Sherman (56) studied rats on diets containing 5 and 20 micrograms of riboflavin per gram of food and found that the tissues of the animals at both these levels were stabilized at a maximum riboflavin content. These workers wondered whether an intake higher than at the level at which the riboflavin content of the tissues is stabilized may result in a higher riboflavin content of the offspring at birth or during infancy. A later report by Ellis, Zmachinsky and Sherman (10) of work with rats on three levels of riboflavin intake (3.0, 6.5 and 10.0 micrograms of riboflavin per gram of air dry food mixture) stated that although the lowest level seemed adequate to support life, the highest level appeared to offer additional benefits such as more favorable growth while on the family diet and greater ability to live on a diet low in riboflavin and thiamine.

Unna and Grelin (55) found excessive doses of riboflavin administered orally to dogs and rats non-toxic, while excessive amounts given by intraperitoneal injection produced death due to kidney concretions formed. The
difference in toxicity displayed by the oral and intra-peritoneal administration of the riboflavin was explained by these authors by the supposition that the low solubility of riboflavin prevented the absorption of excessive amounts of riboflavin from the gastro-intestinal tract.

Two groups of rats were studied by Milla (27) to find the effect of temperature on the riboflavin requirement. One group was kept in a room at 60° F. and the other group was kept in a tropical-like environment at 90° and 70 percent relative humidity. At the end of the six-week study the conclusion was reached that the temperatures resulted in no significant difference in riboflavin requirement.

Bacterial synthesis of riboflavin in man does occur according to Najjar and coworkers (29) who found that the riboflavin excretion in the feces of 12 boys (10 to 16 years of age) remained the same during the riboflavin deficient period as during the control period. The riboflavin excretion in urine and feces was five or six times the amount ingested. It was shown by these workers that riboflavin can be absorbed from the large intestine since a rapid rise in the excretion of riboflavin in the urine was noted following an enema containing 20 milligrams of riboflavin. Succinylsulfathiazole did not affect the amount of riboflavin excreted in the feces, indicating
that possibly the riboflavin-producing organisms are not susceptible to this drug.

Interest has been shown by Sure and Dicheck (49) in the role of riboflavin as a factor in the economy of food utilization by rats. Their control animals gained 56 to 1300 percent more in weight than the riboflavin deficient litter mates. The fat content of the rats showed the greatest increase and there was considerable protein gained but the change in ash content was too small and variable to be significant. Sure (50) reported that the food intake of riboflavin deficient rats, unlike the thiamine deficient ones, did not show any definite trend. He found that there might be a moderate reduction, no change, or an increased food intake in the later stages of riboflavin deficiency.

Sarett, Klein and Perlzweig (37) found that the urinary excretion of riboflavin by dogs and rats showed an inverse relationship to the level of protein intake. The periods of low protein intake resulted in the highest excretion of riboflavin. The inter-relationship of thiamine and riboflavin is not well understood, but Sure (51) found that great losses of urinary riboflavin was produced by a chronic sub-optimum level of thiamine fed to rats.
CHAPTER II

PURPOSE OF THIS INVESTIGATION

The purpose of this investigation was to study and compare the urinary excretion of riboflavin by human subjects on controlled diets containing different levels of riboflavin during two experimental periods.
CHAPTER III

EXPERIMENTAL

PLAN OF THE EXPERIMENT

While the three college women who were selected to be subjects were still on their self-chosen diets, urine samples were collected for three days preceding the study. The day before the first experimental period, the women were given a 10 mg test dose of riboflavin. The controlled diet was then started for two experimental periods of two-weeks each. The test dose was repeated the day between the first and second experimental periods and again at the completion of the study. During the first period, a total of 2 mg of riboflavin was given to the subjects in their diet and supplement. This was decreased to 1.5 mg in the second period, by decreasing the riboflavin supplement and keeping the riboflavin in the diet at the same constant level.

Analysis was made of the riboflavin content of the food ingested and of the urine excreted. The staple foods were analyzed immediately preceding the study, and each time a new supply of any food was purchased. Urine analysis was done on the twenty-four hour samples for the three days preceding the study; also for alternate days of
the first week of each period and for every day during the second week of the periods.

DETERMINATION OF RIBOFLAVIN IN FOOD

A modified Conner and Straub (7) method was used for the analysis of the food. Fifteen to 30 grams of food were ground in the Waring blender with 200 ml of 2% acetic acid for 3 minutes. One half the amount was weighed, and 10 ml of 5% Pslodase solution added. The samples were incubated overnight in an oven at 37°C.

Extracts were filtered through dry filter paper, discarding the first few milliliters.

Fifteen ml of the extracts were pipetted into dark brown bottles. One ml of 4% KMnO₄ was added, the bottles stoppered and shaken vigorously for 1½ minutes. Three ml of 5% H₂O₂ were added and the bottles again shaken vigorously for 1½ minutes. The solutions were then poured into cuvettes and when the bubbles had disappeared, galvanometer readings were made in the fluorometer with the fluorescein standard set at 40.

The solutions were then exposed to the sun or sun lamp for an hour and readings again made. This blank corrected for fluorescent substances other than riboflavin. An "enzyme blank" (a solution containing all the reagents used except the material being analyzed) is also treated
in the same way and the reading subtracted.

A curve of reference was used to determine the amount of riboflavin present in the solution. It was obtained by setting the fluorometer at 40 with the fluorescein solution, and reading the galvanometer with different solutions of known riboflavin content. The curve was fitted according to the method of least squares. A straight line relationship exists between the fluorometer readings and the concentration of the riboflavin solution.

DETERMINATION OF RIBOFLAVIN IN THE URINE

The urine was collected in dark bottles to which 5 ml of glacial acetic acid and 5 ml of toluene had been added. After measuring the volume excreted and thoroughly mixing the samples, a portion was stored in the refrigerator until the analysis was completed.

The Conner and Straub (7) method was used for the analysis of the urine. The riboflavin in 15 ml or other suitable aliquot at pH 4.5 was adsorbed on a column of Florisil as prepared by Ferreebee (15). The urine was passed through the column at the rate of 1 ml per minute, and the column washed with hot distilled water. The riboflavin was then eluted with 20% pyridine in 2% acetic acid. The eluate was made up to 50 ml volume.
Fifteen ml of the eluate were pipetted into a dark bottle, and the analysis completed by the procedure used for the food.

The standard curve of reference used for the urine analysis was made with known amounts of riboflavin in a solution of 20% pyridine in 2% acetic acid.

**REAGENTS AND EQUIPMENT USED**

2% acetic acid: made frequently from a stock solution of 20% acetic acid.

3% Polidase Solution: 3 grams of Polidase dissolved in 100 ml of sodium acetate - acetic acid buffer solution at pH 4.5. Made daily.

Sodium acetate-acetic acid buffer: 55 ml glacial acetic acid and 110.5 grams NaC₂H₃O₂·3H₂O made up to one liter with distilled water.

4% K₂MnO₄: 4 grams of K₂MnO₄ were dissolved in 100 ml of distilled water. Made daily.

3% H₂O₂: Made by diluting 50% H₂O₂ (Superoxol), one part to nine parts distilled water. Diluted immediately before using.

Fluorescein Standard: 10 mg of sodium fluorescein were dissolved in one liter of distilled water. For the working standard 10 ml of the above solution were diluted to one liter. Kept in a brown bottle in the refrigerator.

Florisil: Prepared according to Ferreebe (13), washed with 2% acetic acid, rewashed in distilled water, and then dried.

20% Pyridine in 2% Acetic Acid: Made by mixing one part pyridine to four parts 2% acetic acid.

Photofluorometer: A Coleman Electronic Photofluorometer, Model 12, was used with filter B₂ between the lamp and sample and filter PC₂ between the phototube and the sample.
Exchange tubes: Tubes of one centimeter diameter which are widened at one end to hold about 25 ml of liquid, and a capillary tube at the other end regulates the flow of liquid to about 1 ml per minute. A plug of glass wool supports the one gram column of Florisil.

Incubator oven: Maintained at about 37ºC.

DESCRIPTION OF SUBJECTS

The three subjects were all college women carrying full schedules, and considered moderately active and in good health. JJ and NS were both nineteen-year-old sophomores. JJ was 5 feet, 6 inches tall and weighed 121 pounds, while NS was 5 feet, 9½ inches tall, weighing 157½ pounds. AR, a twenty-two year old senior, weighed 108 pounds and was 5 feet, 3 inches tall.

EXPERIMENTAL DIET

The diet remained constant throughout the experimental periods. The foods in the diet, along with the thiamine, riboflavin and caloric content, are listed in Table I. Soda crackers were given ad libitum. Starting in the latter third of the first period and following a report by Drummond (9) that coffee contained riboflavin, coffee was limited to one cup per meal. JJ drank tea rather than coffee. In addition to 0.342 mgs of riboflavin, the diet supplied approximately 55 gms of protein, 195 mgs of calcium, 8 mgs of iron, 7432 I.U. of vitamin A,
Table I. The Experimental Diet Showing Thiamine, Riboflavin and Caloric Content

<table>
<thead>
<tr>
<th>Food</th>
<th>Amount in Grams</th>
<th>Calories</th>
<th>Thiamine mg</th>
<th>Riboflavin mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grapefruit juice</td>
<td>150</td>
<td>61</td>
<td>0.115</td>
<td>0.006</td>
</tr>
<tr>
<td>Farina</td>
<td>100</td>
<td>59</td>
<td>-----</td>
<td>0.015</td>
</tr>
<tr>
<td>Butter</td>
<td>80</td>
<td>615</td>
<td>-----</td>
<td>0.008</td>
</tr>
<tr>
<td>String beans {canned}</td>
<td>67</td>
<td>28</td>
<td>0.042</td>
<td>0.027</td>
</tr>
<tr>
<td>Apple {raw}</td>
<td>127</td>
<td>60</td>
<td>0.051</td>
<td>0.022</td>
</tr>
<tr>
<td>Ground beef</td>
<td>100</td>
<td>144</td>
<td>0.147</td>
<td>0.058</td>
</tr>
<tr>
<td>White rice</td>
<td>100</td>
<td>88</td>
<td>0.009</td>
<td>0.015</td>
</tr>
<tr>
<td>Carrots {canned}</td>
<td>100</td>
<td>45</td>
<td>0.035</td>
<td>0.010</td>
</tr>
<tr>
<td>Peaches and Juice</td>
<td>115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peanut butter</td>
<td>30</td>
<td>67</td>
<td>-----</td>
<td>0.007</td>
</tr>
<tr>
<td>Granulated sugar - 1 Tb.</td>
<td>30</td>
<td>176</td>
<td>0.165</td>
<td>0.036</td>
</tr>
<tr>
<td>Shortbread cookie</td>
<td>50</td>
<td>76</td>
<td>0.003</td>
<td>0.009</td>
</tr>
<tr>
<td>Biscuits, 7</td>
<td>747</td>
<td></td>
<td>0.120</td>
<td>0.060</td>
</tr>
<tr>
<td>Coffee, 400 cc</td>
<td>---</td>
<td></td>
<td></td>
<td>0.069</td>
</tr>
</tbody>
</table>

Totals:                    | 2236            | 0.740    | 0.342       |

Supplement                |                  | 1.600    |             |

2.340
0.740 mgs of thiamine, 36 mgs of ascorbic acid and 15.42 mgs of nicotinic acid. The figures for the foods, other than those analyzed in the laboratory for riboflavin, were taken from the tables by Taylor (53), except the nicotinic acid values which are from Chaney and Ahlborn's Nutrition (4). The only variations in the diets of the three subjects were the number of biscuits and quantity of butter eaten. The biscuits were made with unenriched flour and without milk so their riboflavin content had no significant effect on the total riboflavin intake. JJ ingested an average of 2235 calories per day, AR, 1947 calories and NS, 2452 calories.

SUPPLEMENTS

The supplement on each day of the first experimental period was 1.6 mgs each of riboflavin and thiamine, and 50 mgs of ascorbic acid. Ascorbic acid was given because analysis of the grapefruit juice in the laboratory showed that it contained considerably less of the vitamin than the average value given in the table. The riboflavin supplement was decreased to 1.2 mgs during the second experimental period, but the ascorbic acid and thiamine level were kept the same. The calcium content of the diet was increased by the addition of 20 gms of calcium carbonate to one day's supply of biscuits.
CHAPTER IV

RESULTS AND DISCUSSION

The data on the excretion of urinary riboflavin for this study are presented in Table II. As can be seen, the daily variations on both the self chosen diets and the constant diet of the experimental periods were great. It should be noted that the subjects maintained their state of good health and physical vigor. AR had a little difficulty in maintaining her weight. She weighed 108 pounds at the beginning of the study and after 26 days weighed 104\(\frac{1}{2}\) pounds, but at the end of the study she weighed 106 pounds. NS varied between 156 and 158 pounds throughout the investigation, starting at 157\(\frac{1}{2}\) pounds and ending at 158 pounds. JJ started at 121 pounds and varied not more than 2\(\frac{1}{2}\) pounds during the study.

During the three days on the self chosen diet the daily urinary excretion of riboflavin by JJ varied from 442 to 1180 mcg, the excretion by AR varied from 449 to 1400 mcg, while NS excreted from 424 to 518 mcg of riboflavin. The excretions of 442, 449, and 424 mcg of riboflavin daily are lower than the normal excretions of 500 to 800 mcg reported by Strong et al (48) or 700 to 1700 mcg by Ferrobee (13), but all the figures are higher than the averages of 357 mcg reported by Sebrell and coworkers.
Table II. Daily Excretion of Riboflavin by Subjects on a Constant Diet with Supplements of Different Amounts of Riboflavin

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>Date</th>
<th>JJ</th>
<th>AR</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplement</td>
<td>Date</td>
<td>1944</td>
<td>Mcg.</td>
<td>Averages</td>
</tr>
<tr>
<td>Self-chosen diet</td>
<td>10/17</td>
<td>701</td>
<td>1400</td>
<td>424</td>
</tr>
<tr>
<td></td>
<td>10/18</td>
<td>442</td>
<td>449</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>10/19</td>
<td>1180</td>
<td>997</td>
<td>518</td>
</tr>
<tr>
<td>10.0 mg test dose</td>
<td>10/20</td>
<td>8552</td>
<td>15361</td>
<td>7605</td>
</tr>
<tr>
<td>2.0 mg riboflavin</td>
<td>10/22</td>
<td>956</td>
<td>1360</td>
<td>827</td>
</tr>
<tr>
<td></td>
<td>10/24</td>
<td>706</td>
<td>376</td>
<td>339</td>
</tr>
<tr>
<td></td>
<td>10/26</td>
<td>501</td>
<td>1192</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>10/28</td>
<td>759</td>
<td>622</td>
<td>332</td>
</tr>
<tr>
<td></td>
<td>10/30</td>
<td>907</td>
<td>603</td>
<td>777</td>
</tr>
<tr>
<td></td>
<td>10/31</td>
<td>295</td>
<td>423</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>11/1</td>
<td>257</td>
<td>641</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>11/2</td>
<td>853</td>
<td>383</td>
<td>383</td>
</tr>
<tr>
<td></td>
<td>11/3</td>
<td>209</td>
<td>310</td>
<td>583</td>
</tr>
<tr>
<td>10.0 mg test dose</td>
<td>11/4</td>
<td>3531</td>
<td>6741</td>
<td>3252</td>
</tr>
<tr>
<td>1.5 mg riboflavin</td>
<td>11/6</td>
<td>429</td>
<td>429</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>11/8</td>
<td>156</td>
<td>225</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>11/10</td>
<td>351</td>
<td>329</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>11/12</td>
<td>257</td>
<td>164</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>11/13</td>
<td>88</td>
<td>88</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>11/14</td>
<td>248</td>
<td>198</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>11/15</td>
<td>223</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>11/16</td>
<td>172</td>
<td>312</td>
<td>312</td>
</tr>
<tr>
<td></td>
<td>11/17</td>
<td>348</td>
<td>348</td>
<td>327</td>
</tr>
<tr>
<td>10.0 mg test dose</td>
<td>11/18</td>
<td>2494</td>
<td>5123</td>
<td>1240</td>
</tr>
</tbody>
</table>

* Average is the average of the last five days of the period.
(39) and 236 to 270 mcg by Axelrod et al. (2).

During the first experimental period when 2.0 mg of riboflavin was ingested per day, the urinary excretion of riboflavin by JJ varied from 956 mcg to 209 mcg. Her average daily excretion for the last five days was 310 mcg. The excretion of riboflavin by AR during the first period varied from 1360 to 368 mcg, with an average for the last five days of 574 mcg daily. NS had riboflavin excretions from 824 to 90 mcg with an average excretion during the last five days of 216 mcg per day.

When the riboflavin ingestion was 1.5 mg per day for the second experimental period, JJ's excretion ranged from 429 to 156 mcg, with a daily average of 247 mcg for the last five days. The urinary excretion of AR ranged from 329 to 89 mcg with an average for the last five days of 244 mcg per day. A range from 230 to 105 mcg of riboflavin excreted per day was shown by NS during this second period, and her average daily excretion for the last five days of the period was 148 mcg. In all cases the average excretions in the second period were lower than those in the first, being on the average 62, 350 and 67 mcg less respectively for the three subjects.

The recovery of test doses of riboflavin has been thought by many workers (2, 12, 59) to be more significant of the nutritional status of the subject than the daily
excretion. Data on the test dose response of the subjects are found in Table III. The response (or percent of the ingested riboflavin which was excreted in the urine) after the self chosen diets was high in all cases, although the response differed for the various subjects. JJ gave a response of 85.5%, AR 133.6% and NS 76.0%. The response to the test dose after the first experimental period was about one half of the previous response by AR and less than half in JJ and NS. A further decrease in the response was found after the second experimental period, so that at the end of the study 24.1%, 49.6% and 12.0% of the riboflavin ingested by JJ, AR and NS respectively was excreted in the urine. The responses of the subjects to the second and third test doses are not strictly comparable to the first because the percent of the first is based on the 10.0 mg dose of riboflavin without knowing the riboflavin content of the self chosen diets of the subjects, while the total riboflavin ingestion (0.342 mg in diet plus 10.0 mg dose) was considered in calculating percent response after the experimental periods. The 12.0% response is very low and presumably too low; and the responses of the other two subjects are considerably lower than their previous responses, but whether they indicate a deficiency cannot be known by our present standards.
<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>JJ</th>
<th>%</th>
<th>AR</th>
<th>%</th>
<th>NS</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self-chosen diet</td>
<td>8552</td>
<td>13,361</td>
<td>7605</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 mg riboflavin</td>
<td>3331</td>
<td>6,741</td>
<td>3252</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 mg riboflavin</td>
<td>2494</td>
<td>5,123</td>
<td>1240</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
From the results it can be seen that a 2.0 mg level or a 1.5 mg level of riboflavin intake was adequate to maintain these three subjects in their usual state of health for two week periods. It is probable that the subjects were depleting their tissue stores of riboflavin as indicated by the low daily excretions and low response to the test doses. Keys (22) states that no functional impairment results from complete absence of riboflavin for 30 days and he suggests that 1.0 mg of riboflavin per day is adequate for 6 months to a year for a person consuming 3000 calories. In view of Keys' work, it might be said that the subjects of this study were getting adequate amounts of riboflavin, at least for the short period of the study.

Considering the riboflavin intake from the standpoint of mg per 1000 calories of food ingested, it is found that during the first period JJ received 0.89 mg per 1000 calories, AR 1.03 mg per 1000 calories and NS 0.82 mg per 1000 calories. During the second experimental period JJ, AR and NS ingested 0.67 mg, 0.77 mg and 0.61 mg of riboflavin respectively per 1000 calories of food. The diet which Keys et al (21) used on their study of normal young men averaged 0.31 mg of riboflavin per 1000 calories. Their study lasted for five months, but they found after a few weeks that the daily urinary excretion of riboflavin and also the recovery of saturation test
doses remained at an average of 12.0% of the intake. This is the same percent excretion that NS reached on this study at the end of the second experimental period, although she was ingesting 0.61 mg of riboflavin per 1000 calories. Williams and coworkers (59) found that 0.35 mg of riboflavin per 1000 calories was associated with tissue depletion, but an intake of 0.8 mg of riboflavin per 1000 calories was not. They thought 0.5 mg was approximately close to the daily requirement necessary for maintaining tissue stores. Although the riboflavin intakes of JJ, AR and NS were all higher than 0.5 mg per 1000 calories, they all showed signs of tissue depletion, especially NS whose test dose response was only 12.0% of her intake. Differences in diet composition, size of subjects, and activities are factors which may account for some variation in the studies by different groups of workers.

Sebrell, Butler, Wooley and Isbell (39) found that 0.35 mg of riboflavin per kg of body weight was not enough to meet the adult requirement. The subjects in this investigation showed lower excretions of riboflavin when the intakes amounted to 0.021, 0.027 and 0.031 mg per kg than when the respective intakes were 0.028, 0.036 and 0.041 mg per kg, and the excretions on these latter levels of intake were considerably lower than on the self-chosen diets. These results are in line with the above findings of
Table IV. Milligrams of Riboflavin Ingested per 1000 Calories

<table>
<thead>
<tr>
<th>Subject</th>
<th>Riboflavin Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0 mg</td>
</tr>
<tr>
<td>JJ</td>
<td>0.99</td>
</tr>
<tr>
<td>AR</td>
<td>1.03</td>
</tr>
<tr>
<td>NS</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Table V. Milligrams of Riboflavin Ingested per Kilogram of Body Weight

<table>
<thead>
<tr>
<th>Subject</th>
<th>Weight in Kg</th>
<th>Riboflavin Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.0 mg</td>
</tr>
<tr>
<td>JJ</td>
<td>55</td>
<td>0.036</td>
</tr>
<tr>
<td>AR</td>
<td>49</td>
<td>0.041</td>
</tr>
<tr>
<td>NS</td>
<td>72</td>
<td>0.028</td>
</tr>
</tbody>
</table>
Sebrell et al. Therefore, on this basis, even the higher level of intake, 2.0 mg per day, does not represent optimal intake.

The extent of biosynthesis of riboflavin in the intestines of these subjects was not known, but if biosynthesis occurs as Najjar and coworkers (29) found, the variation of urinary excretion of riboflavin between subjects may be partially explained.
CHAPTER V

SUMMARY AND CONCLUSIONS

Three subjects were maintained on a constant diet low in riboflavin. The daily urinary excretion of riboflavin was measured during two experimental periods when known supplements of riboflavin were added, first at a 2.0 mg level of intake and secondly at a 1.5 mg level.

The urinary excretion of riboflavin varied a great deal from day to day and between subjects. The averages for the last five days of the first experimental period were 310, 574 and 216 mcg and for the last five days of the second period the respective averages were 248, 244 and 149 mcg. The response to 10.0 mg test doses of riboflavin showed decreasing percentages of riboflavin excreted. On the 2.0 mg level of intake the responses were 32.2%, 65.2% and 31.5%. After the 1.5 mg level of intake, lower test dose responses of 24.1%, 49.8% and 12.0% respectively were obtained. It is interesting to note that ES who excreted the smallest proportion of the test dose was the largest subject, while AR who had the highest proportionate response was the smallest subject.

The low daily excretions and low response to the test doses indicate that the subjects were depleting their tissue stores of riboflavin. With the present knowledge
of riboflavin function and requirement, definite conclusion of deficiency or adequacy cannot be made. At least for two of the subjects the 2.0 mg intake of riboflavin is not an optimum intake and the 1.5 mg intake seems to be low for all three subjects.

Limitations to the conclusions which can be drawn from this study are that the data are only from three subjects, for short periods of study, and at just two levels of riboflavin intake. More data as a result of further investigation on the subjects could readily be used. Until more definite knowledge is obtained, the recommendations of the National Research Council (31) of 2.2 mg for a moderately active woman might be considered to offer a margin of safety and that the lower levels of intake can be used for a period of two weeks without apparent harm to the individual.
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