URINARY EXCRETION OF RIBOFLAVIN BY HUMAN
SUBJECTS ON CONTROLLED DIETS

by

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CHAPTER I

INTRODUCTION

HISTORICAL BACKGROUND

Riboflavin was the first vitamin to be recognized as a pro-enzyme, and was the first member of the vitamin B_2 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 Blyth 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 Koschara and by Kuhn, Györgyi and Wagner-Jauregg. The latter group recognized

the identity of vitamin B_2 and riboflavin activity. By
1935, both the Kuhn and Karrer groups made known the con-
stitution 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:

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 (33) 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. Ribo-
flavin enzyme systems also act in the oxidation of d-amino
acids to their corresponding keto acids (36). 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 phos-
phorylated 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 (36).

Heiman (16) is convinced that riboflavin is con-
cerned 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 oxida-
tion-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 pre-
vious 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 de-
ficiency. 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 character-
istic syndrome for riboflavin deficiency has evolved. This
syndrome includes cheilosis (40,41,26), a sharkskin appear-
ance 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 con-
dition with crystalline riboflavin. Treatment with nico-
tinic 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 mentions that cases of cheilosis have been known to respond to pyridoxine, nicotinic acid, and in some cases of hemorrhagic lesions, ascorbic acid brought about improvement.

The common ocular symptoms are bulbar conjunctivis, 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 deficient rats has provoked much disagreement (15). Day, Darby and Cosgrove (8) produced nutritional cataract in 81 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 intramuscular 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 riboflavin and those provided an adequate diet do not develop

cataracts, but small amounts of riboflavin below the ade-
quate 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 scal-
ing 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, 75.8 percent of a public school
group and 38.4 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 mani-
festations 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 (13), Najjar (28), 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 (13) 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 $KMnO_4$ oxidation to destroy interfering substances and H_2O_2 to decolorize the excess permanganate, but Ferrebee (13) 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 (28) 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 difficul-
ties 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. Isbell 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 (60). 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 1/5, 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, Earle and Zeman (47) have

found no considerable loss of riboflavin occurred on storage, cooking and preservation of foods. They were surprised 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 understanding 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 ingestion of liver which is a rich source of riboflavin. Strong, Feeney, Moore and Parsons (48) 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 micrograms per day was excreted by seven women on an institution diet who were studied by Sebrell, Butler, Wooley and Isbell (39). Najjar 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 excre-
tion 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, Lewis and Alden (12) recommend the
measurement of excretion per milliliter as the most con-
stant value for comparison since they found there was bet-
ter 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 ribo-
flavin. 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 0.3 micrograms or less per milliliter being

excreted.

The daily urinary excretion reflects the daily in-
take 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 re-
covery 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. Feder 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 ex-
cretion 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
683 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 satura-
tion 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 (39) found that
an intake of 0.085 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:

	Man (70 kg)	Woman (56 kg)
Moderately active	2.7 mgs	2.2
Very active	3.3	2.7
Sedentary	2.2	1.8
		Pregnancy 2.5
		Lactation 3.0

Children up to 12 years:

Under 1 year	0.6
1 to 3 years	0.9
4 to 6 years	1.2
7 to 9 years	1.5
10 to 12 years	1.8

Children over 12 years:

Girls 13 to 16 years 2.0 mgs.
     16 to 20 years 1.8

Boys 13 to 15 years 2.4
     16 to 20 years 3.0

FACTORS INFLUENCING RIBOFLAVIN REQUIREMENT

That the body could accumulate a reserve of ribo-
flavin 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 Greslin (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 solu-
bility of riboflavin prevented the absorption of excessive
amounts of riboflavin from the gastro-intestinal tract.

Two groups of rats were studied by Mills (27) to
find the effect of temperature on the riboflavin require-
ment. One group was kept in a room at 68° 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 temper-
atures 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 ribo-
flavin 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 Dichock (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 blendor with 200 ml of 2% acetic acid for 3 minutes. One half the amount was weighed, and 10 ml of 3% Polidase 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% $KMnO_4$ was added, the bottles stoppered and shaken vigorously for $1\frac{1}{2}$ minutes. Three ml of 3% H_2O_2 were added and the bottles again shaken vigorously for $1\frac{1}{2}$ 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 a-mount of riboflavin present in the solution. It was ob-tained 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 fluoro-meter 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 3 ml of toluene had been added. After measuring the volume excreted and thoroughly mixing the samples, a portion was stored in the refriger-ator 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 Ferrebee (13). 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 $NaO_2H_3O_2.3H_2O$ made up to one liter with distilled water.

4% $KMnO_4$: 4 grams of $KMnO_4$ were dissolved in 100 ml of distilled water. Made daily.

3% H_2O_2: Made by diluting 30% H_2O_2 (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 Ferrebee (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 B_2 between the lamp and sample and filter PC_2 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 37°C.

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, 9$\frac{1}{4}$ inches tall, weighing 157$\frac{1}{2}$ 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

Food	Amount in Grams	Calories	Thiamine Mgs.	Riboflavin Mgs.
Grapefruit juice	150	61	0.113	0.006
Farina	100	59	------	0.015
Butter	80	615	------	0.008
String beans (canned)	67	28	0.042	0.027
Apple (raw)	127	80	0.051	0.022
Ground beef	100	144	0.147	0.058
White rice	100	88	0.009	0.015
Carrots (canned)	100	45	0.085	0.010
Peaches and	113			
Juice	30	67	------	0.007
Peanut butter	30	176	0.165	0.036
Granulated sugar – 1 Tb.		50	------	------
Shortbread cooky		76	0.008	0.009
Biscuits, 7		747	0.120	0.060
Coffee, 400 cc		---	------	0.069
Totals:		2236	0.740	0.342
Supplement			1.600	
			2.340	

0.740 mgs of thiamine, 88 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 diffi-culty in maintaining her weight. She weighed 108 pounds at the beginning of the study and after 26 days weighed 104½ pounds, but at the end of the study she weighed 106 pounds. NS varied between 156 and 158 pounds throughout the investigation, starting at 157½ pounds and ending at 158 pounds. JJ started at 121 pounds and varied not more than 2½ 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 ribo-flavin. The excretions of 442, 449, and 424 mcg of ribo-flavin daily are lower than the normal excretions of 500 to 800 mcg reported by Strong et al (48) or 700 to 1700 mcg by Ferrebee (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

SUBJECT		JJ		AR		NS	
Supplement	Date 1944	Mcg.	Average*	Mcg.	Average*	Mcg.	Average*
Self-chosen diet	10/17	701		1400		424	
	10/18	442		449		450	
	10/19	1180		997		518	
10.0 mg test dose	10/20	8552		13361		7603	
2.0 mg riboflavin	10/22	956		1360		824	
	10/24	706		378		339	
	10/26	501		1192		168	
	10/28	769		622		332	
	10/29	907		603		777	
	10/30	295		423		90	
	10/31	573		368		154	
	11/1	257		641		306	
	11/2	217		853		388	
	11/3	209	310	583	574	143	216
10.0 mg test dose	11/4	3331		6741		3252	
1.5 mg riboflavin	11/6	429		151		176	
	11/8	156		225		202	
	11/10	381		329		116	
	11/12	257		164		230	
	11/13			88			
	11/14			248		198	
	11/15	223				105	
	11/16	172		312			
	11/17	348	248	327	244	143	149
10.0 mg test dose	11/18	2494		5123		1240	

* 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 88 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, 330 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 86.5%, 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 re-
sponses, but whether they indicate a deficiency cannot be
known by our present standards.

Table III. Urinary Excretion of Riboflavin in Response to 10.0 Mg Test Dose

SUBJECT	JJ		AR		NS	
Response after:	Mcg.	%	Mcg.	%	Mcg.	%
Self-chosen diet	8552	85.5	13,361	133.6	7603	76.0
2.0 mg riboflavin	3331	32.2	6,741	65.2	3252	31.5
1.5 mg riboflavin	2494	24.1	5,123	49.6	1240	12.0

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 0.55 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

Subject	Riboflavin Intake	
	2.0 mg	1.5 mg
JJ	0.89	0.67
AR	1.03	0.77
NS	0.82	0.61

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

Subject	Weight in Kg	Riboflavin Intake	
		2.0 mg	1.5 mg
JJ	55	0.036	0.027
AR	49	0.041	0.031
NS	72	0.028	0.021

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 those 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 31.6%. After the 1.5 mg level of intake, lower test dose responses of 24.1%, 49.6% and 12.0% respectively were obtained. It is interesting to note that NS 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 conclu-
sion 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 mgs 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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