AN ABSTRACT OF THE THESIS OF

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Title The Concentration of Riboflavin in the Serum and Urine of Human Subjects on a Controlled Diet.

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The study reported in this thesis is part of an investigation designed to determine the metabolism of thiamine and riboflavin in human subjects who were maintained on controlled diets. Part of this investigation was made during a 30-day experimental period in 1950 and the other part, during a 30-day experimental period in 1951. In the studies of both years, the subjects were maintained on a diet which was adequate in all nutrients except thiamine and riboflavin. With respect to thiamine, each of the 30-day studies was divided into two experimental periods of 15 days each. During the first 15-day period, the National Research Council's recommended allowance of 500 mcg. thiamine per 1000 calories per person per day was tested and in the second period, each subject received 300 mcg. thiamine per 1000 calories daily in the diet. The daily intake (about 1.2 mg.) of riboflavin was constant throughout the whole study.

This thesis is a report of the riboflavin phase of the study only.

Four women were selected as subjects for each year's 30-day experiment. In general, they were healthy throughout the entire period of the study.

Daily micro determinations of the concentration of free (+FMN) and total riboflavin in the serum and daily macro determinations of riboflavin excreted in 24-hour collections of urine were made.

Based on the results of the studies of both years, the mean concentration of free (+FMN) serum riboflavin for seven subjects (data for one subject were omitted) was 1.43 and ranged between 0.47 to 3.50 mcg. per cent regardless of the different levels of thiamine intake; the mean concentration of total serum riboflavin was 3.22 and ranged from 2.36 to 5.30 mcg. per cent.

The mean concentration of free (+FMN) riboflavin in the serum was slightly, but not significantly lower on the period of restricted thiamine intake. The mean concentration of total riboflavin increased 0.43 mcg. per cent in the period of restricted thiamine intake; this increase was statistically significant. This phenomenon may have been due to the effect of thiamine on the utilization of riboflavin in metabolism; i.e., a decrease in thiamine intake reduces the requirement for riboflavin and is reflected in an increased concentration of riboflavin in the serum.

The statistical analysis indicated that the variation among individuals in serum riboflavin concentration is statistically significant. There was no significant day-to-day variation in the riboflavin value in serum during the period of study.

The mean daily urinary excretion of riboflavin of three subjects (data for one subject were omitted) in the 1950 study was 382 mcg. per day, and that of four subjects in the 1951 study, 376 mcg. per day. The riboflavin output was about 32 per cent of the ingested vitamin in both years' studies. The riboflavin excretion per gram of creatinine ranged from 248 to 474 mcg.

THE CONCENTRATION OF RIBOFLAVIN IN THE SERUM AND URINE OF HUMAN SUBJECTS ON A CONTROLLED DIET

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THE CONCENTRATION OF RIBOFLAVIN IN THE SERUM AND URINE OF HUMAN SUBJECTS ON A CONTROLLED DIET

CHAPTER I

INTRODUCTION

The estimation of nutritional status with respect to riboflavin has been made mostly by the evaluation of urinary excretion of riboflavin in 24-hours, one-hour during fasting, and in response to a test dose. Only a few estimations of riboflavin have been made on blood. Axelrod, Spies, and Elvehjem (1941) reported that there was no difference between the concentration of riboflavin in whole blood in normal subjects and that in deficient subjects. Strong et al. (1941) determined the concentration of riboflavin in the whole blood of the human, the rat, the calf, the dog, and the hog. They found that the concentration of riboflavin in the blood of human beings on an unrestricted diet was 0.49 mcg. per ml. of whole blood; that the concentration of riboflavin in the whole blood of the rat and of the calf was about the same as in the human, but that in the dog and the hog it was twice as high. However, the information concerning riboflavin levels in blood as indicative of the nutritional status in regard to this vitamin is incomplete.

The micro-method, proposed by Burch, Bessey, and Lowry (1948), for the determination of riboflavin in serum, has made it possible to extend the studies on riboflavin in blood. They showed that the free and combined forms, FMN (flavin-mononucleotide) and FAD (flavin-adenine-dinucleotide), could be determined by this method. Based on

unpublished data, obtained by Holt, for a few subjects, deficient in riboflavin, Burch and her coworkers suggested "that the total riboflavin in man may be too stable to be of value, but that the free serum riboflavin may prove useful as a measure of nutritional status."

The purpose of the investigation reported in this thesis was to determine the daily concentration of free (+FMN) and total riboflavin in the serum and the daily excretion of riboflavin in the urine of normal women subjects on a controlled diet with an adequate intake of this vitamin. The relationship of thiamine intake to the riboflavin concentration in the serum was also observed. The intake of riboflavin was constant throughout the experiment, but as far as the thiamine intake was concerned, the experiment was divided into two periods: during the first period the subjects received the amount of thiamine (500 mcg. per 1000 calories) recommended by the Food and Nutrition Board of the National Research Council, and during the second period the subjects received 300 mcg. of thiamine per 1000 calories.

CHAPTER II

LITERATURE REVIEW

HISTORICAL BACKGROUND

Riboflavin, which formerly was designated as "vitamin G" by American biochemists and as "vitamin B_2" by British and German workers, is a water-soluble, yellow pigment with green fluorescence (Booher, 1933, and Chick, Copping and Roscoe, 1930). The first chemical research was carried on in 1879, when Blyth reported that he obtained a yellow-green fluorescent pigment in whey and called it lactochrome.

Early in the 20th century nutrition research was begun on animals. Osborne and Mendel (1913) recognized a water-soluble, growth-promoting substance in milk, and McCollum and Kennedy (1916) named one of those rat growth-promoting factors "water-soluble B". At that time it was uncertain whether the water-soluble, growth-promoting substance effective for rats and the anti-beriberi vitamin were identical. In 1919, Mitchell pointed out that it was doubtful that the factors for the maintenance of life and growth and for the prevention of multiple neuritis were identical. In 1920, Emmett and Luros found that these factors were not equally susceptible to destruction by heat, and further investigations confirmed their findings. Vitamin G, or B_2, was designated as a heat-stable fraction of the vitamin B-complex (Smith and Henrick, 1926, and Sherman, 1926).

A concentrate of yellow-green fluorescent pigment was obtained by Bleyer and Kallman (1925), and also some of its properties were determined.

In 1926, Goldberger and Lillie's experiments with rats produced a deficiency syndrome which resembled human pellagra. Although this syndrome had been considered to be caused by the lack of the pellagra preventive (P-P) factor, Goldberger and Lillie proved it to be largely due to riboflavin deficiency.

A method, using the rat as the experimental animal for the determination of vitamin G in various materials, was proposed by Bourquin and Sherman (1931). It was used extensively for testing the potency of foods and pharmaceuticals. The potency was expressed as the "Bourquin and Sherman unit", each unit equalling 3 mcg. of riboflavin.

Warburg and Christian (1932) obtained a yellow oxidation enzyme from yeast and also found that this enzyme possessed a protein and a pigment component. The latter component was the chemically active group of the enzyme. In the next year Ellinger and Koschara (1933), Booher (1933), and Kuhn, Gyorgy and Wagner-Jauregg (1933) isolated the pure form of the yellow-green fluorescent pigment from different materials, such as milk, liver, kidney, urine, muscle, yeast, and egg white; and found this yellow pigment to be related to the Warburg and Christian yellow enzyme, to a water-soluble rat growth-promoting factor, and also to vitamin G or B_2. The chemical group name of "flavins" was suggested for this pigment and the prefixes ova-(from egg), lacto-(from milk),

and hepato-(from liver) to indicate their origins.

Kuhn et al. (1935) and Karrer et al. (1935) independently synthesized the vitamin and found the activities of the synthetic vitamin to be identical with those of the naturally occurring vitamin.

Sebrell and Butler (1938) first described induced human riboflavin deficiency due to the intake of a diet deficient in riboflavin.

PHYSIOLOGY OF RIBOFLAVIN

Enzymatic activities

One of the most important known functions of riboflavin in living tissue is its participation in enzyme systems which regulate cellular oxidations. These enzyme systems are associated with intermediate enzymatic action in carbohydrate, amino acid, and/or fat metabolism. Warburg and Christian (1932) first reported an enzyme which contained riboflavin. It was known as the "Warburg and Christian yellow enzyme", and was concerned in the oxidation of hexose-phosphoric acid. Riboflavin was also reported to be present in the enzyme systems of amino acid oxidase, xanthine oxidase, and succinic acid dehydrogenase (Axelrod and Elvehjem, 1941).

Blood regeneration

Gyorgy et al. (1938) found the regeneration of hemoglobin to be hastened by riboflavin. They demonstrated that anemic dogs fed lactoflavin (natural or synthetic) in daily doses of 1.7 to 10.0 mg., or 0.1 to 0.5 mg. per kilogram of body weight, definitely gained in hemoglobin. Spector et al. (1943) produced anemia in dogs by

feeding them a synthetic diet devoid of riboflavin, and brought about recovery by administration of riboflavin. Waisman (1944) reported anemia in monkeys as one of the results of a riboflavin-deficient diet, since erythrocyte and hemoglobin levels fell to anemic stage shortly after the appearance of the dermatitis. On the other hand, Sebrell and Onstott (1938) found that anemia occurred frequently in riboflavin-deficient dogs but did not respond to riboflavin therapy. Keys et al. (1944) failed to obtain any signs of anemia in the young men who were on a diet containing as little as 0.31 mg. of riboflavin per 1000 calories per day for 84 days.

Visual function

Finding riboflavin deficiency associated with dimness of vision, impairment of visual acuity, and photophobia, Heiman (1942) suggested that riboflavin may function in cone vision as carotene functions in rod vision. He pointed out that riboflavin is concerned in the visual process by its functions and property as follows:

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.

Factor for normal growth

Many investigators have found that riboflavin is a growth-promoting factor. Bourquin and Sherman in 1931, in a study of the growth-promoting effect of vitamin G in rats, found that with

an increased intake in the amount of vitamin G, there was a proportional increase in body weight. In young chicks, prolonged partial riboflavin deficiency led to "curled toe" paralysis and dystonia.

Warkany and Schraffenberger (1944), in their studies on rats, used a basal diet containing yellow corn meal 76, wheat gluten 20, calcium carbonate (C.P.)3 and sodium chloride (C.P.) 1 per cent. This diet was supplemented by 60 I.U. of vitamin D as viosterol every 10 days. They found congenital malformations of the offspring of rats when the maternal basal diet was supplemented with the vitamin B-complex excluding riboflavin. They also showed that when the maternal diet was supplemented with riboflavin, the congenital malformations were prevented; however, supplements of thiamine hydrochloride, niacin, pyridoxine, and calcium pantothenate were not preventive.

ASSESSMENT OF THE STATE OF NUTRITION WITH RESPECT TO RIBOFLAVIN

Clinical examination

Eye

Corneal vascularization was the first morphological manifestation of riboflavin deficiency observed in rats by Bessey and Wolbach (1939). The same symptom was noted in man by Kruse et al. (1940). It was usually accompanied by photophobia, lacrimation, and burning and itching of the eyes. Visual fatigue, dimness of vision, and a sensation of roughness of the eyes were also reported. As the first change in corneal

lesion, it was noted that capillaries of the limbus arising at the temporal and/or nasal side of the anterior ciliary vessels extended into the superficial layers, and anastomosed to form tiers of loops. The corneal lesion consisted of the injection and proliferation of the vessels of the limbus and in the early stages could be seen by the aid of a slit lamp or other instrument. Later circumcorneal injection was visible. Superficial ulcerations and both superficial and interstitial opacities, of the diffuse or patchy type, of the cornea might also occur. Sydenstricker, Sebrell, Cleckly and Kruse (1940) also found blepharitis, conjunctivitis, iritis, and corneal vascularization in their patients. They described the lids as red, swollen, and matted together with a sticky exudate.

Some of the early investigators considered corneal vascularization as a specific evidence of riboflavin deficiency and reported this nutritional disorder as based on slight changes of the blood vessels of the limbic plexus. However, in other cases, it was shown that this syndrome was also caused by other dietary deficiencies or excesses, and by many types of trauma and infection. Totter et al. (1942), Albanese and Buschke (1942), and Albanese, Randall and Holt (1943) reported that ocular lesions in rats were produced by a tryptophane-deficient diet. Bessey and Wolbach (1939) observed corneal vascularization in vitamin A deficiency in rats. Totter et al.

(1942) demonstrated that a lysine-deficient diet also caused a similar syndrome. An intake low in sodium (Follis et al., 1942) and low in zinc (Follis et al., 1941) and large doses of nicotinic acid (Gregory, 1943) were reported as causing corneal lesions.

Based on results obtained in a nutrition survey, Anderson and Milam (1945) reported that there was no correlation between the dietary intake of riboflavin and symptoms of corneal vascularization.

Many investigators, studying human beings maintained on diets presumably deficient in riboflavin, have failed to obtain the symptom of corneal vascularization (Sebrell and Butler, 1938 and 1939, and Williams et al., 1943). Pett (1943) found that a large percentage of cases with this syndrome did not respond to riboflavin therapy.

There is good evidence that corneal vascularization was produced by riboflavin deficiency. This, however, cannot be considered as the sole criterion of ariboflavinosis. It has been suggested that circumcorneal lesion may be considered a symptom of ariboflavinosis only when the clear cornea is invaded by capillaries of the limbus. This invasion always occurs bilaterally but not always in equally advanced stages in both eyes. The changes involve the whole circumference of the cornea. Circumcorneal infection may not appear until a late stage, when deep vascularization and corneal opacities

occur. This circumcorneal lesion responds to riboflavin therapy within a few days (Parsons, 1944).

Cataract was reported produced by riboflavin deficiency in rats, mice, chickens, and monkeys by Day et al. (1931 and 1934), who prevented and cured the condition by adequate riboflavin administration. However, Bessey and Wolbach (1939) could not confirm this finding. Mitchell et al. (1938) found that a galactose-containing diet produced cataracts which did not respond to riboflavin therapy. Cataract due to riboflavin deficiency in human beings has not been demonstrated.

Mouth

Cheilosis, one of the symptoms caused by riboflavin deficiency has been reported by many workers (Sebrell and Butler, 1938 and 1939, and Sydenstricker, Kelly, and Weaver, 1941). In general, the lesions of the lips begin at the angles of the mouth as small, red, painful spots, macerating and fissuring. The lips appear dry and chapped, and shallow ulcerations and crusting may occur in severe cases. The lesions are usually covered with yellowish crusts. When the crusts are removed, a reddish non-bleeding surface is apparent.

Lesions of the mouth in themselves cannot be considered a specific syndrome of riboflavin deficiency. It may be that cheilosis is not a manifestation of riboflavin deficiency alone, since improvement has resulted from treatment with vitamin B_6, niacin, the entire B-complex, and iron (Machella,

1942 and Smith and Martin, 1940). Jeghers (1943) listed other possible causes: the effects of lipstick, dental plates, chewing gum, mouth washes, cigarette holders, throat lozenges, reeds of musical instruments, and sun exposure.

The condition of the tongue also indicated riboflavin deficiency. The epithelium over the papillae appeared flattened and edematous. The papillae took on a mushroom shape which gave the surface of the tongue a granular appearance. Pain, a burning sensation of the tongue, and even difficulty in swallowing sometimes resulted. With this syndrome the tongue became purplish-red or magenta in color, due to filation of the capillaries, with stagnant blood under the changed epithelium (Jeghers, 1942).

Skin

A fine, scaly, slightly greasy desquamation on a mildly erythematous base in the nasolabial folds, on the alae nasi, in the vestibule of the nose, and on the ears was found in women subjects who were on a riboflavin deficient diet (Sebrell and Butler, 1938). In an experiment on dogs, the skin of the abdomen and hind legs was scaly, with accompanying loss of weight (Street et al., 1941).

Biochemical or physiological tests

Studies on urinary excretion of riboflavin

24-hour excretion

Daily urinary excretion of riboflavin was thought by many

investigators to be indicative of the nutritional status with respect to this vitamin. In normal persons the 24-hour excretion of riboflavin reflects the dietary intake of this vitamin; the amount has been found to be about 150 to 2,000 mcg. daily. If the diet has remained relatively constant at either low or high levels, the excretion is indicative of tissue depletion or saturation. In a study on two 5-year-old boys, Oldham et al. (1944) found that a constant excretion of 105 to 117 mcg. of riboflavin indicated an adequate intake for these children. Sebrell, Butler, Wooley, and Harris (1941) in a study of normal adults suggested that the excretion of about 200 mcg. in 24 hours was the lower limit of normal, and Copping (1945) considered that an excretion of less than 200 mcg. in 24 hours indicated an inadequate intake. However, in 1945, Hagedorn, Kyhos, Germek, and Sevringhaus noted that men, who for 2 years or more had been eating not more than 0.5 mg. riboflavin per day, excreted 50 to 120 mcg. daily, and gave no physical indication of riboflavin deficiency. Najjar and Holt (1941) stated that the 24-hour output of riboflavin reflected only the immediate dietary intake, but was not an accurate measure of nutritional status with respect to riboflavin.

Horwitt et al. (1950) suggested that a 24-hour urinary excretion of less than 100 mcg. of riboflavin indicated that the recent diet provided less than the minimum requirement of this vitamin. They considered that an excretion below 50 mcg.

per day showed that the individual had been on a diet deficient in riboflavin for some time.

Load test

Another method of obtaining information on tissue depletion or saturation of riboflavin is the use of a test dose, usually administered either orally or parenterally. Oldham et al. (1944) in a study on children suggested that the return of 20 per cent of a test dose indicated a satisfactory nutritional status with regard to riboflavin. Feder, Lewis and Alden (1944) suggested that a return of 35 per cent of the test dose indicated a normal return. In 1946, Davis et al., in a study of 12 women subjects, found that only 4 of the 12 reached 20 per cent return of the test dose; one of the four had 35 per cent return; and their average finding for the normal subjects was about 15 per cent.

Fasting excretion

Excretion of riboflavin in one hour during fasting has been considered a good means of assessing nutritional status, with respect to riboflavin, by many workers (Holt and Najjar, 1942, Johnson et al., 1945, and Kark et al., 1947). They suggested that an excretion below 20 mcg. during this period of time is evidence of a deficiency state. Oldham et al. (1944) suggested that a one-hour fasting excretion of 9 mcg. was satisfactory evidence of adequacy of riboflavin intake for the children. Davis et al. (1946) in their study of adult

women found that the average excretion on a riboflavin intake of 290 mcg. per 1000 calories was 6 mcg.; the excretion was increased to 11 mcg. when the riboflavin intake was increased to 490 mcg. per 1000 calories. The same value was found when the intake was increased to 660 mcg. per 1000 calories.

Feder, Lewis, and Alden (1944) suggested that one-hour fasting excretions be calculated on a unit volume basis, but Davis et al. (1946) reported that the per hour values were more constant than the values per unit volume.

Studies on blood or blood fractions

Little work has been done on blood for evaluating the nutritional status with respect to riboflavin. Most data have been obtained on analyses of whole blood. Axelrod, Spies, and Elvehjem (1941) reported that there was no difference between the concentration of riboflavin in the blood of normal and in deficient subjects. For normal subjects the average value was 0.42 mcg. per ml., ranging from 0.35 to 0.45 mcg. per ml. Strong et al. (1941) found that, in normal subjects on an unrestricted diet, the concentration of riboflavin in whole blood was 0.49 mcg. per ml.

In 1948, Burch, Bessey and Lowry proposed a micro-method for the determination of riboflavin in fractions of blood; namely, serum, white cells and platelets, as well as red cells. They reported that for well-nourished adults the concentration of free riboflavin in serum was 0.8 mcg. per 100 ml., ranging

from 0.3 to 1.3 mcg. per cent, the concentration of riboflavin in the white blood cells and platelets was 252, ranging from 227 to 293 mcg. per cent, and the concentration in the red cells was 22.4 mcg. per 100 grams, ranging from 18.0 to 26.2 mcg. per 100 grams.

Estimation of past dietary intake

Past dietary intake has usually been used in surveys on nutritional status. Anderson and Milam (1945) made a survey of nutritional status among children in Durham, North Carolina. They took 7-day food intake records on each person and calculated the average daily intake of various nutrients including riboflavin. They found no correlation between the riboflavin intake and the incidence or severity of the corneal vascularization.

Relationship between riboflavin and creatinine excretion

Excretion of creatinine is presumed to be constant in normal persons, averaging about 1 gram per 110 pounds (50 kilograms) of body weight. The amount of riboflavin excreted per day can be roughly estimated from the amount of riboflavin excreted per gram of creatinine. This method is convenient when it is impossible to collect specimens during fixed intervals; it may correct findings in regard to size and age of individuals. The excretion of 400 mcg. or more daily, per gram of creatinine, is considered normal, and an excretion of less than 200 mcg. per gram of creatinine is unsatisfactory (Aykroyd et al., 1948).

HUMAN REQUIREMENTS OF RIBOFLAVIN

Evidence for assessing the desirable allowance of riboflavin is still rather incomplete. Based on different studies, various amounts have been suggested by many investigators. In 1941, Sebrell et al. suggested that a daily intake of riboflavin, 0.035 to 0.06 mg. per kilogram of body weight and roughly 0.9 mg. per 1000 calories, or about 3 mg. per day, was adequate for an adult. In the same year, Strong and co-workers (1941) studied the daily urinary excretion of subjects on unrestricted diets and found that the 24-hour excretion of riboflavin ranged from 500 to 800 mcg. However, when the intake of this vitamin was restricted to 1 to 2 mg. per day, the daily excretion rapidly decreased to 50 to 150 mcg. Since this amount, i.e., 1 to 2 mg. per day, was considered insufficient to meet the daily requirement, they increased the riboflavin intake from 2 up to 5 mg.; whereupon the excretion promptly increased. Based on the above studies, the Food and Nutrition Board of the National Research Council (1941) recommended an allowance of 2.5 mg. per day for active women, and for active men, 3.0 mg. per day, or 0.9 mg. of riboflavin per 1000 calories. Later investigations have shown that the above recommendation was too high. Oldham et al. (1944) studied two 5-year-old boys and reported that 0.53 mg. per 1000 calories appeared to be an adequate amount for them. Williams, Mason, Cusick and Wilder (1943) suggested that 0.5 mg. of riboflavin per 1000 calories was approximately the minimal daily requirement for the adult. They also found that, on an intake as low as 0.35 mg. per

1000 calories for 288 days, there was no clinical evidence of deficiency; but there was some tissue depletion. In another case, Keys et al. (1944) noted that active men maintained on a diet containing 0.31 mg. of riboflavin per 1000 calories (0.99 mg. per day) for five months showed no physiological handicap. In regard to this finding, however, Parsons (1944) pointed out that, judging from urinary excretion throughout the period, there appeared to be a slow depletion of tissue stores in these men.

From the previous studies, Copping (1945) suggested that 0.5 mg. of riboflavin per 1000 calories, or 0.029 mg. per kilogram of body weight, or 1.5 to 2.0 mg. per day was adequate for an adult. An additional amount was needed for the actively growing child and for the adult during pregnancy and lactation. On the basis of increasing evidence, the Food and Nutrition Board of the National Research Council (1945) revised the recommended allowance for riboflavin to 0.6 and 0.7 mg. per 1000 calories for moderately active women and men, respectively.

Davis et al. (1946) estimated an intake of 0.5 mg. of riboflavin per 1000 calories as satisfying the needs of adult women. This conclusion confirmed the findings of Williams et al. (1943).

In 1948, the Food and Nutrition Board of the National Research Council pointed out that the evidence from experiments with rats showed that no increased destruction of riboflavin occurred as the caloric consumption was increased. It seemed desirable that the recommended allowance should not be based on the caloric consumption

but rather on weight or some function thereof. Therefore the recommended allowance of riboflavin in adults was revised to 1.5 mg. for women and 1.8 mg. for men per day.

FACTORS AFFECTING RIBOFLAVIN REQUIREMENT

Many factors have been reported as affecting the requirement of riboflavin. The synthesis of riboflavin by intestinal flora, depending upon the nature of the food, has been reported by many investigators. Hathaway and Lobb (1946) found that the urinary excretion of riboflavin in subjects maintained on a diet of natural foods was 2.8 times greater than on a synthetic diet. Mannering et al. (1944) demonstrated that when carbohydrate, in the form of dextrin or cornstarch was used in the diet, rats needed less riboflavin; however, the substitution of sucrose, cellulose, or lard did not have this effect. This experiment showed that dextrin or cornstarch increased the intestinal synthesis and, therefore, decreased the riboflavin requirement.

Sarett, Klein, and Perlzweig (1942) found that the urinary excretion of riboflavin by both dogs and rats showed an inverse relationship to the level of protein intake. The period of low protein intake resulted in the highest excretion of this vitamin. This might indicate that an increase in the protein intake increased the amount of riboflavin required for metabolism. Sarett and Perlzweig (1943) demonstrated that the concentration of riboflavin in the liver varied directly with the amount of protein consumed.

The interrelationship of thiamine and riboflavin has been demonstrated by many workers. Supplee et al. (1942) found that thiamine was concerned in the mobilization of riboflavin from the tissues to the liver. Sure and Ford (1942) found a marked increase in the output of riboflavin in rats when the diet was restricted in thiamine. Davis et al. (1946) showed that the return of the test dose increased from 3.1 to 4.2 per cent from the period of an average ~~riboflavin~~ thiamine intake of 0.14 to an intake of 0.20 mg. per 1000 calories. Possibly, this phenomenon indicated that an increase in thiamine intake may have had some effect on the utilization of riboflavin.

On the basis of previous work on the role of riboflavin as a factor in the economy of utilization of food by rats, Sure and Dicheck (1941) found that their control animals gained 56 to 1300 per cent more in weight than litter mates which were in a state of riboflavin deficiency. The fat content of the rats showed the greatest gain and the protein content a considerable increase, but the change in ash content was too small and too variable to be significant.

The active growing child, the pregnant or lactating woman, and persons under physiological or pathological stress need a greater supply of riboflavin (National Research Council, 1948).

CHAPTER III

EXPERIMENTAL

PLAN OF EXPERIMENT

The study reported in this thesis was planned to determine the nutritional status with respect to thiamine and riboflavin, using adult women as subjects.

The daily intake of riboflavin was constant throughout the study. Two different levels of thiamine intake were used: during the first 15 days, the thiamine intake approximated 1000 mcg. per day and during the rest of the period (15 days) the thiamine intake approximated 600 mcg. per day.

In the 1950 study, the experiment began April 8, and ended May 11, and in the 1951 study, it began January 19 and ended February 17. The thiamine phase of the study was divided into two periods. The first* period was of 15 days' duration and was designed to test the National Research Council's recommended allowance of 500 mcg. of thiamine per 1000 calories per person per day. During this period, each subject took 400 mcg. of thiamine hydrochloride every morning before breakfast in order to meet the recommended allowance. The second period was of 15 days' duration, and each subject received only 300 mcg. of thiamine per 1000 calories daily and was supplied by the diet alone. Daily

* The first period of the 1950 study was extended to 19 days because during the first 4 days the Farrand fluorometer was erratic so the data for those days were not included.

determinations of the concentration of thiamine in the whole blood and in the urine were made. The details of this part of the experiment are not included in this thesis.

For the riboflavin phase of the study, daily determinations of the concentration of free and total riboflavin in serum and of riboflavin in urine were made. The creatinine excretion was also determined daily.

Daily fasting blood samples were collected each morning before breakfast from a finger prick. Blood samples were collected for the analyses of hematocrit, blood thiamine, and serum riboflavin. The micro-method of Burch, Bessey and Lowry (1948) was used for determining free and total serum riboflavin. The daily riboflavin excretion in urine was determined by the macro-method of Burch, Bessey and Lowry (1948).

Collection of urine

The urine was collected for each 24-hour period. Immediately following each voiding, the urine was measured and the volume recorded on the label on the bottle. In the 1950 study, the urine was then divided into two equal parts. One part was preserved with 2 per cent by volume of glacial acetic acid and stored in a brown bottle. The other part was stored in an ordinary plain bottle without a preservative. Both bottles were kept in a refrigerator or a cool place. At the end of each 24-hour period the two samples were measured and the total volumes recorded. Each sample was then shaken to assure thorough mixing. The preserved urine was used for

the thiamine and riboflavin determinations, and the unpreserved sample was used for the creatinine determination.

Later experiments showed that preservation of urine with 2 per cent by volume of glacial acetic acid had no effect on creatinine determination. In the 1951 study, therefore, each voiding was preserved with 2 per cent by volume of glacial acetic acid.

Collection of blood

The daily fasting blood samples were collected each morning before breakfast from a finger pricked with a Bard-Parker blade.

For hematocrit determination free flowing blood was collected in 4-inch lengths of 3 c.mm. diameter glass tubing which had been treated with heparin. A small vial was used for collecting the blood to be analyzed for thiamine. The blood sample for serum riboflavin determination was collected in a small vial also, but the blood could be squeezed or "milked" from the finger if necessary. Following the collection of the sample for serum riboflavin determination, the vials were covered with black paper and allowed to stand in a dark room for 30 minutes until clotting had taken place. The vials were then centrifuged at full speed in a clinical centrifuge for 15 minutes, and the serum was used for analysis.

DESCRIPTION OF SUBJECTS

Subjects of the 1950 study

Four graduate students, three from China and one from Korea,

were selected as subjects in this investigation. Three of the subjects were apparently normal and in good health and carried on their ordinary school work during the time of the study. Subject HHY had had an abdominal tumor removed a few months previously and was undergoing X-ray treatment for prevention of excessive scar tissue formation. At the time for the study she was nervous and depressed, suffering occasional recurring pains in the lower abdomen and lower part of the right breast. She was taking multiple vitamin pills and other medical treatment under a doctor's prescription. Despite this, she had a good appetite and worked as usual.

Age, height, weight and weight range for each experimental subject is shown in Table 1.

Table 1

AGE, HEIGHT, WEIGHT, AND WEIGHT RANGE OF EACH EXPERIMENTAL SUBJECT IN THE 1950 STUDY

Subject	Age	Height	Mean Weight	Weight Range
	yr.	in.	lb.	lb.
MLW	35	65.5	122	120.0-124.0
KD	25	62.5	106	105.0-108.0
SWW	24	60	106	105.0-106.5
HHY	37	64	128	126.0-129.5

Subjects of the 1951 study

Four adult women served as subjects for the 1951 study. They were all apparently healthy and carried on their regular activities.

Age, height, weight and weight range of each experimental subject is shown in Table 2.

Table 2

AGE, HEIGHT, WEIGHT AND WEIGHT RANGE OF EACH EXPERIMENTAL SUBJECT IN THE 1951 STUDY

Subject	Age	Height	Mean Weight	Weight Range
	yr.	in.	lb.	lb.
MLW	35	65.5	122	121.0-123.5
HAL	31	68	140	138.5-142.0
CAS	44	58.5	153	151.0-154.0
RBD	29	62	105	102.5-106

DIET

A modification of the diet of Gifft and Hauck (1946) was used for this study and it was adequate in all respects with the exception of thiamine and riboflavin. The daily intake (about 1.2 mg.) of riboflavin was constant throughout the study. The food was analyzed for riboflavin and thiamine and the results are shown in Tables 3 and 4. The basal diet contained about 1000 calories (Table 3), and additions to the basal diet were planned in units (Table 4). Each unit contained about 500 calories. Each subject had free choice in selecting the number of

Table 3

COMPOSITION OF THE BASAL DIET

Food	Amount	Calories**	Thiamine*		Riboflavin*		Protein**	Fat**	Carbohydrate**
			1950	1951	1950	1951			
	gm.		mcg.	mcg.	mcg.	mcg.	gm.	gm.	gm.
Milk, evaporated	100	139	75	75	336	373	7.0	7.9	9.9
Carrots, canned	100	30	23	18	32	22	0.5	0.4	6.1
Beef, round	100	177	47	77	110	27	19.5	11.0	-
Wheat germ	6	24	150	129		20	1.5	0.6	3.1
Pears, canned	100	75	8	10	21	12	0.2	0.1	18.4
Peaches, canned	100	75	13	11	25	18	0.4	0.1	18.2
Green beans, canned	100	22	35	45	57	67	1.0	0.1	4.2
Orange juice	166	80	100	127	141	68	1.0	0.3	18.4
Cream of wheat	30	108	17	19	17	-	3.3	0.2	23.2
Egg, E.P.	54	96	43	51	187	264	7.8	7.0	0.4
Cheese	30	120	15	14	161	181	7.5	9.7	0.6
Totals		946	526	576	1087	1052	49.7	37.4	102.5

* The thiamine and riboflavin values were obtained by chemical analyses in this laboratory.

** Values were calculated from the table of "Composition of Foods - Raw, Processed, Prepared," by Bureau of Human Nutrition and Home Economics, Agriculture Research Administration, Agriculture Handbook No. 8, 1950.

Table 4

UNIT ADDITION TO THE BASAL DIET

Food	Amount	Calories**	Thiamine*		Riboflavin*		Protein**	Fat**	Carbohydrate**
			1950	1951	1950	1951			
	gm.		mcg.	mcg.	mcg.	mcg.	gm.	gm.	gm.
Biscuits	55	186	17	14	11	8	4.5	5.9	28.7
Cookies	48	211	15	12	43	51	2.9	6.1	36.0
Butter	15	110	-	-	3	4	0.1	12.2	-
Sugar	10	40	-	-	-	-	-	-	10.0
Totals		547	32	26	57	63	7.5	24.2	74.7

1950

For 3 subjects:
Total riboflavin intake from food = 1201 mcg. daily
Total thiamine intake from food = 590 mcg. daily
Total calorie intake from food = 2038 daily

For subject HHY:
Total riboflavin intake from food = 1258 mcg. daily
Total thiamine intake from food = 619 mcg. daily
Total calorie intake from food = 2587 daily

1951

For all four subjects:
Total riboflavin intake from food = 1178 mcg. daily
Total thiamine intake from food = 628 mcg. daily
Total calorie intake from food = 2038 daily

* The thiamine and riboflavin values were obtained by chemical analyses in this laboratory.

** Values were calculated from the table of "Composition of Foods - Raw, Processed, Prepared," by Bureau of Human Nutrition and Home Economics, Agriculture Research Administration, Agriculture Handbook No. 8, 1950.

units to supplement the basal diet to meet her personal appetite and physiological needs, but the decision had to be made during the first three days, since it was necessary that the subjects eat a constant amount of food throughout the entire study. In the 1950 study, three subjects, MLW, KD, and SWW, took two units in addition to the basal diet; whereas subject HHY took three units. In the 1951 study all four subjects took two units in addition to the basal diet. Coffee and tea without cream and sugar were the only foods allowed ad libitum. Drummond and Moran (1944) stated that tea contained 9 micrograms of riboflavin per gram and coffee contained 1.7 micrograms per gram. All subjects took coffee but no tea (except HHY who occasionally had a cup of green tea) during the study. The riboflavin intake from coffee in any case was insignificant.

The menu for the 1950 study was as follows:

Breakfast	Lunch	Dinner
Orange juice	Cheese	Beef (found, mixed with wheat germ)
Egg	String beans	Carrots
Cream of wheat	Peaches	Pears
Coffee	Biscuits	Biscuits
Sugar	Butter	Butter
Evaporated milk	Cookies	

The menu for the 1951 study was about the same as above except that the wheat germ was served at breakfast only.

RECIPES

Biscuit Mix		Cookie Mix	
Flour, unenriched	660 grams	Flour, unenriched	450 grams
Shortening, Crisco	180 grams	Brown Sugar	450 grams
Salt	1 tb.	Shortening, Crisco	250 grams
Baking powder, Royal	55 grams	Eggs	2
Water	400 cc.	Salt	1 tsp.
		Soda	½ tsp.
		Vanilla	1 tb.
Bake at 450° F. for 12 minutes		Bake at 375° F. for 10 minutes	
900 grams approximately per recipe of mix 55 grams per person per meal 110 grams per person per day for four persons Mix lasted two days (4 meals) with four people at each meal. 20 X recipe for a 30-day period		96 grams per person per day 384 grams per day (4 people) 15 X recipe for a total of 30 days	
Sift flour, salt and baking powder in sieve four times. Add shortening and blend. Yields approximately 450 grams of mix. Keep in refrigerator. Weigh out 225 grams, add 100 cc. of water and mix about 50 strokes. Knead on unfloured board approximately 150 strokes. Roll out and cut in squares. Bake on ungreased tin. Yields approximately 250 grams baked.		Blend sugar and shortening. Add egg and vanilla. Mix well. Sift in salt, soda and flour and blend. Make into rolls (4-5 long ones) on unfloured board. Keep in refrigerator. Slice off as needed and bake on ungreased tins. Yields approximately 2-2½ rolls for 2 cookie sheets for each day.	

DETERMINATION OF RIBOFLAVIN IN FOOD

The modified method of Conner and Straub

The riboflavin of beans, carrots, pears, biscuits, cream of wheat, orange juice, and cookies was determined by using the modified method (Davey, 1945) of Conner and Straub (1941). The details of the method are as follows:

Equipment

1. Conical centrifuge tubes with glass stoppers (reaction vessels)
2. Calibrated optical tubes, pyrex, 10 x 75 mm.
3. Funnels
4. Filter paper
5. Syringe pipettes for 1 and 3 ml. capacity
6. Stop watch
7. Waring blendor
8. Farrand fluorometer
9. AH-5 mercury vapor lamp

Reagents

1. 2 per cent acetic acid solution : made fresh daily from a 20 per cent stock solution
2. 3 per cent polidase, freshly prepared : 3 grams of polidase dissolved and made up to 100 ml. with sodium acetate-acetic acid buffer solution at pH 4.5
3. Sodium acetate-acetic acid buffer pH 4.5 : 55 ml. glacial acetic acid and 66.64 gm. sodium acetate made

up to one liter with redistilled water

4. 4 per cent potassium permanganate : 4 gm. of potassium permanganate dissolved and diluted to 100 ml. with redistilled water

5. 3 per cent hydrogen peroxide : freshly prepared from Superoxol

6. Fluorescein standard solutions:

   Fluorescein stock solution: 10 mg. fluorescein dissolved in 5 ml. of 95 per cent alcohol and about 50 ml. of 0.1 N sodium acetate-acetic acid buffer solution of pH 4.5 and then made up to volume in a 1 liter volumetric flask, with 0.1 N sodium acetate-acetic acid buffer solution of pH 4.5.

A series of fluorescein standard solutions was prepared as follows:

Fluorescein	Standard	A = 10 c.mm.	stock solution	+ 10 ml.	0.1 N sodium acetate-acetic acid buffer, pH 4.5
"	"	B = 500 c.mm.	solution A	+ 1 ml.	" " " " " " " " "
"	"	C = 200 c.mm.	solution A	+ 1 ml.	" " " " " " " " "
"	"	D = 100 c.mm.	solution A	+ 1 ml.	" " " " " " " " "
"	"	I = 20 c.mm.	stock solution	+ 10 ml.	" " " " " " " " "
"	"	II = 30 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	III = 50 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	IV = 60 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	V = 100 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	VI = 150 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	VII = 200 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	VIII = 300 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	IX = 500 c.mm.	" "	+ 10 ml.	" " " " " " " " "
"	"	X = 1000 c.mm.	" "	+ 10 ml.	" " " " " " " " "

All these standards were placed in optical tubes and capped with waxed stoppers.

All reagents (except fluorescein standards) were kept in the refrigerator when they were not in use.

Method

Fifteen to 30 gm. of food were ground in the Waring blendor with 200 ml. of 2 per cent acetic acid for 3 minutes. One half the amount was weighed into a flask and 10 ml. of 3 per cent polidase solution were added. The contents of the flask were well mixed; then the flask was covered with aluminum foil and incubated at 37° C overnight.

The following procedure was performed in a darkened room: The extracts were filtered through dry filter paper, discarding the first few milliliters. Fifteen ml. of the extract were pipetted into a conical centrifuge tube. One ml. of 4 per cent potassium permanganate was added and the tube was capped and shaken vigorously for 1½ minutes. Three ml. of 3 per cent hydrogen peroxide were then added, and the tube was again shaken vigorously for 1½ minutes. One ml. of the above solution was pipetted into each of three 10 x 75 mm. calibrated optical tubes. The galvanometer reading, R_1, was made in the Farrand fluorometer. Readings were made by setting the fluorometer at 75 with the fluorescein standard V. If the reading was too high, beyond the scale, or too low, another suitable standard was used; but the final reading was converted to the setting at 75 with fluorescein standard V.

Following the readings, the optical tubes were capped with parafilm and exposed directly to the sunlight or to an AH-5 mercury vapor lamp for an hour, and then the reading, R_2, was read as blank. This blank corrected for any interfering substances giving fluorescence, other than riboflavin. A reagent blank substituting

redistilled water for the sample was treated and read in the same manner to obtain whatever fluorescence the reagents might render. Correction was made for the reagent blank.

A standard curve was obtained (see next paragraph) using different solutions of known riboflavin content. The readings were taken by setting the fluorometer at 75 with fluorescein standard V. The amounts of riboflavin in the food samples were evaluated from the curve (Figure 1 and Table 5). The method of calculation is shown in Table 6.

Determination of riboflavin standard curve for food analysis

1. Riboflavin stock solution : 20 mg. crystalline riboflavin dissolved and diluted to one liter with 0.01 N hydrochloric acid.
2. First dilution : 10 ml. of the above stock solution was made up to 100 ml. with 2 per cent acetic acid. The concentration of this solution was 2 mcg. of riboflavin per ml.
3. Working solutions : dilution of the above "first dilution" with 2 per cent acetic acid to the following concentrations:
    a. 1 ml. of "first dilution" to 200 ml. so there was 1 mcg. per 100 ml. or 0.01 mcg. per ml.
    b. 1 ml. of "first dilution" to 100 ml. so there were 2 mcg. per 100 ml. or 0.02 mcg. per ml.
    c. 2 ml. of "first dilution" to 100 ml. so there were 4 mcg. per 100 ml. or 0.04 mcg. per ml.

One ml. of each working solution was transferred to an optical tube, and the initial reading was taken with the instrument setting of 75 with fluorescein standard solution V. Then, the solution was irradiated with an AH-5 mercury vapor lamp for an hour and the solution re-read with the same instrument setting. Thus, a blank reading was obtained. The initial reading minus the blank reading gave the corrected reading (Table 5).

Table 5

RIBOFLAVIN STANDARD CURVE

Concentration of riboflavin standard	Mean corrected reading
mcg. %	
1	17.75
2	34.63
4	69.44

The modified method of Kodicek and Wang

Preliminary work using the modified method of Conner and Straub gave high blank readings for foods containing large amounts of fat and protein. As a result, these foods: egg, meat, cheese, milk and butter were analyzed by our modification of the method of Kodicek and Wang (1949). The details of this method are as follows:

Equipment

1. Centrifuge tubes (50 ml. capacity)

FIGURE 1

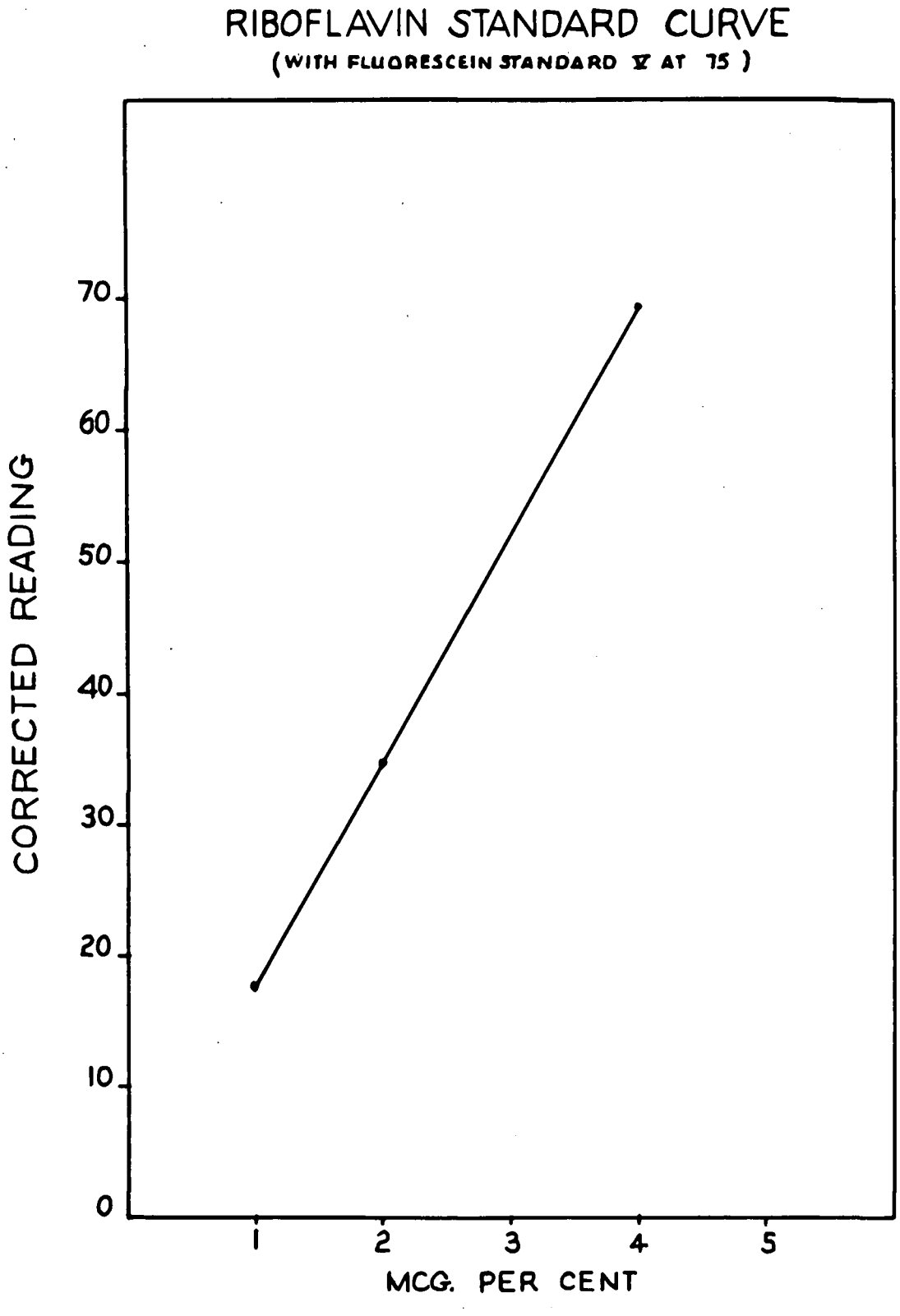

RIBOFLAVIN STANDARD CURVE
(WITH FLUORESCEIN STANDARD Y AT 75)
CORRECTED READING
70
60
50
40
30
20
10
0
1
2
3
4
5
MCG. PER CENT

Table 6

CALCULATION OF RESULTS FOR FOODS ANALYZED ACCORDING TO THE MODIFIED METHOD OF CONNER AND STRAUB

Samples		Reading with fluorescein standard V set at 75		R_1-R_2		Reading of sample - Reading of Reagent Blank	Concentration of riboflavin in aliquot	Concentration of riboflavin in sample
		R_1	R_2 (blank)					
							mcg. %	mcg. %
H_2O		0.75						
Reagent Blank	1	45.00	18.00	27.00				
	2	46.25	18.25	28.00	27.33			
	3	46.00	19.00	27.00				
Peach	1	82.00	22.75	59.25				
	2	82.00	24.00	58.00	58.42	31.09	1.78*	18.7**
	3	82.00	24.00	58.00				

* Read from curve (Figure 1)

** Calculated from the formula

$$\frac{\text{mcg. \% riboflavin in aliquot}}{\text{gm. \% sample in aliquot}} \times 100 = \text{riboflavin mcg. \% in sample}$$

30 gm. of sample were used, therefore,

$$\frac{1.78}{9.50} \times 100 = 18.7 \text{ mcg. \% riboflavin in peach}$$

2. Conical centrifuge tubes with glass stopper (reaction vessels)
3. Calibrated optical tubes, 10 X 75 mm.
4. Erlenmeyer flasks, 125 ml. capacity
5. Stop watch
6. Waring blendor
7. Farrand fluorometer
8. AH-5 mercury vapor lamp
9. Centrifuge

Reagents

1. 0.1 M hydrochloric acid
2. 2 per cent sodium hydroxide
3. 25 per cent metaphosphoric acid, freshly prepared
4. 4 per cent potassium permanganate
5. 3 per cent hydrogen peroxide, freshly prepared from Superoxol
6. Chloroform, redistilled
7. Petroleum ether
8. Sodium dithionite (hydrosulfite) solution : 0.5 gm. sodium dithionite (hydrosulfite) and 0.6 gm. sodium bicarbonate dissolved in 10 ml. water. Prepared immediately before use.
9. Riboflavin standard solution: 25 mg. crystalline riboflavin in 50 ml. redistilled water and 1 ml. glacial acetic acid. The solution was mixed with a

further 500 ml. redistilled water (at 50°), and shaken until the riboflavin was completely dissolved. Then it was made up to 1 liter with redistilled water and ethanol so that the final concentration of ethanol was 20 per cent. The solution was kept in a brown bottle in the refrigerator. The internal standard solution which was made from the above standard solution contained 5 mcg. of riboflavin per ml. : 10 ml. of the standard solution was diluted to 50 ml. with 20 per cent ethanol and one drop of concentrated hydrochloric acid.

10. One per cent acetic acid
11. Hydrion paper

Method

The high fat and protein-rich foodstuffs were analyzed by the modified method of Kodicek and Wang (1949). Except for milk which was in liquid form and, therefore, not washed with petroleum ether, each food sample (6 to 30 gm.), containing about 15 mcg. of riboflavin, was washed twice with light petroleum (petroleum ether). The liquid was poured off and the remaining petroleum ether was evaporated in a water bath. The sample was ground with 50 ml. of 0.1 N hydrochloric acid in a Waring blendor for 3 minutes. Half of the amount was weighed out in an Erlenmeyer flask and then heated in a boiling water bath for 30 minutes. At the end of this period the sample was

cooled, and 2.5 ml. of 25 per cent metaphosphoric acid were added. The contents of the flask were well mixed by agitation. The flask was allowed to stand for 10 minutes before the contents were transferred to a centrifuge tube. The tube was centrifuged for 15 minutes at full speed. The supernatant was decanted into a small beaker and the residue was re-extracted with 17 ml. of one per cent acetic acid. (It was not necessary to heat it.) The tube was then centrifuged again. The extracts were pooled together and brought up to a volume of 50 ml. with redistilled water. Ten ml. of the sample were washed with an equal amount of redistilled chloroform by shaking vigorously 1½ minutes in a glass-stoppered conical centrifuge tube. The layers were separated quickly by centrifugation. A 5 ml. aliquot of the aqueous layer was transferred to a small beaker and oxidized with 4 per cent potassium permanganate, drop by drop with continuous stirring. The pink color was allowed to disappear completely before adding the next drop. The addition of potassium permanganate was stopped when the faint pink color lasted for 30 seconds; then 1 to 2 drops of 3 per cent hydrogen peroxide were added to stop the oxidation and to decolorize the excess potassium permanganate. After five minutes the aliquot was neutralized to pH 5.5 to 6.0 with 2 per cent sodium hydroxide (using hydrion paper as the indicator). The final solution was made up to 15 ml. with redistilled water. One ml. of the aliquot was transferred to

each of three 10 X 75 mm. optical tubes, and the apparent riboflavin content was measured by reading the optical density in a Farrand fluorometer.

Two aliquots of the triplicate sample were measured by following the modification of the Kodicek and Wang method. After the initial reading, R_1, was made; 10 c.mm. of the internal standard were added to one tube, and 10 c.mm. of redistilled water were added to the other tube. Both tubes were tapped with a finger to insure mixing, care being taken not to touch the lower part of the tube. The second reading, R_2, was made. The R_2 reading of the first tube minus the R_2 reading of the second tube gave the reading for the internal standard, 0.05 mcg. per tube. The third reading, the blank, was obtained by adding 10 c.mm. of sodium dithionite (hydrosulfite) solution to each tube. The initial reading, R_1, minus the blank reading gave the reading for the riboflavin content in the sample. The amount of riboflavin in the solution was calculated from the ratio value of the internal standard. Since the reading for the internal standard varied slightly for different samples, the third tube was read without the internal standard but evaluated by using the standard curve. After the initial reading, R_1, was obtained, the tube was either capped with parafilm and irradiated by the AH-5 mercury vapor lamp for an hour, or 10 c.mm. sodium hydrosulfite were added to each tube and then read again to obtain the blank reading. The reading

for riboflavin in the aliquot was calculated from R_1 minus the blank reading to give the corrected reading. This corrected reading was used to determine the amount of riboflavin per ml. of the solution, using the standard curve. All readings were read at the same instrument setting or converted to the same setting. The riboflavin values in food were calculated in both ways, i.e., 1. using the internal standard as well as 2. reading from the standard curve, and the results agreed closely, but the latter method was finally used. The method of calculation is shown in Table 7.

The results of food analyses for riboflavin are given in tables 3 and 4.

DETERMINATION OF FREE AND TOTAL RIBOFLAVIN IN SERUM

Equipment

1. 10 X 75 mm. pyrex test tubes
2. 10 X 75 mm. pyrex test tubes which were calibrated for the optical use

   All tubes were boiled in 1:1 concentration nitric acid for half an hour and rinsed 8 times with tap water and 8 times with redistilled water, then boiled with redistilled water for half an hour.
3. Constriction pipettes : 10, 100, 200, 800 c.mm.
4. Syringe pipette for 2 ml. capacity
5. Farrand fluorometer (Farrand Optical Company, Inc., Bronx Boulevard and East 238th Street, New York 66)

Table 7

CALCULATION OF RESULTS FOR FOOD ANALYZED ACCORDING TO THE MODIFIED METHOD OF KODICEK AND WANG

Samples		Reading with fluorescein standard V set at 75		R'_2* (Blank)	$R_1-R'_2$	Concentration of riboflavin in aliquot	Concentration of riboflavin in sample
		R_1	R_2				
						mcg. %	mcg. %
H_2O		0.75					
Egg IV_2	1	51.00	2.50	2.53	48.47	2.77**	277***
	2	51.00	2.25	2.27	48.73	2.79	279
	3	51.00	2.50	2.53	48.47	2.77	277

* $R'_2 = R_2 + (R_2 \times 0.01)$

** Read from the curve (Figure 1)

*** 6 gm. of egg were used for each sample, therefore, mcg. per cent riboflavin in sample $= \frac{2.77}{0.01} = 277$

6. Wire rack which had been fitted with strings so that the test tubes would stand upright
7. Centrifuge (The International Clinical centrifuge with regular micro heads)
8. Incubator which could be set at 37°C
9. Blade (Bard-Parker, No. 11)
10. Agitator made according to the directions of Lowry (1950)
11. Parafilm

Reagents

1. 100 per cent trichloroacetic acid solution : 100 gm. of redistilled (under diminished pressure) trichloroacetic acid diluted to 100 ml. with redistilled water. From this a 5 per cent solution was prepared every other day.
2. 2.4 N dipotassium acid phosphate solution
3. Riboflavin standard solution : 20 mg. of crystalline riboflavin dissolved and diluted to 1000 ml. with 0.1 N hydrochloric acid. From this the internal standard was prepared daily: 1 ml. of stock solution diluted to 100 ml. with 0.1 N hydrochloric acid. Thus the internal standard contained 0.2 mcg. riboflavin per ml. or 2 mmcg. in 10 c.mm.
4. Sodium hydrosulfite solution : 0.5 gm. sodium hydrosulfite was dissolved in 5 ml. of 5 per cent sodium bicarbonate and was prepared just before use. This reagent was kept in a small beaker (20 ml. capacity) in ice water in order to delay oxidation by air. It is not stable for more than ½ hour.

Method

The micro-method of Burch, Bessey and Lowry (1948) was used for the determination of riboflavin in serum. The analysis was carried out in a darkened room equipped with red lamps. Since the amount of aliquot was not sufficient to read in the cuvette, the amount was doubled; 100 c.mm. of serum were delivered with a constriction pipette into 2.0 ml. of 5 per cent trichloroacetic acid in a 10 X 75 mm. pyrex test tube which was kept cold in an ice bath. The contents were well mixed using a buzzer. Tubes were allowed to stand at 0 to 5° C (in ice water) for 15 minutes and then were centrifuged in the refrigerator for 15 minutes at full speed. Then 0.8 ml. of the supernatant was quickly transferred to each of two calibrated optical tubes, the first of which contained 0.2 ml. of 2.4 M dipotassium acid phosphate. The other tube was reserved for the determination of total riboflavin as described below. The apparent riboflavin content (A) of the tube containing the neutralized extract was measured in the Farrand fluorometer within one to two hours of neutralization. All tubes were kept covered with a dark paper in order to prevent contamination and destruction of the riboflavin by light. The tubes were carefully wiped with a slightly damp linen cloth followed by a dry linen cloth and then the three tubes were read with the same instrument setting at 75 using fluorescein standard A. If the readings were too low or beyond the scale, a more suitable fluorescein standard was used. The final readings were: an initial reading, R_1, a second reading, R_2, after

the addition of an internal riboflavin standard (10 c.mm. equivalent to 2 mmcg. of riboflavin), and a reduced reading, R_3, after the addition of 10 c.mm. of sodium hydrosulfite solution. The solution was mixed by tapping with a finger. A complete reagent blank solution, in triplicate, was treated in the same manner. A reading for a tube containing redistilled water was used as a measure of scattered light and possible fluorescence from the tube itself.

The second tube of filtrate was capped with parafilm, and was allowed to hydrolyze in the incubator at 37° C overnight. It was then neutralized with 0.2 ml. of 2.4 M dipotassium acid phosphate, and the total riboflavin content (B) was measured as described above.

Calculation of results

According to Burch, Bessey and Lowry, (1948), the readings R_2 and R_3 were corrected for the dilution resulting from the addition of the internal standard and the reducing agent, and they were designated R'_2 and R'_3. The R'_2 is one per cent of R_2 plus R_2; and R'_3 is 2 per cent of (R_3-H_2O) plus (R_3-H_2O) and plus H_2O. (The reading for redistilled water was subtracted from the sample reading before correcting for dilution, since the contribution from scattered light would not be affected by dilution).

$$\text{micrograms \% riboflavin} = \left(\frac{\text{micrograms riboflavin added}}{\text{ml. serum in aliquot}}\right)\left(\frac{R_1-R'_3}{R'_2-R_1}\right)(100)$$

The above figure was corrected for the reagent blank which had been

treated and calculated in the same manner as the sample (Table 8).

Since flavin-adenine-dinucleotide (FAD) gives above 14 per cent fluorescence, before the analysis, the

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

and, therefore

micrograms % of free (+FMN) riboflavin = A - 0.14 FAD

= 1.163 A - 0.163 B

DETERMINATION OF RIBOFLAVIN IN URINE

Equipment

1. Test tubes, 1.5 X 12 cm. test tubes
2. Parafilm
3. Calibrated optical tubes, pyrex, 10 X 75 mm.
4. Syringe pipettes for 0.4, 1, and 7 ml. capacity
5. Test-tube racks
6. Centrifuge
7. Farrand fluorometer

Reagents

1. 3.25 M Sodium acetate-acetic buffer pH 4.6
2. Ammonium sulfate (previously washed 3 or 4 times with 95 percent alcohol and ether to remove fluorescent substances)
3. Benzyl alcohol, C.P., redistilled, and saturated with water
4. 45 per cent ethyl alcohol which was 0.1 N in acetic acid and 0.1 N in sodium acetate
5. 4 per cent potassium permanganate

Table 8

CALCULATION OF RIBOFLAVIN IN SERUM

a. Free (+ FMN) riboflavin in serum

Samples		Reading with fluorescein standard V set at 75 R_1	R_2	R_3	R_3-H_2O	R'_2	R'_3	R'_2-R_1	$R_1-R'_3$	$\frac{R_1-R'_3}{R'_2-R_1}$	Riboflavin in Serum Without Correction	Corrected from Reagent Blank
											mcg. %	mcg. %
Redistilled H_2O		3.00										
Reagent blank	1	17.00	41.50	17.00	14.00	41.92	17.28	24.92	-0.28			
	2	17.25	41.75	17.75	14.75	42.17	18.05	24.92 25.00	-0.80 -0.62			
	3	17.00	41.75	17.50	14.50	42.17	17.79	25.17	-0.79	-0.025	-0.13	
MLW	1	25.00	48.00	19.50	16.50	48.48	19.83	23.48	5.17	0.22	1.17	
	2	25.00	48.50	19.75	16.75	48.99	20.09	23.99	4.91	0.20	1.06 1.13	1.26 (A)
	3	25.00	49.00	19.25	16.25	49.49	19.58	24.99	5.42	0.22	1.17	

b. Total riboflavin in serum

Redistilled H_2O		3.00										
Reagent blank	1	13.25	38.00	14.25	11.25	38.38	14.48	25.13	-1.23			
	2	13.25	38.00	14.25	11.25	38.38	14.48	25.13 25.13	-1.23 -0.97			
	3	13.25	38.00	13.50	10.50	38.38	13.71	25.13	-0.46	-0.039	-0.21	
MLW	1	54.25	82.00	22.25	19.25	82.82	22.64	28.57	31.61	1.11	5.88	
	2	47.25	74.00	20.75	17.50	74.74	21.11	27.74	28.89	0.93	4.93 5.30	5.51 (B)
	3	49.25	74.00	24.25	21.25	74.74	24.68	25.49	24.57	0.96	5.09	

Free Riboflavin = 1.163 (A) - 0.163 (B)

= 1.47- 0.90

= 0.57 mcg. % of free (+ FMN) riboflavin in serum

6. 30 per cent hydrogen peroxide (Superoxol)
7. Riboflavin internal standard solution : 10 ml. of stock solution (20 mg. crystalline riboflavin per 1000 ml.) made up to 100 ml. with redistilled water. 10 c.mm. of internal standard (containing 20 mmcg. riboflavin in 10 c.mm.) were used for each tube.

Method

Urinary riboflavin determinations were made following the macro-method of Burch, Bessey and Lowry (1948). The preserved urine was removed from the refrigerator and left standing in a dark room for about an hour before sampling in order for it to reach room temperature. In general, the urine was diluted 1 to 5. Four ml. of diluted urine sample plus 0.4 ml. of 3.25 M sodium acetate-acetic acid pH 4.6 buffer were transferred to a 1.5 X 12 cm. test tube and oxidized with one ml. of 4 per cent potassium permanganate by shaking the tube for one minute right after the addition of potassium permanganate. Oxidation was stopped by the addition of 0.1 ml. of hydrogen peroxide; this also bleached the excess of potassium permanganate. Then 4 gm. of washed ammonium sulfate were added and mixed by agitation. Three ml. of redistilled benzyl alcohol, saturated with water, were added to extract the riboflavin. The tube was shaken vigorously for 1½ minutes and then centrifuged for 10 minutes to accelerate the separation of the alcohol layer. Then one ml. of this alcohol extract was transferred to a 1.5 X 12 cm. test tube which contained 7 ml. of 45 per cent alcohol buffer,

and the solution was mixed with the aid of a buzzer. One ml. of this solution was transferred to a 10 X 75 mm. calibrated optical tube and the riboflavin measurement was made in the same manner as for serum riboflavin.

The method of calculation is shown on the following page and in Table 9.

Calculation of results

The following is an example of the calculation of the micrograms of riboflavin excreted in a 24-hour collection of urine which was diluted 1 to 5 for analysis:

$$\text{Mcg. riboflavin excreted in 24 hours} = (8)(3)(5)\left(\frac{\text{micrograms riboflavin added}}{\text{ml. diluted urine sample used}}\right)\left(\frac{R_1-R'_3}{R'_2-R_1}\right)^{*} \text{ (volume of urine)}$$

$$=(120)\left(\frac{0.02 \text{ mcg. riboflavin added}}{4 \text{ ml.}}\right)\left(\frac{R_1-R'_3}{R'_2-R_1}\right) \text{ (volume of urine)}$$

$$=(120)\ (0.005)\left(\frac{R_1-R'_3}{R'_2-R_1}\right) \text{ (volume of urine)}$$

$$=(0.6)\left(\frac{R_1-R'_3}{R'_2-R_1}\right) \text{ (volume of urine)}$$

* The value for $\frac{R_1-R'_3}{R'_2-R_1}$ is corrected for the reagent blank as shown in Table 9.

Table 9

CALCULATION OF RIBOFLAVIN IN URINE

Samples		Reading with fluorescein standard V set at 75			R_3-H_2O	R'_2	R'_3	R'_2-R_1	$R_1-R'_3$	$\frac{R_1-R'_3}{R'_2-R_1}$		Urine Volume	Riboflavin daily excretion
		R_1	R_2	R_3								ml. per 24 hrs.	mcg. per 24 hrs.
H_2O		0.75											
Reagent blank	1	2.00	42.00	2.25	1.50	42.42	2.28	40.42	-0.28	-0.01			
	2	2.25	43.00	2.50	1.75	43.43	2.54	41.18	-0.29	-0.01	0.01		
	3	2.00	43.00	2.50	1.75	43.43	2.54	41.43	-0.54	-0.01			
HAL	1	41.00	83.00	6.00	5.25	83.83	6.11	42.83	34.89	0.81			
	2	41.00	83.00	6.00	5.25	83.83	6.11	42.83	34.89	0.81	0.81	1511	372**
	3	41.00	83.00	6.00	5.25	83.83	6.11	42.83	34.89	0.81	(0.82*)		

* The figure was corrected for the reagent blank.

** Mcg. riboflavin excreted in 24 hours $= \left(\frac{R_1-R'_3}{R'_2-R_1}\right)(0.6)(1511)$

STUDY OF THE EFFECT OF A 2 MG. ORAL TEST DOSE OF RIBOFLAVIN ON RIBOFLAVIN CONTENT OF URINE AND SERUM

Approximately two weeks after the completion of the study in 1951, a 2 mg. oral test dose of riboflavin was given to each experimental subject. The solution was made by dissolving and diluting 20 mg. riboflavin to 500 ml. with redistilled water. The test dose, 50 ml. of the above solution, containing 2 mg. of riboflavin, was given to each subject immediately after the collection of the fasting blood sample and the one-hour fasting urine sample. Water, soda crackers, and black coffee were the only foods allowed until the end of the test dose study. Blood and urine samples were collected at one-half hour intervals for the first hour and thereafter at hourly intervals for 5 hours following the ingestion of the test dose.

CHAPTER IV

RESULTS AND DISCUSSION

SERUM RIBOFLAVIN FOR THE EIGHT SUBJECTS DURING THE EXPERIMENTS OF 1950 AND 1951

The results of the determination of the daily concentration of free (+FMN) and total riboflavin in the serum and in the 24-hour urine collections for 30 days for eight women on a controlled diet, regardless of the two levels of thiamine intake, have been recorded in Tables 10, 11, 12 and 13 and Figures 2 and 3. The mean values for total riboflavin for MLW, KD, SWW, and HHY were 2.93, 3.02, 5.30, and 3.51, respectively; and the mean values of free (+FMN) riboflavin were 1.00, 1.24, 3.50, and 1.49, respectively. For subjects MLW, HAL, CAS, and RBD the total riboflavin values were 2.61, 3.43, 2.86, and 2.36; and the mean values for free (+FMN) were 0.72, 1.44, 1.63, and 0.47, respectively. Although HHY took a high dosage of vitamin pills every day, her serum riboflavin concentration, both in total and free (+FMN) forms, was close to that of the rest of the group. SWW was apparently much higher in serum riboflavin concentration than were the others of the group. Horwitt et al. (1950) claimed that there were important individual variations in the amount of riboflavin excreted by some of their subjects, even though the riboflavin intake was controlled and in this study there were individual variations in serum riboflavin.

Table 10

DAILY CONCENTRATION OF TOTAL AND FREE (+ FMN) RIBOFLAVIN IN THE SERUM OF FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET FOR 30 DAYS IN 1950

Day of Experiment		MLW		KD		SWW		HHY	
		Serum Riboflavin		Serum Riboflavin		Serum Riboflavin		Serum Riboflavin	
		Total	Free (+ FMN)	Total	Free (+ FMN)	Total	Free (+ FMN)	Total	Free (+ FMN)
		mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %
1	4-13-1950	2.62	1.91	2.91	2.36	5.75	2.55	3.88	1.89
2	14	2.59	0.84	3.11	1.22	5.74	3.69	3.65	1.54
3	15	3.33	0.61	3.19	0.95	6.10	3.45	3.95	1.07
4	16	2.04	1.30	2.39	1.53	5.06	3.48	3.01	1.88
5	17	3.01	1.18	3.14	1.19	5.47	3.11	3.54	1.55
6	18	2.99	1.06	2.90	1.25	5.57	3.84	3.47	1.47
7	19	3.21	0.98	3.05	1.48	5.30	3.89	3.00	1.78
8	20	3.30	0.77	3.19	0.91	5.77	3.46	3.78	1.58
9	21	2.61	0.78	2.19	0.99	5.15	3.39	3.03	1.21
10	22	2.61	0.81	2.65	1.01	4.94	2.64	2.81	1.05
11	23	2.70	1.10	3.09	1.21	5.46	3.66	2.93	1.06
12	24	2.29	1.15	2.06	1.23	4.18	3.74	2.74	1.55
13	25	2.15	1.09	2.22	1.24	3.97	3.79	2.49	1.81
14	26	2.22	1.19	2.57	1.18	4.22	3.65	3.49	1.65
15	27	2.35	1.06	3.16	1.52	3.62	-	3.41	1.95
16	28	3.57	0.84	2.77	1.20	4.76	3.23	3.59	1.46
17	29	2.11	1.18	3.06	1.56	3.54	4.23	1.72	-
18	30	3.19	0.56	3.68	0.81	6.25	3.35	4.16	0.94
19	5- 1-1950	-	-	4.20	1.01	-	-	4.72	1.07
20	2	3.37	0.87	3.23	1.25	5.44	3.42	3.71	1.31
21	3	3.00	1.13	2.85	1.25	5.13	3.39	3.22	1.72
22	4	3.01	0.89	3.02	1.15	5.87	3.23	3.82	1.64
23	5	3.05	1.34	2.98	1.63	5.88	3.83	3.46	1.74
24	6	3.03	0.95	3.00	1.06	5.82	3.49	4.06	1.61
25	7	3.26	0.76	3.28	1.56	5.57	3.50	4.01	1.34
26	8	3.32	0.89	3.14	1.36	5.42	3.43	3.55	1.47
27	9	3.28	0.82	2.90	1.12	5.39	3.39	3.58	1.50
28	10	3.66	0.85	3.58	0.77	6.15	3.52	4.17	1.37
29	11	3.35	1.10	3.42	1.23	6.06	3.79	4.04	1.61
30	12	3.74	0.93	3.65	1.11	6.23	3.89	4.36	1.38
	Average	2.93	1.00	3.02	1.24	5.30	3.50	3.51	1.49

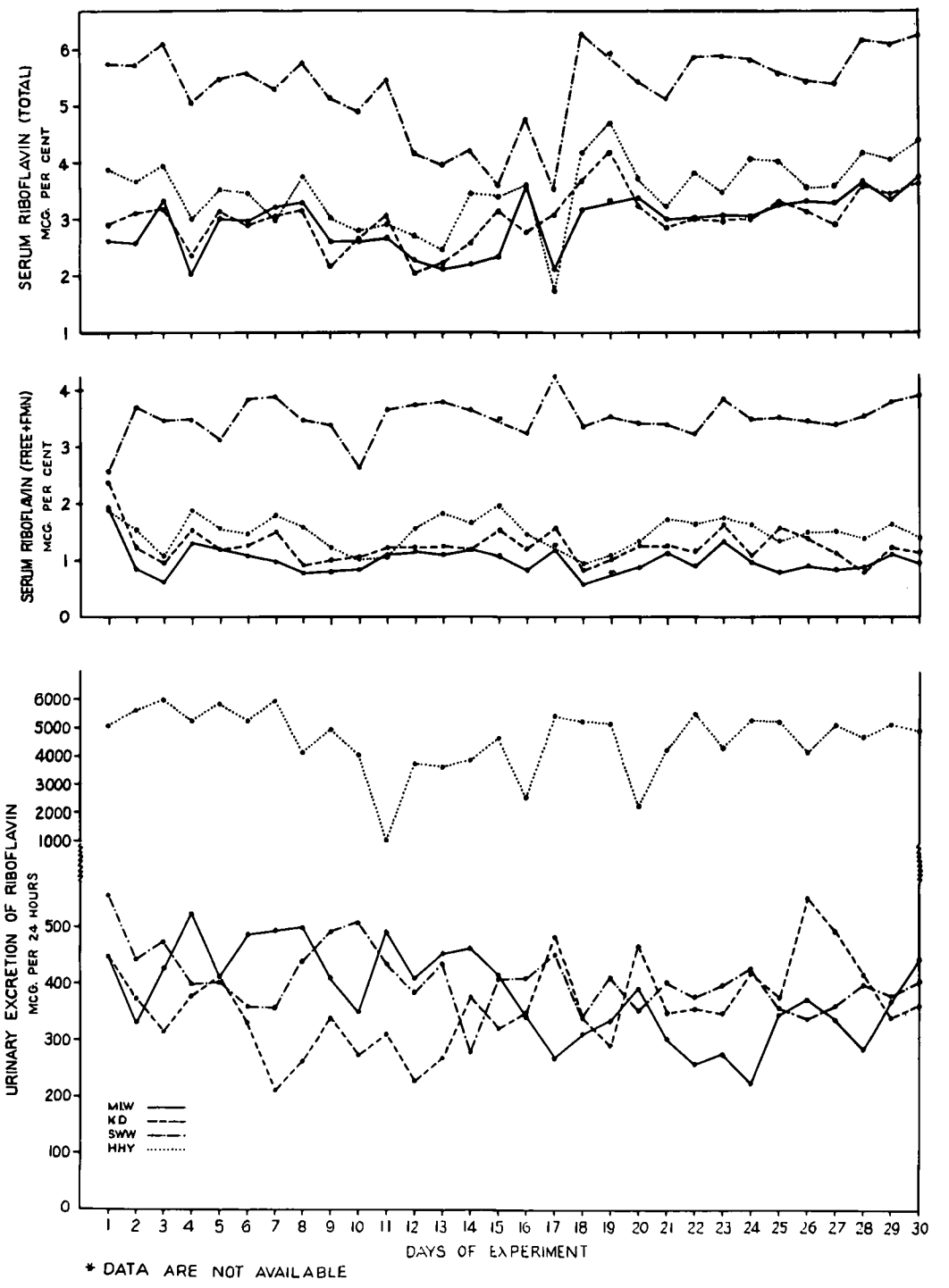
Table 11

DAILY EXCRETION OF RIBOFLAVIN AND CREATININE IN THE URINE, THE EXCRETION OF RIBOFLAVIN IN TERMS OF MCG. PER ML. OF URINE AND THE VOLUME OF URINE EXCRETED BY FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET FOR 30 DAYS IN 1950

Day of Experiment	Date	MLW				KD				SWW				HHY			
		Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine	Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine	Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine	Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine
		mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.	mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.	mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.	mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.
1	4-13-1950	446	1924	0.23	1.04	448	820	0.55	1.07	554	1000	0.55	0.89	5039	1768	2.85	1.06
2	14	330	1140	0.29	1.00	374	1126	0.33	1.08	442	734	0.60	0.77	5582	1784	3.13	1.06
3	15	426	1383	0.31	1.00	315	1406	0.22	1.11	473	882	0.54	0.85	5939	1474	4.03	1.11
4	16	522	1410	0.37	1.07	373	1180	0.32	1.14	398	756	0.53	0.78	5221	1940	2.69	1.11
5	17	410	1575	0.26	1.06	410	1060	0.39	1.10	404	1080	0.37	0.86	5809	1770	3.28	1.06
6	18	487	1740	0.28	1.08	331	1070	0.31	1.10	358	1073	0.33	0.81	5250	1757	2.99	1.06
7	19	492	1080	0.46	1.09	211	1104	0.19	0.90	356	990	0.36	0.89	5912	1494	3.96	1.01
8	20	499	980	0.51	1.08	262	1290	0.20	1.11	439	940	0.47	0.83	4119	1227	3.36	1.06
9	21	410	2030	0.20	1.12	337	1530	0.22	1.11	491	1111	0.44	0.85	4956	1763	2.81	1.07
10	22	350	1444	0.24	1.06	274	1130	0.24	1.11	507	1050	0.48	0.91	4056	1661	2.44	1.06
11	23	491	2042	0.24	1.13	311	1292	0.24	1.11	435	842	0.52	0.89	1031	1800	0.57	1.08
12	24	408	1400	0.29	1.12	229	1490	0.15	1.10	383	944	0.41	0.78	3765	1660	2.27	1.07
13	25	452	1555	0.29	1.08	267	1256	0.21	1.17	435	962	0.45	0.93	3663	1500	2.44	1.06
14	26	462	1050	0.44	1.06	376	1130	0.33	1.13	279	838	0.33	0.73	3860	2166	1.78	1.11
15	27	413	1280	0.32	1.05	320	1010	0.32	1.07	407	1260	0.32	0.81	4678	1611	2.90	1.09
16	28	342	1185	0.29	0.90	347	1443	0.24	1.09	409	1116	0.37	0.87	2561	1893	1.35	1.05
17	29	269	1252	0.21	0.98	484	1052	0.46	1.11	452	1292	0.35	0.89	5439	1530	3.56	1.06
18	30	310	1070	0.29	0.95	339	810	0.42	1.09	342	790	0.43	0.89	5283	1426	3.71	1.04
19	5- 1-1950	334	1800	0.19	1.01	293	1440	0.20	1.08	411	980	0.42	0.85	5176	1570	3.30	1.10
20	2	391	1429	0.27	1.03	467	1330	0.35	1.24	351	1000	0.35	0.83	2285	1727	1.32	1.06
21	3	302	2146	0.14	1.05	349	1240	0.28	1.08	401	1115	0.36	0.84	4278	1681	2.55	1.05
22	4	258	1750	0.15	1.08	306	1454	0.21	1.21	377	743	0.51	0.88	5557	1469	3.78	1.18
23	5	278	1751	0.16	1.09	349	1040	0.34	1.12	399	1030	0.39	0.93	4318	1999	2.16	1.16
24	6	225	1527	0.15	1.04	420	1084	0.39	1.12	426	1243	0.34	0.93	5321	1429	3.72	1.11
25	7	345	1311	0.26	1.05	377	1060	0.36	0.86	357	702	0.51	0.86	5275	1596	3.30	1.10
26	8	374	1688	0.22	1.10	554	1850	0.30	1.15	339	1131	0.30	0.82	4172	1289	3.24	0.87
27	9	338	1380	0.24	1.02	495	1390	0.36	1.05	361	1234	0.29	0.88	5169	1696	3.05	1.05
28	10	284	1270	0.22	1.05	419	1085	0.39	1.06	399	1273	0.31	0.87	4722	2055	2.30	1.09
29	11	368	1350	0.27	1.12	343	1380	0.25	1.10	380	1270	0.30	0.92	5173	1360	3.80	1.14
30	12	444	2300	0.19	1.12	361	1364	0.26	1.07	454	1126	0.40	0.92	4980	1505	3.31	1.07
	Average	382		0.27	1.05	358		0.30	1.09	407		0.41	0.86	4620		2.87	1.07
Riboflavin Excreted in Urine Expressed in Terms of Per Cent of Intake		32				30				34							

FIGURE 2

DAILY CONCENTRATION OF TOTAL AND FREE (+FMN) RIBOFLAVIN IN THE SERUM AND THE URINARY EXCRETION OF RIBOFLAVIN FOR FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET FOR 30 DAYS IN 1950



* DATA ARE NOT AVAILABLE

Table 12

DAILY CONCENTRATION OF TOTAL AND FREE (+ FMN) RIBOFLAVIN IN THE SERUM OF FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET FOR 30 DAYS IN 1951

Day of Experiment		MLW		HAL		CAS		RBD	
		Serum Riboflavin		Serum Riboflavin		Serum Riboflavin		Serum Riboflavin	
		Total	Free (+ FMN)	Total	Free (+ FMN)	Total	Free (+ FMN)	Total	Free (+ FMN)
		mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %
1	1-19-1951	2.70	1.14	3.82	1.78	2.84	2.15	2.63	0.96
2	20	2.49	0.73	3.49	1.47	2.37	1.80	1.98	0.45
3	21	2.45	0.71	3.30	1.84	2.40	1.73	1.73	0.51
4	22	2.52	0.79	3.36	1.63	2.36	1.86	2.20	0.41
5	23	2.39	0.63	3.19	1.49	2.46	1.60	1.74	0.33
6	24	2.22	0.89	3.12	1.70	2.79	1.81	2.23	0.50
7	25	2.16	0.75	3.36	1.63	2.50	2.02	2.19	0.79
8	26	2.23	0.78	2,61	1.75	2.57	1.87	1.83	0.57
9	27	2.66	0.64	3.29	1.73	3.33	1.42	2.43	0.59
10	28	2.57	0.73	3.26	1.45	3.07	2.06	2.51	0.51
11	29	2.52	0.82	3.12	1.55	3.00	1.60	2.57	0.56
12	30	2.66	1.01	3.09	1.52	3.29	1.74	2.40	0.45
13	31	2.51	0.67	3.54	1.28	2.67	1.74	2.16	0.24
14	2- 1-1951	2.29	0.66	3.52	1.32	2.88	1.53	2.23	0.21
15	2	2.12	0.77	3.35	1.45	2.94	1.75	2.16	0.32
16	3	2.74	0.83	3.70	1.35	2.94	1.52	2.46	0.46
17	4	2.87	0.56	3.62	1.38	2.79	1.48	2.96	0.57
18	5	2.87	0.67	3.52	1.24	3.21	1.54	2.30	0.43
19	6	2.86	0.60	3.58	1.22	3.01	1.44	2.64	0.38
20	7	2.61	0.68	3.58	1.14	2.85	1.51	2.69	0.36
21	8	2.92	0.56	3.54	1.17	3.01	1.50	2.62	0.39
22	9	2.73	0.64	3.36	1.29	3.09	1.47	2.54	0.32
23	10	2.84	0.76	3.47	1.39	2.93	1.43	2.52	0.35
24	11	2.78	0.68	3.63	1.48	2.89	1.58	2.65	0.59
25	12	2.86	0.78	3.44	1.44	3.11	1.68	2.49	0.46
26	13	2.72	0.49	3.73	1.29	2.97	1.53	2.70	0.63
27	14	2.63	0.65	3.38	1.48	2.87	1.56	2.08	0.45
28	15	2.72	0.60	3.94	1.37	3.01	1.11	2.59	0.30
29	16	2.57	0.78	3.25	1.35	2.73	1.71	2.28	0.49
30	17	2.96	0.66	3.73	1.14	3.01	1.25	2.36	0.41
	Average	2.61	0.72	3.43	1.44	2.86	1.63	2.36	0.47

Table 13

DAILY EXCRETION OF RIBOFLAVIN AND CREATININE IN THE URINE, THE EXCRETION OF RIBOFLAVIN IN TERMS OF MCG. PER ML. OF URINE AND THE VOLUME OF URINE EXCRETED BY FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET FOR 30 DAYS IN 1951

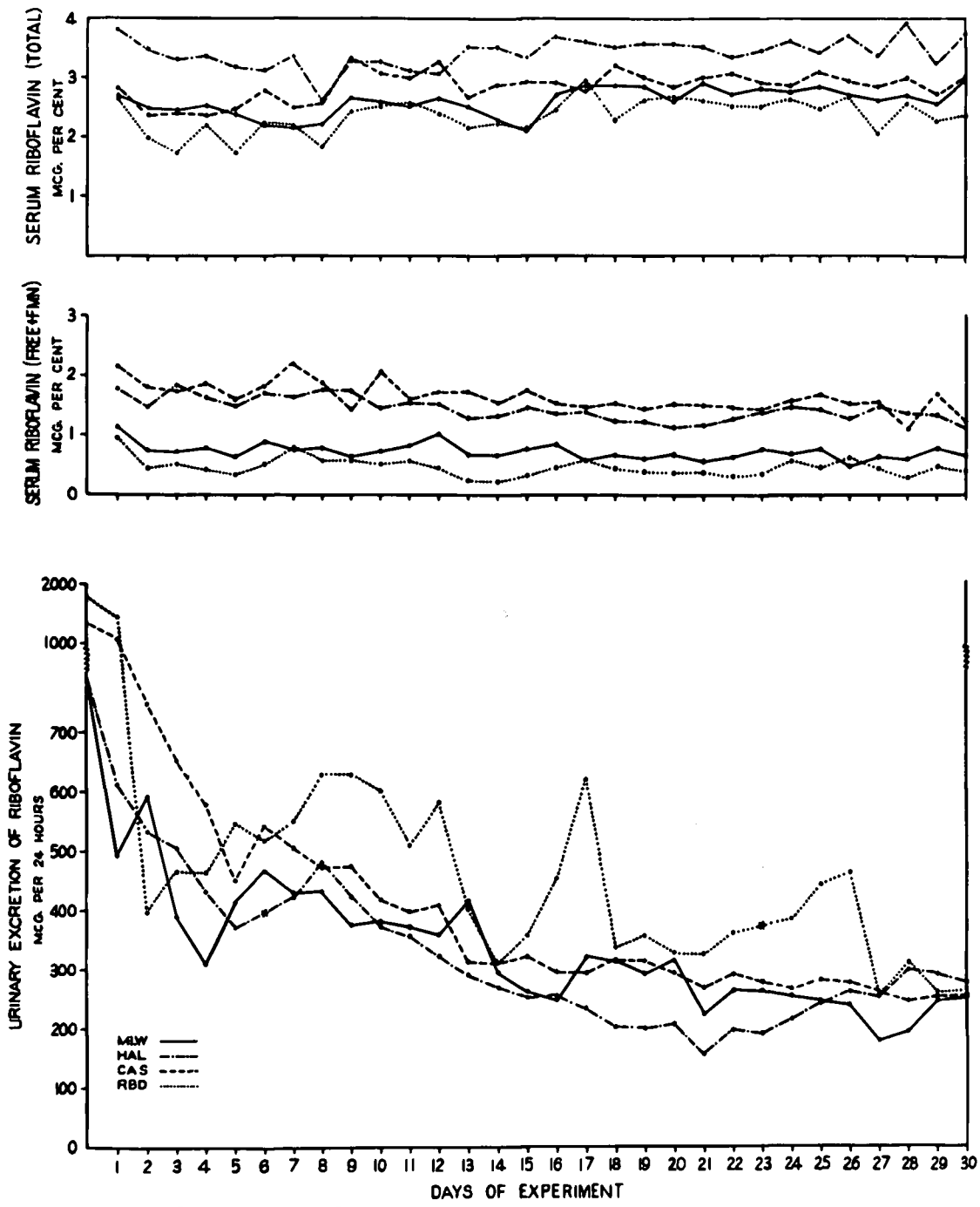
Day of Experiment	Date	MLW				HAL				CAS				RBD			
		Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine	Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine	Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine	Urinary Riboflavin	Urine Volume	Urinary Riboflavin	Urinary Creatinine
		mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.	mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.	mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.	mcg. per 24 hrs.	ml. per 24 hrs.	mcg. per ml.	gm. per 24 hrs.
Previous Day	1-18-1951	787	957	0.82	1.10	788	806	0.98	1.18	1326	1727	0.77	1.51	1843	1200	1.54	1.08
1	19	494	1645	0.30	1.05	615	1829	0.34	1.21	1076	2086	0.52	1.55	1417	952	1.49	1.07
2	20	593	1163	0.51	1.13	532	2162	0.25	1.24	748	2309	0.32	1.54	397	601	0.66	1.06
3	21	390	1587	0.25	1.03	506	1918	0.26	1.28	651	2171	0.30	1.54	467	1217	0.38	1.13
4	22	310	1754	0.18	1.01	433	1642	0.26	1.28	578	2571	0.23	1.50	463	1456	0.32	1.01
5	23	415	1257	0.33	1.11	372	1511	0.25	1.25	-*	-*	-*	-*	547	1703	0.32	1.10
6	24	466	1451	0.32	1.12	-*	-*	-*	-*	542	3284	0.17	1.52	517	2052	0.25	1.10
7	25	429	1858	0.23	1.12	423	1485	0.29	1.20	505	2632	0.19	1.42	550	1745	0.32	1.11
8	26	432	1180	0.37	1.12	480	1720	0.28	1.26	472	2861	0.17	1.45	628	1563	0.40	1.09
9	27	375	1289	0.29	1.12	423	1933	0.22	1.23	474	2050	0.23	1.46	627	1331	0.47	1.10
10	28	382	1652	0.23	1.13	373	1945	0.19	1.21	418	2678	0.16	1.45	600	1626	0.37	1.09
11	29	371	1157	0.32	1.14	356	1694	0.21	1.28	398	2211	0.18	1.47	510	1505	0.34	1.08
12	30	358	1474	0.24	1.21	321	1622	0.20	1.28	408	2303	0.18	1.53	580	1361	0.43	1.16
13	31	415	1270	0.33	1.15	291	1833	0.16	1.26	312	2421	0.13	1.53	403	1199	0.34	1.18
14	2- 1-1951	295	1346	0.22	1.16	269	2083	0.13	1.25	310	1721	0.18	1.51	310	1290	0.24	1.09
15	2	261	1451	0.18	1.18	252	1471	0.17	1.29	323	2292	0.14	1.50	356	1563	0.23	1.04
16	3	249	1535	0.16	1.15	251	2350	0.11	1.27	295	2733	0.11	1.48	454	1611	0.28	1.24
17	4	321	1320	0.24	1.17	232	2092	0.11	1.29	294	2387	0.12	1.51	620	1915	0.32	1.08
18	5	312	1651	0.19	1.13	203	1648	0.12	1.29	315	2563	0.12	1.57	336	533	0.63	1.08
19	6	291	1277	0.23	1.10	200	1855	0.11	1.27	313	2127	0.15	1.59	356	1580	0.23	1.12
20	7	315	1362	0.23	1.10	207	2095	0.10	1.27	295	2805	0.11	1.57	329	1445	0.23	1.08
21	8	224	1526	0.15	1.07	156	2161	0.07	1.28	269	2719	0.10	1.56	325	1408	0.23	1.08
22	9	265	1633	0.16	1.12	198	1942	0.10	1.27	291	2767	0.11	1.56	361	1717	0.21	1.10
23	10	264	1872	0.14	1.13	191	2360	0.08	1.27	278	2723	0.10	1.56	-*	-*	-*	-*
24	11	255	1216	0.21	1.09	215	2392	0.09	1.35	267	2697	0.10	1.55	385	1529	0.25	1.11
25	12	246	1640	0.15	1.14	243	2323	0.10	1.32	281	2792	0.10	1.62	443	1306	0.34	1.12
26	13	240	769	0.31	1.13	263	2308	0.11	1.26	277	2265	0.12	1.58	464	1646	0.28	1.08
27	14	180	1496	0.12	1.09	253	2055	0.12	1.31	261	2420	0.11	1.54	255	1326	0.19	1.07
28	15	195	1547	0.13	1.13	300	2082	0.14	1.35	246	2282	0.11	1.56	311	1569	0.20	1.10
29	16	246	1867	0.13	1.07	292	2432	0.12	1.28	253	2406	0.11	1.53	261	1894	0.14	1.04
30	17	250	1949	0.13	1.11	277	2335	0.12	1.32	254	2550	0.10	1.48	265	1973	0.13	1.06
	Average**	328		0.23	1.12	315		0.17	1.27	393		0.16	1.53	467		0.35	1.10
Riboflavin Excreted in Urine Expressed in Terms of Per Cent of Intake		28				27				33				40			

* The samples were spilled and data were not available.

** Analysis made prior to the diet was not included in the calculation.

FIGURE 3

DAILY CONCENTRATION OF TOTAL AND FREE (+FMN) RIBOFLAVIN IN THE SERUM AND THE URINARY EXCRETION OF RIBOFLAVIN FOR FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET FOR 30 DAYS IN 1951



* DATA ARE NOT AVAILABLE

TOTAL AND FREE (+FMN) RIBOFLAVIN IN THE SERUM DURING TWO PERIODS ON DIFFERENT LEVELS OF THIAMINE INTAKE

The mean concentration for each subject of total and free (+FMN) riboflavin in the serum during two periods on different levels of thiamine intake as well as the differences between the periods are presented in Tables 14 and 15. The mean was calculated from the data for the last ten days only, of each period. Values for the first five days were omitted in order to allow for adjustment to the new level of thiamine intake. During the first period the thiamine intake was approximately 500 mcg. per 1000 calories; that of the second period was approximately 300 mcg. per 1000 calories. Higher values for total serum riboflavin were observed for all subjects in this second period. For HHY who had been taking vitamin pills every day during the study, values for both serum and for urinary riboflavin were omitted in the final comparison and in the statistical analysis. The mean differences of total riboflavin in serum for the other seven subjects ranged from 0.06 to 0.93 mcg. per cent. However, the concentration of free (+FMN) riboflavin in the serum, in six out of the seven subjects was lower in the second period. The mean differences for free (+FMN) riboflavin, between the two periods for MLW*, KD, SWW, MLW, HAL, CAS, and RBD were +0.03, -0.02, +0.01, +0.11, +0.20, +0.27, and +0.30 mcg. per cent, respectively. Although the concentration of free (+FMN) riboflavin increased when the thiamine intake was decreased, the differences apparently were small and

* MLW was a subject for both the 1950 and 1951 studies.

Table 14

MEAN SERUM RIBOFLAVIN [TOTAL AND FREE (+ FMN)] FOR THREE SUBJECTS ON A CONTROLLED DIET WITH TWO LEVELS* OF THIAMINE INTAKE FOR 15 DAYS EACH IN 1950

	MLW		KD		SWW	
	Serum Riboflavin		Serum Riboflavin		Serum Riboflavin	
	Total	Free (FMN)	Total	Free (FMN)	Total	Free (FMN)
	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %
First period	2.64	1.00	2.71	1.20	4.82	3.56
Second period	3.27	0.97	3.18	1.22	5.75	3.55
Difference between two periods	-0.63	+0.03	-0.47	-0.02	-0.93	+0.01

* During the first 15 days each subject received a total of 998 mcg. of thiamine daily.

During the second 15 days each subject received a total of 598 mcg. of thiamine daily.

Table 15

MEAN SERUM RIBOFLAVIN [TOTAL AND FREE (+ FMN)] FOR FOUR SUBJECTS ON A CONTROLLED DIET WITH TWO LEVELS OF THIAMINE* INTAKE FOR 15 DAYS EACH IN 1951

	MLW		HAL		CAS		RBD	
	Serum Riboflavin		Serum Riboflavin		Serum Riboflavin		Serum Riboflavin	
	Total	Free (+ FMN)	Total	Free (+ FMN)	Total	Free (+ FMN)	Total	Free (+ FMN)
	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %	mcg. %
First Period	2.39	0.77	3.32	1.54	2.90	1.75	2.27	0.47
Second Period	2.77	0.66	3.55	1.34	2.96	1.48	2.48	0.44
Difference Between Two Periods	-0.38	+0.11	-0.32	+0.20	-0.06	+0.27	-0.21	+0.03

*During the first 15 days each subject received a total of 1028 mcg. of thiamine daily.
During the second 15 days each subject received a total of 628 mcg. of thiamine daily.

insignificant.

Supplee et al. (1942) found that thiamine mobilized riboflavin from the tissues to the liver. Sure and Ford (1942) reported a marked increase in the output of riboflavin in rats when the diet was restricted in thiamine. Davis et al. (1946), on the contrary, found a higher percentage of return of the test dose in the period of higher intake of thiamine. In the study reported in this thesis, the interrelationship of these two vitamins in metabolism appeared to be thus: the lower the thiamine intake, the less the need for riboflavin and the higher its retention in the serum.

RIBOFLAVIN CONTENT OF THE SERUM OF 29 NORMAL ADULTS

For comparison with the data on the subjects on the controlled diet, serum samples of a group of 29 normal students and faculty members of Oregon State College were analyzed for riboflavin content (Table 16). Analyses were made in triplicate for free (+FMN) and total serum riboflavin.

The mean values for free (+FMN) and total serum riboflavin reported by Burch et al. (1948) for 13 subjects and those obtained in this laboratory for 29 subjects as well as those for the subjects on the controlled diet may be seen in Table 17.

Table 16

CONCENTRATION OF TOTAL AND FREE (+FMN) RIBOFLAVIN AND FAD OF THE SERUM OF 29 NORMAL ADULTS

Subject No.	Serum Riboflavin		
	Total	Free (+FMN)	FAD
	mcg. %	mcg. %	mcg. %
1	2.64	0.77	1.87
2	5.67	0.56	5.11
3	2.67	0.12	2.55
4	3.07	0.41	2.66
5	2.39	0.23	2.16
6	3.16	1.81	1.35
7	3.63	0.55	3.08
8	2.04	0.47	1.57
9	2.63	1.30	1.33
10	2.57	0.80	1.77
11	2.23	0.37	1.86
12	2.23	0.51	1.72
13	2.93	0.64	2.29
14	2.15	0.75	1.40
15	4.67	3.12	1.55
16	2.66	1.17	1.49
17	5.49	3.87	1.62
18	2.13	0.32	1.81
19	5.19	1.72	3.47
20	2.36	0.85	1.51
21	1.91	0.60	1.31
22	2.06	1.40	0.66
23	1.60	0.73	0.87
24	2.34	0.55	1.79
25	3.09	1.08	2.01
26	1.58	0.10	1.48
27	3.76	2.40	1.36
28	2.05	0.68	1.37
29	3.04	1.38	1.66
Average	2.89	1.01	1.89
Range	1.58-5.67	0.10-3.12	0.66-5.11

Table 17

COMPARISON OF DATA FOR SERUM RIBOFLAVIN VALUES OBTAINED IN THIS LABORATORY WITH THOSE REPORTED BY BURCH et al. (1948)

Studies	Serum Riboflavin			
	Total		Free (+FMN)	
	Mean	Range	Mean	Range
	mcg. %	mcg. %	mcg. %	mcg. %
13 Subjects (Burch et al., 1948)	3.2	2.6-3.7	0.8	0.3-1.3
29 Subjects (Study in this laboratory)	2.89	1.58-5.67	1.01	0.10-3.12
7 Subjects (Study on the controlled diet in this laboratory)	3.22	2.36-5.30	1.43	0.47-3.50

INTERPRETATION OF STATISTICAL ANALYSES

From the statistical analyses presented in Tables 20, 21, 22, and 23 (in the appendix), the indication is that the differences among the individual persons in respect to the content of riboflavin in the serum are significant. This is true for both the free (+FMN) and the total form.

The change of the thiamine intake did not affect the concentration of free (+FMN) riboflavin in the serum significantly, but it did affect the total riboflavin of the serum significantly.

There was no significant day-to-day variation in the total and free riboflavin values of the serum during any one level of thiamine intake.

Davis et al. (1946) observed from their studies on the urinary excretion of riboflavin, that approximately 10 days were required to adjust to a new level of intake, with the exception of one subject who required 6 weeks to make a similar adjustment. In the 1951 study (Figure 3) the data indicated that a longer period of time might have been needed for the adjustment to the new level of thiamine intake since a downward trend was noted until the 14th day, with urine riboflavin values for subject RBD showing sharp peaks every few days. Subject RBD, however, suffered occasionally from migraine headaches during the experiment. Before the experiment, she took BEXEL (multiple vitamin pills containing iron) and Brewer's yeast tablets every day for approximately two months. She stopped taking the vitamin pills, eighteen days before the beginning

of the study and the Brewer's yeast tablets, three days before the experiment began. During the study (January 19) she suffered from a headache and vomited her breakfast and the vitamin B_1 supplement. She spent the remainder of the day in bed, eating only her portions of pears and meat. On January 30, she developed a slight cold which resulted in a sore throat on January 31 and February 1. February 4, she was ill with nausea and diarrhea and vomited after lunch. On February 13, she suffered from a migraine headache, again. For these reasons, a statistical analysis of the data of the 24-hour urinary riboflavin values for the two levels of thiamine intake might not constitute a valid comparison.

URINARY EXCRETION OF RIBOFLAVIN

The mean daily urinary excretion of riboflavin for three subjects during the entire period in the 1950 study was 382 mcg. ranging from 358 to 407 mcg.; the mean riboflavin excretion for the four subjects in the 1951 study was 376 mcg., ranging between 315 to 467 mcg. The mean riboflavin output for all was 32 per cent of the intake during the 1950 and 1951 studies.

Hathaway et al. (1946) found that, when the intake of riboflavin was 1330 mcg. per day, the urinary excretion on a natural diet ranged from 174 to 229 mcg. Davis et al. (1946) observed that, when the daily riboflavin intake was 660 mcg. per 1000 calories, the average 24-hour excretion of 6 subjects ranged between 214 to 345 mcg. Swaminathan (1942) estimated the daily urinary riboflavin

excretion, on an intake of 1200 to 1500 mcg. per day, to be 25 to 30 per cent of riboflavin ingested.

The mean urinary riboflavin excretion per gram of creatinine for each of the seven subjects is recorded in Table 18. The means ranged between 248 to 474 mcg. per gram of creatinine. According to Aykroyd et al. (1948) if the excretion of riboflavin is less than 200 mcg. per gram of creatinine, the intake of riboflavin may be considered unsatisfactory. The mean urinary riboflavin values of all seven subjects were above this level.

Feder, Lewis, and Alden (1944) stated that the amount of riboflavin per ml. of urine showed less variation than the total amount excreted, but Hathaway and Lobb (1946) found that urinary riboflavin excretion showed no relation to urinary volume. The results reported in this thesis are in agreement with those of Hathaway and Lobb.

RESPONSE TO 2 MG. ORAL DOSE OF RIBOFLAVIN

The results of the response of four subjects to a 2 mg. oral dose of riboflavin indicated that the peak of total riboflavin in the serum occurred between one-half hour and one hour after the dose was taken. An increase of 97 to 147 per cent was noted in the concentration of total riboflavin in the serum (Table 19 and Figure 4). However, for free (+FMN) riboflavin, the peak, for all four subjects, occurred one-half hour after the test dose was taken, or a 249 to 393 per cent increase. The highest output of riboflavin in the urine occurred at the end of the first hour (Table 23 and

Table 18

THE MEAN URINARY EXCRETION OF RIBOFLAVIN, OF CREATININE AND OF RIBOFLAVIN PER GRAM OF CREATININE FOR EACH OF SEVEN SUBJECTS

Subjects	Mean Urinary Excretion		
	Riboflavin	Creatinine	Riboflavin per gram of creatinine
	mcg. per 24 hr.	gm. per 24 hr.	mcg.
MLW*	382	1.05	364
KD	358	1.09	328
SWW	407	0.86	474
MLW**	328	1.12	293
HAL	315	1.27	248
CAS	393	1.53	257
RED	467	1.10	424

* MLW in the 1950 study.
** MLW in the 1951 study.

Table 19

CONCENTRATION OF TOTAL AND FREE (+FMN) RIBOFLAVIN IN THE SERUM AND URINARY EXCRETION OF RIBOFLAVIN OF FOUR SUBJECTS FOLLOWING A 2 MG. ORAL DOSE OF RIBOFLAVIN

Time	MLW			HAL			CAS			RBD		
	Serum Riboflavin Total	Serum Riboflavin Free (+FMN)	Riboflavin Excreted in Urine	Serum Riboflavin Total	Serum Riboflavin Free (+FMN)	Riboflavin Excreted in Urine	Serum Riboflavin Total	Serum Riboflavin Free (+FMN)	Riboflavin Excreted in Urine	Serum Riboflavin Total	Serum Riboflavin Free (+FMN)	Riboflavin Excreted in Urine
	mcg. %	mcg. %	mcg. per hr.	mcg. %	mcg. %	mcg. per hr.	mcg. %	mcg. %	mcg. per hr.	mcg. %	mcg. %	mcg. per hr.
Fasting 7:00 A.M.	2.73	1.15	12	3.46	1.61	14	2.68	1.60	15	2.60	1.01	36
7:30 A.M.	5.27	4.64	139 / 304 = 443	6.83	5.62	56 / 260 = 316	6.15	6.08	147 / 276 = 423	5.75	4.98	152 / 345 = 497
8:00 A.M.	5.56	2.57		6.09	3.44		5.04	3.17		6.42	3.36	
9:00 A.M.	4.84	1.34	385	5.22	1.95	185	4.68	1.85	77	4.20	1.55	355
10:00 A.M.	3.09	1.42	115	4.19	1.98	78	3.83	2.07	67	3.71	1.66	178
11:00 A.M.	3.26	0.99	53	4.43	1.71	51	4.03	2.24	45	3.05	1.02	149
12:00 A.M.	3.08	0.84	48	3.90	1.70	46	3.51	1.72	50	2.92	0.99	64

FIGURE 4

CONCENTRATION OF TOTAL AND FREE (+FMN) RIBOFLAVIN IN THE SERUM AND THE URINARY EXCRETION OF RIBOFLAVIN FOR FOUR SUBJECTS FOLLOWING A 2 MG. ORAL DOSE OF RIBOFLAVIN

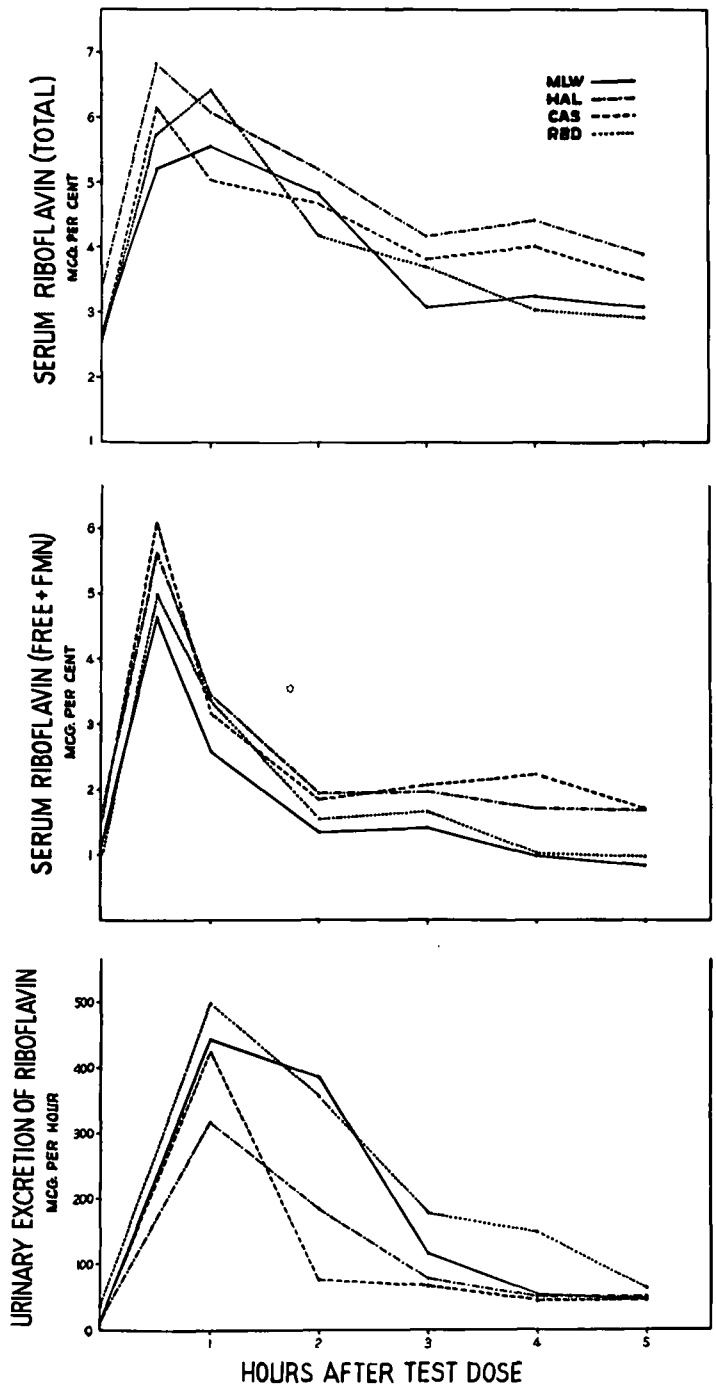


Figure 4). The values for both serum and urinary riboflavin showed an abrupt decrease after the peak had been reached. At the end of the 5th hour, the riboflavin values for serum and urine were almost at the original level.

CHAPTER V

SUMMARY

The daily micro determination of free (+FMN) and total riboflavin in the serum and the daily macro determination of riboflavin in 24-hour collections of urine of eight normal women on a controlled diet for the studies of 1950 and 1951 are described.

The mean concentration of free (+FMN) serum riboflavin for seven of the eight subjects was 1.43, and ranged from 0.47 to 3.50 mcg. per cent; the mean concentration of total serum riboflavin was 3.22, and ranged from 2.36 to 5.30 mcg. per cent. The data for one subject (HHY) who received some supplements and medications were not included in calculating the mean values or in the ranges given.

The mean concentration of free (+FMN) riboflavin in the serum was slightly lower in the periods of restricted thiamine intake; however, the difference was not statistically significant. The increase (0.43 mcg. per cent) in the mean concentration of total riboflavin in the serum during the period of restricted thiamine intake, was statistically significant. This phenomenon may be due to the effect of thiamine on the utilization of riboflavin in metabolism; i.e., a decrease in thiamine intake decreases the requirement for riboflavin and is reflected in an increased concentration of riboflavin in the serum.

The statistical analyses of the data indicated that there are individual differences among persons in the riboflavin content of serum. The change of the thiamine intake affected the total serum

riboflavin concentration significantly.

There was no significant day-to-day variation in the free or total riboflavin content of the serum during each 15-day period of study.

On a constant riboflavin intake the mean daily urinary riboflavin excretion of three subjects in the 1950 study was 382 mcg. per day, and that of four subjects in the 1951 study was 376 mcg. per day. The riboflavin output was about 32 per cent of the ingested vitamin in both years' studies. The riboflavin excretion per gram of creatinine ranged from 248 to 474 mcg.

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APPENDIX

DIRECTIONS FOR THE EXPERIMENTAL SUBJECTS IN THE 1950 STUDY

A. The study will begin officially on Saturday, A.M. April 8 between 6:15 and 6:30 A.M. Each person will report to the laboratory at that time daily. If all goes well, the experiment will end with the completion of analyses on May 8, 1950.

B. The study will be divided into 2 parts of 15 days each.

Period I. Supplements of crystalline thiamine will be given each day to bring the total thiamine intake up to the recommended allowance of the National Research Council. The basal diet will contain 300 micrograms per 1000 calories.

Period II. The basal diet will not be supplemented this period. Each person will, therefore, receive 300 micrograms of thiamine for every 1000 calories consumed.

The total daily ascorbic acid intake will meet the National Research Council Recommended Allowance.

C. The basal diet contains about 1000 calories. No foods (except coffee or tea without cream and sugar) will be allowed <u>ad libitum</u>.

D. Blood samples will be taken daily.

E. Urine collections for 24-hour periods will begin after the first voiding on the first day and will include the first voiding on the following day. You will preserve one-half each collection with 2 per cent, by volume, of glacial acetic acid. This preservative is stable.

F. Do not take aspirin, vitamin pills or <u>any</u> medicine during the course of the entire experiment.

(Subject HHY is taking some medication and supplements by prescription and although her regime will not be the same as the others, it will be recorded throughout the study.)

DIRECTIONS FOR THE EXPERIMENTAL SUBJECTS IN THE 1951 STUDY

A. The study will begin officially on Friday, A.M. January 19 between 6:15 and 6:30 A.M. Each person will report to the laboratory at that time daily. If all goes well, the experiment will end with the completion of analyses on Sunday, February 18. (For Rachel Dube and Mei-ling Wu, on February 19.)

B. The study will be divided into 2 parts of 15 days each.

Period I. Supplements of crystalline thiamine will be given each day to bring the total thiamine intake up to the recommended allowance of the National Research Council. The basal diet will contain 300 micrograms per 1000 calories.

Period II. The basal diet will not be supplemented during this period. Each person will, therefore, receive 300 micrograms of thiamine for every 1000 calories consumed.

The total daily ascorbic acid intake will meet the National Research Council Recommended Allowance.

C. The basal diet plus 2 units contains about 2000 calories. No foods (except coffee without cream and sugar) will be allowed ad libitum.

D. Blood samples will be taken daily.

E. Urine collections for 24-hour periods will begin after the first voiding on the first day and will include the first voiding on

the following day. You will preserve each collection with 2 per cent, by volume, of glacial acetic acid. This preservative is stable. Please use tampons to prevent contamination of urine with blood during menstrual periods.

F. Do not take aspirin, vitamin pills or any medicine during the course of the entire experiment.

STATISTICAL ANALYSES

SERIAL CORRELATION

The serial correlation is a method of testing the randomness of a set of observations. The serial correlation coefficient is the correlation coefficient between adjacent observations such as the yields of adjacent plots or the observations made at adjacent time periods. Let $x_1, x_2, \ldots, x_n$ be the N observations of a sample, where x_i is the i^{th} observation. The serial correlation coefficient of this sample is

$$R = \frac{\sum (x_i - \bar{x})(x_{i+1} - \bar{x})}{\sum (x - \bar{x})^2} = \frac{\sum x_i x_{i+1} - (\sum x)^2/N}{\sum x^2 - (\sum x)^2/N}$$

The serial correlation coefficient measures the randomness of a sequence of observations. It ranges from -1 to +1. A high negative serial correlation coefficient indicates that the observations go above and below their mean too frequently. A high positive correlation indicates that the observations follow a downward or upward trend. A low coefficient indicates randomness.

TABLE OF 5% AND 1% POINTS OF DISTRIBUTION OF SERIAL COEFFICIENTS OF RANDOM SAMPLES DRAWN FROM NORMAL POPULATION

N	Negative tail		Positive tail	
	1%	5%	5%	1%
5	-0.798	-0.753	0.253	0.297
6	-0.863	-0.708	0.345	0.447
7	-0.799	-0.674	0.370	0.510
8	-0.764	-0.625	0.371	0.531
9	-0.737	-0.593	0.366	0.533
10	-0.705	-0.564	0.360	0.525
11	-0.679	-0.539	0.353	0.515
12	-0.655	-0.516	0.348	0.505
13	-0.634	-0.497	0.341	0.495
14	-0.615	-0.479	0.335	0.485
15	-0.597	-0.462	0.328	0.475
20	-0.524	-0.399	0.299	0.432
25	-0.473	-0.356	0.276	0.398

The 5% and 1% points are used for 10% and 2% significance levels for two-tailed tests.

SERIAL CORRELATION
(1950 STUDY)

SUBJECT MLW

First Period

Total Riboflavin

The correction term $= \frac{(26.43)^2}{10} = 69.8545$

$$R = \frac{70.7860 - 69.8545}{71.3659 - 69.8545} = \frac{0.9315}{1.5114} = 0.616$$

Free (+FMN) Riboflavin

The correction term $= \frac{(9.99)^2}{10} = 9.9800$

$$R = \frac{10.1174 - 9.9800}{10.2017 - 9.9800} = \frac{0.1374}{0.2217} = 0.620$$

Second Period

Total Riboflavin

The correction term $= \frac{(32.70)^2}{10} = 106.9290$

$$R = \frac{107.0574 - 106.9290}{107.5576 - 106.9290} = \frac{0.1284}{0.6286} = 0.204$$

Free (+FMN) Riboflavin

The correction term $= \frac{(9.66)^2}{10} = 9.3316$

$$R = \frac{9.3054 - 9.3316}{9.6066 - 9.3316} = \frac{-0.0262}{0.2750} = -0.095$$

SERIAL CORRELATION
(1950 STUDY)

SUBJECT KD

First Period

Total Riboflavin

$$\text{The correction term} = \frac{(27.08)^2}{10} = 73.3326$$

$$R = \frac{73.4818 - 73.3326}{75.0178 - 73.3326} = \frac{0.1492}{1.6852} = 0.089$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(12.02)^2}{10} = 14.4480$$

$$R = \frac{14.4900 - 14.4480}{14.7986 - 14.4480} = \frac{0.0420}{0.3506} = 0.120$$

Second Period

Total Riboflavin

$$\text{The correction term} = \frac{(31.82)^2}{10} = 101.2512$$

$$R = \frac{101.3029 - 101.2512}{101.9866 - 101.2512} = \frac{0.0517}{0.7354} = 0.070$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(12.24)^2}{10} = 14.9818$$

$$R = \frac{14.9005 - 14.9818}{15.541 - 14.9818} = \frac{-0.0813}{0.5592} = -0.145$$

SERIAL CORRELATION
(1950 STUDY)

SUBJECT SWW

First Period

Total Riboflavin

$$\text{The correction term} = \frac{(48.18)^2}{10} = 232.1312$$

$$R = \frac{233.8415 - 232.1312}{237.2916 - 232.1312} = \frac{1.7103}{5.1604} = 0.331$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(32.06)^2}{9} = 114.2048$$

$$R = \frac{114.4509 - 114.2048}{115.3808 - 114.2048} = \frac{0.2461}{1.1760} = 0.209$$

Second Period

Total Riboflavin

$$\text{The correction term} = \frac{(57.52)^2}{10} = 330.8550$$

$$R = \frac{330.8021 - 330.8550}{332.0330 - 330.8550} = \frac{-0.0529}{1.1780} = -0.045$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(35.46)^2}{10} = 125.7412$$

$$R = \frac{125.7388 - 125.7412}{126.1676 - 125.7412} = \frac{-0.0024}{0.4264} = -0.006$$

SERIAL CORRELATION
(1951 STUDY)

SUBJECT MLW

First Period

Total Riboflavin

$$\text{The correction term} = \frac{(23.94)^2}{10} = 57.3124$$

$$R = \frac{57.5453 - 57.3124}{57.7120 - 57.3124} = \frac{0.2329}{0.3996} = 0.583$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(7.72)^2}{10} = 5.9598$$

$$R = \frac{5.9581 - 5.9598}{6.0754 - 5.9598} = \frac{-0.0017}{0.1156} = -0.015$$

Second Period

Total Riboflavin

$$\text{The correction term} = \frac{(27.73)^2}{10} = 76.8953$$

$$R = \frac{76.8980 - 76.8953}{77.0331 - 76.8953} = \frac{0.0027}{0.1378} = 0.020$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(6.60)^2}{10} = 4.3560$$

$$R = \frac{4.3351 - 4.3560}{4.4382 - 4.3560} = \frac{-0.0209}{0.0822} = -0.254$$

SERIAL CORRELATION
(1951 STUDY)

SUBJECT HAL

First Period

Total Riboflavin

$$\text{The correction term} = \frac{(32.26)^2}{10} = 104.0708$$

$$R = \frac{104.0205 - 104.0708}{104.7148 - 104.0708} = \frac{0.0503}{0.6440} = 0.078$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(15.38)^2}{10} = 23.6544$$

$$R = \frac{23.7772 - 23.6544}{23.9010 - 23.6544} = \frac{0.1228}{0.2466} = 0.498$$

Second Period

Total Riboflavin

$$\text{The correction term} = \frac{(35.47)^2}{10} = 125.8121$$

$$R = \frac{125.5244 - 125.8121}{126.2089 - 125.8121} = \frac{-0.2877}{0.3968} = -0.725$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(13.40)^2}{10} = 17.9560$$

$$R = \frac{18.0075 - 17.9560}{18.0826 - 17.9560} = \frac{0.0515}{0.1266} = 0.407$$

SERIAL CORRELATION
(1951 STUDY)

SUBJECT CAS

First Period

Total Riboflavin

$$\text{The correction term} = \frac{(29.04)^2}{10} = 84.3322$$

$$R = \frac{84.4049 - 84.3322}{85.0438 - 84.3322} = \frac{0.0727}{0.7116} = 0.102$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(17.54)^2}{10} = 30.7652$$

$$R = \frac{30.6290 - 30.7652}{31.1320 - 30.7652} = \frac{-0.1362}{0.3668} = -0.371$$

Second Period

Total Riboflavin

$$\text{The correction term} = \frac{(29.62)^2}{10} = 87.7344$$

$$R = \frac{87.7042 - 87.7344}{87.8482 - 87.7344} = \frac{-0.0302}{0.1138} = -0.265$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(14.82)^2}{10} = 21.9632$$

$$R = \frac{21.8203 - 21.9632}{22.2678 - 21.9632} = \frac{-0.1429}{0.3046} = -0.469$$

SERIAL CORRELATION
(1951 STUDY)

SUBJECT RBD

First Period

Total Riboflavin

$$\text{The correction term} = \frac{(22.71)^2}{10} = 51.5744$$

$$R = \frac{51.6907 - 51.5744}{51.9919 - 51.5744} = \frac{0.1163}{0.4175} = 0.279$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(4.74)^2}{10} = 2.2568$$

$$R = \frac{2.4057 - 2.2468}{2.5274 - 2.2468} = \frac{0.1589}{0.2806} = 0.566$$

Second Period

Total Riboflavin

$$\text{The correction term} = \frac{(24.83)^2}{10} = 61.6529$$

$$R = \frac{61.5275 - 61.6529}{61.9815 - 61.6529} = \frac{-0.1254}{0.3286} = -0.382$$

Free (+FMN) Riboflavin

$$\text{The correction term} = \frac{(4.39)^2}{10} = 1.9272$$

$$R = \frac{1.9308 - 1.9272}{2.0343 - 1.9272} = \frac{0.0036}{0.1071} = 0.034$$

Table 20

ANALYSIS OF VARIANCE CALCULATIONS

SERUM RIBOFLAVIN (FREE + FMN) OF THREE SUBJECTS MAINTAINED ON A CONTROLLED DIET WITH TWO LEVELS OF THIAMINE INTAKE FOR 15 DAYS EACH IN 1950

Preliminary Calculations

(1) Source of Variation	(2) Total of Squares	(3) No. of items Squared	(4) Observations per Squared Item	(5) Total of Squares per Observation (2) ÷ (4)	(6) Sum of Squares (5)-correction
Correction	10592.5264	1	54	196.1579	0
Diet	5296.3650	2	27	196.1617	0.0038
Person	4828.5094	3	18	268.2505	72.0926
Sub-class	2414.5932	6	9	268.2881	72.1302
Individual observations	271.0332	54	1	271.0332	74.8753
Day	1179.9806	9	6	196.6634	0.5055
D-d, sub-class	593.8454	18	3	197.9485	1.7906
P-d, sub-class	538.4932	27	2	269.2466	73.0887

Table 20 (continued)

Analysis of Variance

Variation Due to:	Sum of Squares	Degrees of Freedom	Mean Square	Remarks
Diet	0.0038	1	0.0038	Not significant
Person	72.0926	2	36.0463	Significant
Day	0.5055	8	0.0632	Not significant
Diet vs. person	0.0338	2	0.0169	
Diet vs. day	1.2813	8	0.1602	
Person vs. day	0.4906	16	0.0307	
Error	0.4677	16	0.0292	
Total	74.8753	53		

Table 21

ANALYSIS OF VARIANCE CALCULATIONS

SERUM RIBOFLAVIN (TOTAL) OF THREE SUBJECTS MAINTAINED ON A CONTROLLED DIET WITH TWO LEVELS OF THIAMINE INTAKE FOR 15 DAYS EACH IN 1950

Preliminary Calculations

(1) Source of Variation	(2) Total of Squares	(3) No. of items Squared	(4) Observations per Squared Item	(5) Total of Squares per Observation (2) ÷ (4)	(6) Sum of Squares (5)-correction
Correction	50055.1129	1	60	834.2519	0
Diet	25234.6177	2	30	841.1539	6.9020
Person	18138.0569	3	20	906.9028	72.6509
Sub-class	9143.5365	6	10	914.3537	80.1018
Individual observations	925.2525	60	1	925.2525	91.0006
Day	5016.8381	10	6	836.1397	1.8878
D-d, sub-class	2547.2291	20	3	849.0764	14.8245
P-d, sub-class	1820.5369	30	2	910.2685	76.0166

Table 21 (continued)

Analysis of Variance

Variation Due to:	Sum of Squares	Degrees of Freedom	Mean Square	Remarks
Diet	6.9020	1	6.9020	Significant
Person	72.6509	2	36.3255	Significant
Day	1.8878	9	0.9098	Not significant
Diet vs. person	0.5489	2	0.2745	
Diet vs. day	6.0347	9	0.6705	
Person vs. day	1.4779	18	0.0821	
Error	1.4984	18	0.0832	
Total	91.0006	59		

Table 22

ANALYSIS OF VARIANCE CALCULATIONS

SERUM RIBOFLAVIN (FREE + FMN) OF FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET WITH TWO LEVELS OF THIAMINE INTAKE FOR 15 DAYS EACH IN 1951

Preliminary Calculations

(1)	(2)	(3)	(4)	(5)	(6)
Source of Variation	Total of Squares	No. of items Squared	Observations per Squared Item	Total of Squares per Observation (2) ÷ (4)	Sum of Squares (5)-correction
Correction	7155.4681	1	80	89.4434	0
Diet	3596.7685	2	40	89.9192	0.4758
Person	2163.8773	4	20	108.1939	18.7505
Sub-class	1088.2865	8	10	108.8287	19.3853
Individual observations	110.4587	80	1	110.4587	21.0153
Day	718.5863	10	8	89.8233	0.3799
D-d, sub-class	362.8349	20	4	90.7087	1.2653
P-d, sub-class	217.9013	40	2	108.9507	19.5073

Table 22 (continued)

Analysis of Variance

Variation Due to:	Sum of Squares	Degrees of Freedom	Mean Square	Remarks
Diet	0.4758	1	0.4758	Not Significant
Person	18.7505	3	6.2502	Significant
Day	0.3799	9	0.0422	Not Significant
Diet vs. person	0.1590	3	0.0530	
Diet vs. day	0.4096	9	0.0455	
Person vs. day	0.3769	27	0.0140	
Error	0.4636	27	0.0172	
Total	21.0153	79		

Table 23

ANALYSIS OF VARIANCE CALCULATIONS

SERUM RIBOFLAVIN (TOTAL) OF FOUR SUBJECTS MAINTAINED ON A CONTROLLED DIET WITH TWO LEVELS OF THIAMINE INTAKE FOR 15 DAYS EACH IN 1951

Preliminary Calculations

(1) Source of Variation	(2) Total of Squares	(3) No. of items Squared	(4) Observations per Squared Item	(5) Total of Squares per Observation (2) ÷ (4)	(6) Sum of Squares (5)-correction
Correction	50895.3600	1	80	636.1920	0
Diet	25494.7250	2	40	637.3681	1.1761
Person	12958.1890	4	20	647.9095	11.7175
Sub-class	6493.8440	8	10	649.3844	13.1924
Individual observations	652.5342	80	1	652.5342	16.3422
Day	5095.7670	10	8	636.9709	0.7789
D-d, sub-class	2556.4396	20	4	639.1099	2.9179
P-d, sub-class	1298.8286	40	2	649.4143	13.2223

Table 23 (continued)

Analysis of Variance

Variation Due to:	Sum of Squares	Degrees of Freedom	Mean Square	Remarks
Diet	1.1761	1	1.1761	Significant
Person	11.7175	3	3.9058	Significant
Day	0.7789	9	0.0865	Not significant
Diet vs. person	0.2988	3	0.0996	
Diet vs. day	0.9629	9	0.1629	
Person vs. day	0.7259	27	0.0269	
Error	0.6821	27	0.0253	
Total	16.3422			