URINARY RIBOFLAVIN EXCRETIONS AND TEST DOSE RETURNS OF HEALTHY WOMEN AS AFFECTED BY DIETARY NITROGEN

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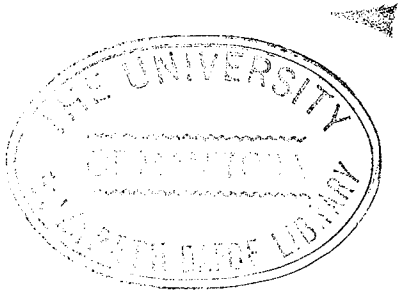

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Five healthy women were maintained for two consecutive 10-day periods on a controlled intake of 1.4 milligrams of riboflavin. For the first period, the nitrogen intake was 3.5 grams and, for the second, 15.4 grams. Twenty-four hour urine and fecal samples were collected. Riboflavin was determined in food and urine samples by a modification of the fluorometric method of Slater and Morell, and nitrogen in food, urine and fecal samples by the Macro-Kjeldahl method.

A 5.0 milligram oral test dose of riboflavin was administered on the morning following each experimental period. Complete urinary collections were then made at 30 minute intervals over a 4-hour fasting period.

All subjects were in negative nitrogen balance on an intake of 3.5 grams and in positive nitrogen balance on 15.4 grams. Riboflavin excretion levels showed large inter-individual variation although day-to-day values for individuals were quite constant. The three heavier, older subjects excreted less riboflavin than the two lighter, younger subjects. Ribo-

flavin excretion for the group averaged 14 per cent of the intake during the period of positive nitrogen balance and 34 per cent during negative nitrogen balance. The drop in riboflavin excretion in the second period was highly significant according to t test. The inverse relationship between nitrogen balance and urinary riboflavin excretion was reflected by high correlation coefficients of -.81, -.70, -.66, -.88 and -.93.

Mean percentage test dose excretions were very similar, 31 and 36 per cent respectively, for the two periods. Individuals, however, varied in their response. No conclusions could be drawn as to the effect of nitrogen balance on the 4-hour return of a 5 milligram test dose of riboflavin. Three subjects showed a lower percentage return, one, a higher, and one no change in the period of negative nitrogen balance than in the period of positive balance.

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To four staff members, aware of the importance of research, I wish to extend my sincere thanks for their willingness to participate as subjects in this study. Without their support and cheerful endurance of the dietary regime, this study would not have been possible.

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INTRODUCTION

The science of nutrition has progressed over the centuries from a study of the single nutriment to an examination of complex nutrient interrelationships. An important phase in this development was the discovery of the fact that, instead of a single energy-producing nutriment, food was composed of four components, namely, carbohydrate, fat, protein and ash. This was later followed by the finding that these four nutriments alone were unable to support growth and development. The concept of accessory food factors then arose, early in the twentieth century. In the period that followed, many vitamins and trace mineral elements were identified and great progress made in the elucidation of their nutritional roles. At the same time, it was also recognized that enzyme structures were a complex of specific amino acids, vitamins and/or minerals and that the endocrine system played an important role in the regulation of energy and nutrient utilization. It has become evident that the almost 50 known nutrients do not function in the organism as single entities but that complex interrelationships exist between many nutrients, enzymes and hormones.

A protein-riboflavin interrelationship is believed to exist. Evidence to date indicates that urinary riboflavin excretion increases when dietary protein is inadequate or of poor quality. Since the presence of certain levels of riboflavin in the urine is widely used, in nutrition surveys, as the

criterion of adequacy of riboflavin intake, incorrect interpretation of data could result in certain instances if the protein-riboflavin interrelationship were ignored. This might occur with undernourished groups, such as the aged, who are on low protein intakes.

The present study was undertaken to obtain further information on the protein-riboflavin interrelationship. A limited number of studies have been conducted to examine this interrelationship and very few of these have been of women. Since wide variations between individuals are characteristic of biological studies, it is essential that hypotheses concerning human metabolic response be supported by the observations of many studies. Biochemical individuality is a factor which must not be overlooked.

This study was undertaken with the following objectives:

1. To observe differences, if any, in the levels of riboflavin excreted by five healthy women, normally consuming diets high in protein and riboflavin, while in positive and negative nitrogen balance.

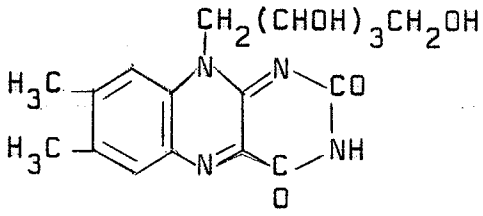
2. To observe the response of five subjects to a test dose of riboflavin after periods of positive and negative nitrogen balance.

REVIEW OF LITERATURE

As early as 1879, the existence of a yellow-green fluorescent pigment in milk whey was observed by A. W. Blyth (22). He called the substance lactoflavin. The function and importance of this pigment in animal and human nutrition, however, was not realized until the 1930's. In 1933, Kuhn, Győrgy and Wagner-Jauregg (22) isolated the pigment from several foods and renamed it riboflavin. Later, these workers demonstrated the growth promoting properties of this pigment in rats. The chemical composition of riboflavin and its eventual synthesis was achieved almost simultaneously by Karrer (60) and Kuhn (60) in 1935.

CHEMICAL NATURE AND DETERMINATION OF RIBOFLAVIN

Riboflavin has the chemical formula 6,7-dimethyl-9-(1'-D-ribityl)-isoalloxazine and the following structure:



It is not soluble in ordinary fat solvents but is soluble to a limited extent in water. Riboflavin is fairly stable in strong mineral acids and oxidizing agents but is sensitive to alkali and to visible or ultra-violet light (19). When exposed to ultra-violet irradiation, it exhibits a yellow-green fluorescence

which is increased considerably in acid or alkaline solution. Alkaline irradiation produces lumiflavin (6,7,9-trimethylisoalloxazine) and neutral or acidic irradiation produces lumichrome (6,7-dimethylalloxazine). These reactions are not directly reversible. Riboflavin is easily reduced by sodium hydrosulfite, hydrogen sulfide in alkaline solution, hydrogen in the presence of a catalyst and by other reducing agents. The reduction product, leucoriboflavin, is a colorless non-fluorescent compound which is readily re-oxidized by atmospheric oxygen.

Fluorometric and microbiological procedures are used in the determination of riboflavin in biological fluids and food. The fluorometric procedure relies upon the fact that the fluorescence of riboflavin is proportional to its concentration under controlled conditions of pH, temperature, and salt concentration (22). The microbiological method depends on the growth stimulation of Lactobacillus casei by riboflavin (53). Although the widely-used microbiological technique is sensitive and specific, very satisfactory results have been obtained from recent modifications of the fluorometric procedures.

Najjar (42) observed in 1941 that two sources of error, inherent in all methods in which fluorescence was measured in aqueous solutions, were turbidity of the solution and the formation of gaseous emulsions. The methods which utilize the addition of potassium permanganate followed by hydrogen peroxide to oxidize interfering pigments and fluorescent

substances result in the formation of minute bubbles of oxygen. These tend to remain dispersed in the medium causing a whitish tint which interferes with the accuracy of the measurement of the fluorescence. Najjar proposed that this difficulty could be avoided by the extraction and measurement of the fluorescence in a non-aqueous medium. He accomplished the extraction through the use of pyridine and butyl alcohol.

Najjar (42) also suggested the use of sunlight or a mercury vapor lamp to destroy the riboflavin in the test sample in order to obtain a blank reading. Reducing agents, such as sodium hydrosulfite, are also used but are not entirely satisfactory since leucoriboflavin, the reduction product, is readily re-oxidized by oxygen. Also, reducing agents decolorize other pigments as well as riboflavin and thus can alter the optical properties of the sample.

Slater and Morell (51) modified the Najjar procedure through the introduction of the 'internal standard' which corrected for the quenching of fluorescence by foreign pigments and other factors. These workers obtained satisfactory results by this method when they compared it with the microbiological method of Snell and Strong (53).

Other methods employ the use of adsorbing agents, such as Florisil, to extract the riboflavin from the test sample (13,18). This procedure reduces the presence of other fluorescent compounds or pigments in the extract and may be advantageous in the analysis of food samples or highly pigmented urine samples.

OCCURENCE AND FUNCTION OF RIBOFLAVIN

Riboflavin is synthesized by most higher plants and a variety of micro-organisms. Though higher animals are unable to synthesize the vitamin themselves, due to the inability of animal tissues to combine D-ribitol and 5,6-dimethylisoalloxazine, it is produced to a variable extent by micro-organisms in the intestinal tract (6).

Najjar and co-workers (44) have reported that the riboflavin derived from bacterial synthesis may be absorbed to some extent by the human organism. Other workers have demonstrated, however, that the amount absorbed is insufficient to maintain normal nutrition since the synthesis occurs predominantly in the large intestine. Thus, higher animals are dependent upon extrinsic sources for their riboflavin supply.

Evidence has been obtained recently which indicates that microbial synthesis may be influenced by the type of diet consumed. This, in turn, may have an effect upon the amount of riboflavin absorbed from the intestinal tract. Mannering, Orsini and Elvehjem (35) found large differences in the fecal riboflavin (which is derived chiefly from bacterial synthesis) when rats were fed diets with varying levels of fat. It appeared that high fat diets inhibited the microbial formation of riboflavin. De and Roy (15) demonstrated that dextrin and corn starch favored bacterial synthesis whereas sucrose did not.

Czaczkes and Guggenheim (14) reported decreased fecal riboflavin and a reduction in the number of viable bacteria in

the feces but a low fat diet produced an increased number.

Work done by Iinuma (28) indicated somewhat similar results in man. One male subject was studied for a period of 6 weeks. The fecal riboflavin excretion on a normal mixed diet was found to be approximately 0.5 mg. daily. When the subject was placed on a vegetable diet, slightly lower in fat content, the fecal riboflavin values increased markedly until about 2.5 mg. were excreted per day. No significant difference in fecal riboflavin was noted on a meat diet which was somewhat higher in fat and protein than the normal diet.

Boyden and Erikson (7) observed a decrease in the fecal riboflavin excretion in preadolescent children consuming a diet providing approximately 50 per cent of the recommended intake for protein. These workers stated that this may have been the result of several factors, including the low lactose, high sucrose, and low protein intake.

Oldham, Lounds and Porter (46), however, in their studies on young women maintained on diets high and low in protein, observed only small and inconsistent differences in the fecal riboflavin values.

Free riboflavin from foods must be phosphorylated before it can be absorbed from the intestinal tract. This is presumably accomplished by an enzymatic reaction in which a secretion from the adrenal glands plays an important role. In the tissues of the body, riboflavin is found mainly in the form of flavoproteins and nucleotides. Free riboflavin occurs only to a

limited extent in the tissues and plasma but is found in larger concentrations in the urine and retina of the eye (60). Animals do not appear to possess a specialized mechanism for the storage of riboflavin. High intakes of riboflavin may result in an increase in tissue content for a short period of time but storage of the extra vitamin is poor because of the low renal threshold of riboflavin (6).

Mammalian tissues have been shown to have a number of flavoprotein enzyme systems each containing a specific protein (apoenzyme) and a riboflavin-containing prosthetic group (co-enzyme), either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). The riboflavin-containing prosthetic group functions as an intermediate carrier of hydrogen in oxidation-reduction reactions in the mitochondria of living cells. Flavo-proteins are classified as flavoprotein oxidases which are capable of utilizing molecular oxygen as a hydrogen acceptor, and flavoprotein dehydrogenases which are capable of reducing substances other than molecular oxygen. Among these flavin-containing enzymes are xanthine oxidase, D-amino acid oxidase, L-amino acid oxidase, glycine oxidase, aldehyde oxidase, glucose oxidase and cytochrome c reductase (4).

Several workers have investigated the effect of dietary riboflavin intake upon the activity of the flavin enzymes. Axelrod and Elvehjem (3) found that xanthine oxidase activity in the liver of rats decreased considerably in riboflavin deficiency. Riboflavin therapy was found effective in restoring the xanthine

oxidase activity to its normal level. These workers also observed that increasing the intake of riboflavin resulted in an increase in the activity of xanthine oxidase and D-amino acid oxidase.

Burch, Lowry, Padilla and Combs (10) noted considerable variation in the sensitivity of the flavin enzymes, in the liver of rats, to riboflavin deficiency. After three weeks of deficiency, D-amino acid oxidase activity decreased to 35 per cent of the control value. After six weeks, glycine oxidase activity was only 20 per cent of the control value whereas L-amino acid oxidase was 70 per cent. Glycolic acid oxidase activity had disappeared almost completely after twelve weeks and xanthine oxidase activity was 35 per cent of the control. The activity of diphosphopyridine nucleotide (DPNH) dehydrogenase decreased only a small amount even in severe riboflavin deficiency. In a second series of experiments, rats depleted in riboflavin for 5 to 6 weeks, were given intraperitoneal injections of riboflavin. This resulted in a substantial increase, within a few hours, in the activity of glycolic acid oxidase, D-amino acid oxidase and xanthine oxidase. The activity of glycine oxidase also increased but took considerably longer. Deij (16) conducted similar experiments and confirmed the findings of these workers.

RIBOFLAVIN LEVELS IN THE BLOOD AND URINE OF HUMANS

The concentration of riboflavin in the blood and urine of humans on varying levels of riboflavin intake has been investigated by many workers. Attempts have been made to correlate the percentage of riboflavin excreted in the urine and the concentrations of free riboflavin and its nucleotides in the blood with the adequacy of the riboflavin intake. Investigations to date have not revealed a relationship between the concentrations of riboflavin in the blood and dietary intake. Difficulties have been encountered in the separation and estimation of the three forms of riboflavin in the various fractions of the blood. In view of this fact, most research workers agree that further work is required in this area.

Suvarnakich, Mann and Stare (55) found the use of free riboflavin in the serum unsatisfactory as a criterion for the estimation of riboflavin adequacy because of its small concentration and difficulty in measurement. They also observed a wide range in the levels of free riboflavin plus FMN in the serum of 141 subjects on unrestricted but nutritionally adequate diets.

Horwitt, Love and Bessy (26) reported that the plasma FAD values did not change significantly in ten subjects during riboflavin restriction over a period of 16 months. Total riboflavin content of the red blood cells, however, was found to be consistently lower in the restricted group than in the control group. White blood cell concentrations of riboflavin did not

differ significantly in the two groups. Beal and Buskirk (5) studied the riboflavin concentration in the red blood cells of children and found no significant relationship between the intake and the level of riboflavin in the red blood cells. These children, however, were consuming riboflavin in excess of the recommended intake. Lower levels of riboflavin may show a relationship to exist.

Horwitt and co-workers (25) concluded from extensive studies on urinary riboflavin excretion that the output of riboflavin was closely related to the dietary intake. They observed that the excretion of riboflavin by human subjects on diets containing varying surpluses of riboflavin differed greatly, but as the intake of the vitamin was reduced, the variations in output became smaller. At low levels of intake, the amounts excreted became quite uniform. In one of their studies, they found that the average urinary excretion of 42 subjects consuming a diet which provided 1.6 mg. riboflavin daily was 434 $\pm$ 185 µg. This represented 25 to 30 per cent of the dietary intake. The average 24 hour excretion of 12 subjects whose dietary intake changed from 1.6 mg. to 0.85 mg. dropped from 399 $\pm$ 138 µg. to 133 $\pm$ 52 µg. after ten weeks. Four weeks later, the average daily excretion reached the plateau level of 76 $\pm$ 38 µg. which persisted with only minor variations for the next two years.

It was also observed that 30 subjects on a daily intake of 1.1 mg. riboflavin for a period of three months excreted an average of 112 $\pm$ 75 µg. daily. Reducing the intake to 0.55 mg.

resulted in a drop in riboflavin excretion to 49 ± 19 µg. after seven days. The authors suggest that this would indicate that no riboflavin reserve could have been accumulated during the three months on the 1.1 mg. diet. Subjects on a daily intake of 0.55 mg., who developed signs of riboflavin deficiency, were given a 6 mg. oral test dose of riboflavin daily. The urinary riboflavin excretion of these subjects increased very rapidly. For one subject, the daily excretion rose from 33 µg. to 1100 µg. in one day and to 2590 µg. in eight days.

Storvick, Wu and Warren (54) observed that subjects on a daily intake of 1.2 mg. of riboflavin for a period of thirty and thirty-four days excreted an average of 390 µg. riboflavin daily. This represented approximately 33 per cent of the intake. Two subjects of larger body build excreted a lower percentage than this during the last ten days of the study. On the basis of this finding, the authors suggested that body size should be considered in the estimation of riboflavin requirement. Administration of a 2 mg. oral test dose of riboflavin two weeks after the close of the study revealed that urinary riboflavin excretion reached a peak at the end of the first hour. After five hours, the excretion was nearly as low as the one hour fasting excretion. Free riboflavin and total riboflavin in the serum also reached a peak one-half to one hour after ingestion of the test dose. Free riboflavin returned to the fasting level by the end of the second hour and total riboflavin by the fifth hour. These findings illustrate that the tissues are unable to

retain extra riboflavin.

Two series of experiments with college women on self-selected and experimental diets were conducted by Brewer, Porter, Ingalls and Ohlson (8). In the first series, on self-selected diets, the group as a whole excreted from 9 to 57 per cent of a 3 mg. test dose of riboflavin within four hours. In the 24-hour period following dosage, four of the twenty subjects excreted less than 20 per cent and 10 subjects excreted more than 30 per cent of the supplement. The highest excretion was 68 per cent. They observed that the average urinary riboflavin excretion for the three days on the self-selected diet was significantly related to the one hour fasting excretion, the four hour excretion after the oral test dose and the 24 hour excretion after the test dose.

In the second series, the average total daily urinary excretion of the subjects on the experimental diets was approximately 10, 20 and 53 per cent for daily intakes of 0.79 to 1.21 mg., 1.62 mg. and 2.23 mg. respectively. As the intake increased, an increase in the percentage of a 2 mg. test dose of riboflavin excreted was also observed.

Morley and Edwards (39) investigated the 24 hour urinary riboflavin excretion of 7 subjects on a diet providing 1.4 mg. riboflavin daily. They observed that the subjects excreted an average of 26 per cent of the daily intake except for one subject whose excretion decreased steadily throughout the period. This subject excreted an average of 13 per cent of the daily intake.

No details on the body size or prior nutritional status of this subject were given and no explanation was advanced for the difference in response.

Kraut, Ramaswamy and Wildemann (32) administered daily test doses of 2, 4, and 10 mg. of riboflavin to each of three subjects and observed that the excretion increased gradually and reached a maximum after one to one and one-half hours. The peak value of excretion was reached sooner with small doses than with large ones. The authors suggest that this is probably due to the increased time required for intestinal absorption. About two-thirds of the riboflavin intake was excreted in four hours.

In a study done by Najjar and Holt (43), it was demonstrated that subjects whose tissues are depleted in riboflavin tend to retain more of a test dose than normal subjects. Healthy subjects given an intravenous injection of 1 mg. riboflavin excreted from 277 to 683 μg. in four hours, while subjects suffering from riboflavin deficiency excreted only 74 to 194 μg. in the same period of time. That is, 28 to 68 per cent of the test dose was excreted by the healthy subjects in comparison with 7 to 19 per cent by depleted subjects.

Tucker, Mickelson and Keys (56) conducted investigations to assess the effects of sleep, work, diuresis, heat, acute starvation, thiamine intake and bed rest on urinary excretion of riboflavin. These workers found that the rate of excretion during the waking hours was very constant for each individual