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

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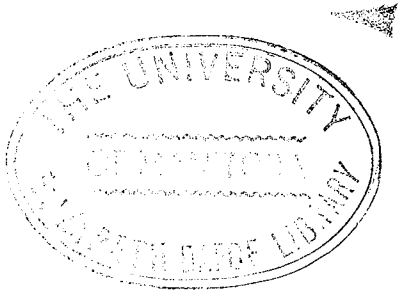

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Five healthy women were maintained for two consecutive 10-day periods on a controlled intake of 1.4 milligrams of riboflavin. For the first period, the nitrogen intake was 3.5 grams and, for the second, 15.4 grams. Twenty-four hour urine and fecal 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.

ACKNOWLEDGEMENTS

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.

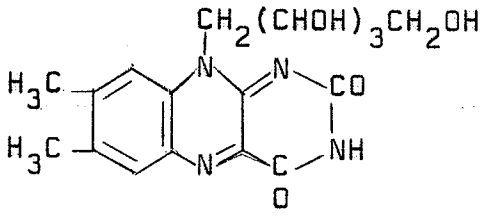
2. To observe the response of five subjects to a test dose of riboflavin after periods of positive and negative nitrogen balance.

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

on any particular day but that the inter-individual values varied by as much as forty-fold. The hourly rate of excretion during sleep was found to decrease significantly. Reduced absorption of riboflavin from the intestine and decreased blood circulation through the kidneys were suggested as possible reasons for this. The investigators found a reduction in the rate of excretion during work and suggested that physical exercise may be associated with the formation of new muscle tissue which incorporates about 3 µg. of riboflavin, per gram of tissue. Water diuresis was found to have no effect on riboflavin excretion by normal subjects at both high and low intakes of riboflavin. They further reported that acute starvation, heat stress, enforced bed rest and thiamine deficiency all led to an increase in riboflavin excretion.

RIBOFLAVIN REQUIREMENT OF HUMANS

Pure riboflavin deficiency, or ariboflavinosis, is characterized by one or several of the following symptoms, cheilosis, glossitis, seborrheic dermatitis and corneal vascularization. The syndrome was first described by Sebrell and Butler (50) in 1938. Their subjects were consuming a diet providing 0.5 mg. riboflavin daily. Horwitt, Hills, Harvey and Liebert (24) confirmed these findings in 1949. They also observed that, unlike thiamine deficiency, there were no neurologic abnormalities and no changes in appetite, attitude or activity in the person with riboflavin deficiency. Biochemically, there was also a sharp

contrast between subjects with riboflavin deficiency and those with thiamine deficiency. A 'double metabolic load' of glucose plus exercise failed to reveal any abnormality in the level of lactic or pyruvic acids in the blood of the riboflavin deficient subject. This was unexpected since these vitamins play an important role in the enzyme systems involved in carbohydrate metabolism.

Horwitt, Liebert, Kreisler and Wittman (23) were unable to produce any symptoms or signs of physical impairment that could be with certainty attributed to lack of riboflavin. Their subjects received an intake of 0.8 to 0.9 mg. of riboflavin per day. Williams, Mason, Cusick and Wilder (58) maintained four women on a diet supplying 0.8 to 0.9 mg. riboflavin daily without finding either subjective or objective signs of deficiency.

Results of work done by Keys, Henschel and Mickelsen (30) indicated that normal young men, experiencing no apparent form of stress, suffered no physiological or clinical handicap by restriction to an intake of 0.31 mg. per 1000 Calories daily for a period of 5 months. These workers stated, however, that over a longer period of time or for very young or aged subjects, larger intakes may prove necessary.

Horwitt, Hills, Harvey and Liebert (25) in 1950, observed that subjects consuming 1.1 mg. riboflavin accumulated no reserve of riboflavin over a period of 3 months. When placed on an intake of 0.5 mg., the excretion dropped from 112 $\pm$ 75 µg. to 49 $\pm$ 19 µg. within seven days. It was also noted that subjects

receiving 1.6 mg. riboflavin daily excreted almost four times as much riboflavin as those subjects receiving 1.1 mg. riboflavin daily.

The optimal intake of riboflavin, that is, the level producing saturation of the tissues, is believed to coincide with the 'critical point' of excretion. This is the point at which excretion increases very rapidly with increasing intakes. The results of the 1950 study by Horwitt et al (25), would tend to indicate that the 'critical point' may be between daily intakes of 1.1 mg. and 1.6 mg. riboflavin.

Due to the important role played by flavoproteins in tissue respiration, the riboflavin requirement has been related to oxygen consumption or energy expenditure, and often expressed in relation to caloric intake under conditions of calorie balance. The Canadian Council on Nutrition has based their recommended intake for riboflavin on its relationship to energy expenditure. The revised standard of 1964 (11) set the recommended level at 0.5 mg. per 1000 Calories for adults.

Recently, riboflavin requirement has also been linked with protein metabolism. It has been demonstrated that riboflavin is essential for growth in animals (9), and evidence has been reported relating nitrogen balance with riboflavin excretion (46,52). Bro Rasmussen (9), in his extensive report on riboflavin requirement, stated that during quantitative or qualitative insufficiency of protein, signs of riboflavin deficiency may be seen at widely varying intakes of riboflavin, from

low intakes up to 1.5 mg. daily. He also stated that there is more evidence for considering the requirement of riboflavin in relation to energy turnover than to protein requirement. Maslenikova (37), however, proposed that the riboflavin requirement be expressed relative to protein requirement and suggested that the riboflavin to protein ratio is 0.035 for children 6 months to 11 years, 0.030 for adolescents, and 0.025 for adults.

FLAVOPROTEINS AS A LABILE PROTEIN RESERVE

The concept of a labile protein reserve developed when it was recognized that tissues are differently affected by changes in nutrition. Some tissues gain or lose substances much more rapidly than others (41). This labile protein reserve is not considered to be a discrete type of protein with only a reserve or storage role. Instead, it is believed to be specific protein complexes. The presence of large stores of amino acids in the body would upset the homeostatic mechanisms and, if any or all proteins were called upon to supply amino acids in times of stress, interference with the basic functional and morphological structures of the body would occur (48).

Bro Rasmussen (9) reported that, in protein deficiency, enzymes vary greatly in lability. Flavoproteins, in general, show the greatest loss of activity. Thus, it has been postulated that some flavoproteins may represent one form of labile protein reserves. During periods of inadequate protein intake, these flavoproteins would be catabolized and riboflavin would be

released. This would result in increased urinary riboflavin excretion. During periods of positive nitrogen balance, riboflavin would also be retained to replace the flavoprotein stores (48).

PROTEIN - RIBOFLAVIN INTERRELATIONSHIP IN ANIMALS

Evidence of the role of riboflavin in the anabolic and catabolic reactions involved in protein metabolism has been obtained in studies with animals. Several workers have reported that the riboflavin content of the livers of animals is dependent on protein intake rather than on riboflavin intake.

Unna et al (57), reported a gradual decline in the riboflavin concentration in the livers of rats fed on an 8 per cent casein diet. After three months, the riboflavin concentration in the liver was almost one-half of that of the control rats fed an 18 per cent casein diet. In spite of the ingestion of liberal amounts of riboflavin, the rats maintained on the low protein diet were unable to retain the riboflavin.

Similar results were obtained by Czaczkes and Guggenheim (14). Rats maintained on a low protein diet showed a greatly reduced organ riboflavin content. Irregardless of the amount of riboflavin administered, the organism appeared unable to maintain a functional level of riboflavin. The rats died of aribоflavinosis after 8-9 weeks on the low protein diet.

Sarett, Klein and Perlzweig (49) investigated the protein-riboflavin interrelationship in dogs and rats. Urinary

excretion of riboflavin in both dogs and rats was found to be highest during the period of low protein intake. Riboflavin excretion also increased during periods of starvation and when casein was replaced by glycine in the diet. The percentage of a test dose of riboflavin excreted by the dogs and rats was higher on the low protein diet than on the high protein diet.

Andrain, Lunner and Terroine (1) maintained adult male rats for a preliminary period on a basal diet adequate in all nutrients but low in protein. This was followed by a period in which the experimental diets contained increased amounts of protein or protein of higher biological value. Riboflavin levels in the urine were greatly reduced when protein intake was increased after the period of low protein intake, but tended to increase again if the high intake was continued. Qualitative improvement of the protein reduced the output of riboflavin in the urine. Riboflavin concentration of the liver was the highest with high intakes of protein.

The effect of animal and vegetable protein on riboflavin excretion was studied by Fen Go-Chen (20). The mean daily output of riboflavin was found to be much greater in rats given gluten, as the sole source of protein, than those given casein. The amount of riboflavin and FAD per gram of liver, however, were not significantly less than in the rats given casein. The addition of lysine and tryptophan to the gluten diet resulted in lower weight gain than the casein diet but the excretion of riboflavin was reduced to the same level.

Evidence which would indicate that riboflavin was involved in the anabolic process of protein metabolism was obtained by Borgstrum and Hammarstren (21), and Mayfield and Hedrick (38). These workers found that, in rats, the intake of riboflavin was directly related to the degree of nitrogen storage and to the ratio of weight gain to food intake. Mayfield and Hedrick calculated the response in terms of the biological value of the dietary intake. They found that the biological value increased with increasing riboflavin intake. Kleiber and Jukes (36) also observed that chicks deficient in riboflavin stored about 30 per cent less of the dietary nitrogen and gained less weight than chicks on an adequate diet.

RIBOFLAVIN EXCRETION AND NITROGEN BALANCE IN HUMANS

An inverse relationship between protein intake and riboflavin excretion was demonstrated in three young women by Oldham, Lounds and Porter (46). Their subjects were maintained on diets providing approximately 5 gm. nitrogen and 18 to 20 gm. nitrogen. Riboflavin intake was approximately 1.0 mg. daily. During periods of strong negative nitrogen balance, the subjects excreted 40 to 60 per cent of the riboflavin intake and, during slightly negative nitrogen balance, 20 to 30 per cent of the intake. Positive nitrogen balance resulted in an immediate drop in riboflavin excretion to approximately 7 per cent of the intake. A test dose of 20 µg. of riboflavin per kilogram of body weight was administered to the subjects following each experimental

period. Two subjects showed lower excretions after periods of positive nitrogen balance than after negative nitrogen balance. The third subject, however, showed no relationship between the test dose return and nitrogen balance.

Smith and co-workers (52) conducted similar studies with seven young men who were maintained on a constant riboflavin intake of 1.6 mg. daily but four different levels of nitrogen. While on the diet containing essentially no nitrogen, the riboflavin excretion ranged from 557 to 997 µg. daily. The riboflavin excretion dropped by approximately 200 µg. when the subjects were placed on 2 and 4 gm. nitrogen diets and about 500 µg. when placed on a 17 gm. nitrogen diet. The mean riboflavin excretion on the 17 gm. nitrogen diet was 247 µg. The percentages of the riboflavin intake excreted on each level of nitrogen were 46, 29, 27 and 17 respectively.

Pollack and Bookman (48) observed that nutritionally normal hospital patients who were in nitrogen equilibrium usually excreted less than 50 per cent of ingested riboflavin and, when in negative nitrogen balance, excreted more than 50 per cent. Studies on post-operative and convalescent patients showed that riboflavin excretion exceeded the intake by 3 to 7 times in some cases in the immediate post-operative period when the loss of nitrogen from the body was the greatest. As nitrogen equilibrium was restored and a positive nitrogen balance achieved, the percentage of riboflavin excreted dropped markedly. In the convalescent patients who were in poor nutritional status at

the beginning of the study, a positive nitrogen balance was associated with a high retention of riboflavin. These workers concluded that, since the excretion of riboflavin during negative nitrogen balance was in excess of the intake, it had to be derived from the breakdown of body riboflavin which was presumably released from flavoproteins.

Windmeuller, Anderson and Mickelsen (59), studied riboflavin excretion during 24-37 hours of fasting. In all but one subject, the riboflavin excretion increased during the fasting period. The increase in micrograms of riboflavin per hour ranged from 15 to 248 per cent of that during the control period and was accompanied by a large excretion of nitrogen. The first indication of an increase in riboflavin excretion was usually seen in the interval 13 to 20 hours after the last meal.

Boyden and Erikson (7) studied riboflavin utilization in relation to nitrogen intake in 35 preadolescent girls on nitrogen intakes ranging from 3 to 14 gm. daily. Forty-five to 68 per cent of the riboflavin intake was excreted daily by the subjects who were on intakes ranging from 1.84 to 2.41 mg. of riboflavin daily. During the period of 3 gm. nitrogen intake, the riboflavin excretion was found to decrease from that excreted on the higher level of intake. This finding differs from that of Oldham et al (46), who found that low intakes of nitrogen resulted in increased riboflavin excretion. The children, however, were not in negative nitrogen balance. The workers suggest that the decrease may indicate lower metabolic demands for

riboflavin with lower nitrogen turnover. If negative nitrogen balance had occurred, riboflavin excretion might have increased.

Derby (17) found no significant relationship between daily nitrogen balance and daily urinary riboflavin excretion within a given individual or between individuals, in 11 adults on a low riboflavin intake for a period of 72 days. However, when period averages of nitrogen balance and urinary riboflavin excretion were correlated, an inverse relationship was found within the group as a whole.

In investigations of the isoleucine and leucine requirements of children, Nakagawa et al (45) found that, when negative nitrogen balance was induced by excluding leucine from the diet, the excretion of riboflavin increased.

Maslenikova and Kosenko (36) studied the urinary riboflavin excretion levels of forty kindergarten children aged 3 to 7 years. They reported that the urinary output of riboflavin ranged from 0.05 to 0.65 mg. with the majority of the values below 0.30 mg. The output of riboflavin in the urine rose with an increase in riboflavin intake but fell with an increase in protein intake. The authors concluded that protein intake must, therefore, be considered in any study of riboflavin requirement.

PROCEDURE

SUBJECTS

Five women, staff members at the University of Manitoba, served as subjects in this study. The subjects ranged in age from 23 to 50 years. They were in good health and, prior to the study, were consuming nutritionally adequate diets as ascertained from dietary histories and three-day food records. The subjects were moderately active and engaged in similar activities. Each subject was aware of the importance of adhering to the dietary regime and made every effort to conform to all restrictions.

Information regarding the age, height, initial weight and mean weight per period of each subject is presented in Table I. Table II gives the average daily nutrient intake for each subject prior to the study as calculated from three-day food records.

DIETARY REGIME

The study consisted of two consecutive 10-day periods during which the riboflavin intake was kept at a constant level of 1.4 mg. per day. During Period I, the diet provided approximately 3.5 g of nitrogen and, during Period 2, approximately 15.4 g of nitrogen.

The first three days of each experimental period were regarded as adjustment to the different level of nitrogen in the

TABLE I

AGE, HEIGHT, INITIAL WEIGHT AND MEAN WEIGHT PER EXPERIMENTAL PERIOD FOR SUBJECTS

Subject	Age	Height	Initial Weight	Average Weight Per Period	
				I	II
	yr.	in.	kg.	kg.	kg.
JS	23	66.5	49.9	49.9	49.5
RD	32	62.5	52.5	52.5	51.9
HL	50	66.8	65.0	64.4	63.1
KY	45	66.0	66.8	67.0	67.0
TW	48	64.8	63.6	63.0	63.1

TABLE II

MEAN NUTRIENT INTAKE OF SUBJECTS ON SELF-SELECTED DIETS PRIOR TO EXPERIMENTAL PERIOD

SUBJ.	CAL.	PRO.	FAT	CARB.	CA.	IRON	VIT.A	THIA.	RIB.	NIA.	VIT.C
		(gm)	(gm)	(gm)	(mg)	(mg)	(I.U.)	(µg)	(µg)	(mg)	(mg)
JS	1864	70	85	208	1051	9.3	10656	977	2152	12.8	45
	*2200	35	-	-	500	10	3700	660	1100	6.6	30
HL	1683	76	84	160	777	8.8	5757	1069	1456	13.5	82
	*2320	45	-	-	500	10	3700	756	1260	7.6	30
KY	1648	69	63	204	567	9.7	4173	1021	1266	10.2	89
	*2320	47	-	-	500	10	3700	756	1260	7.6	30
TW	1690	64	60	231	388	13.4	5567	838	1174	27.5	116
	*2320	44	-	-	500	10	3700	756	1260	7.6	30
RD	1516	86	67	128	726	11.1	3308	1412	1967	11.6	145
	*2134	37	-	-	500	10	3700	640	1067	6.4	30

* Canadian Dietary Standard (11).

diet and were considered separately from the following seven days.

The nutrient content of the experimental diets may be found in Table III. Except for protein, in Period I, the experimental diets met or exceeded the recommended levels for each subject according to the Canadian Dietary Standard. To meet the recommended level for calcium, 300 mg. of calcium, in the form of calcium lactate tablets, were taken daily by each subject. To maintain the riboflavin intake at the constant level of 1.4 mg. daily throughout the study, it was necessary, during Period I, to add 1.0 mg. of crystalline riboflavin to the juice in the diet. The foods in this low protein diet contributed only 0.4 mg. riboflavin daily.

Calories were supplied at a level adequate to closely maintain the initial weight of each subject throughout the entire study. Caloric adjustments were made by each individual through variations in the amounts of sugar, butter, hard candy, jelly and carbonated beverages consumed.

The daily consumption of tea and coffee was regulated during both periods because of the riboflavin content of these beverages. Water and seasoning agents (salt, pepper, paprika and cinnamon) were allowed ad libitum.

The components of the two experimental diets are listed in Table IV. The low protein diet of Period I contained large amounts of canned fruits, fruit juices and vegetables in order to supply adequate bulk and sufficient calories to maintain the

TABLE III

NUTRIENT CONTENT* OF EXPERIMENTAL DIETS

DIET	CAL.	PROT. (gm)	FAT (gm)	CARB. (gm)	CA. (mg)	IRON (mg)	VIT.A (I.U.)	THIA. (µg)	RIB. (µg)	NIA. (mg)	VIT.C (mg)
Low Protein	1637	21.9	42	321	282	10	5715	727	542	6.4	144
		**21.6							**440		
High Protein	1821	101.7	79	168	543	17	5628	960	1519	19.5	110
		**96.3							**1365		

* Calculations based on data from Bowes and Church (12)

** Analyzed values for protein and riboflavin (protein = gm. nitrogen x 6.25)

TABLE IV

EXPERIMENTAL DIETS

LOW PROTEIN DIET		HIGH PROTEIN DIET	
Breakfast:	gm.	Breakfast:	gm.
pineapple juice	125	orange juice	125
applesauce	240	soft boiled eggs	100
bread	20	bread	20
butter	5	butter	5
apple jelly	20		
Lunch:		Lunch:	
apple juice	250	turkey	90
boiled potato	100	celery	30
celery	60	mayonnaise	30
can. green beans	60	tomato - raw	50
lettuce	20	bread	20
Fr. dressing	20	butter	5
butter	10	lettuce	15
can. cherries (2 T. jce.)	120	can. peaches (2 T. jce.)	110
sugar cookie	20	plain cookie	8
Dinner:		Dinner:	
grapefruit-orange jce.	125	round steak	90
ground beef	30	tomato (canned)	60
can. yellow beans	60	onion	3
cabbage	30	frozen peas	60
mayonnaise	20	potato	50
carrot	25	lettuce	15
onion	3	Fr. dressing	10
butter	5	butter	10
can. pears (2 T. jce.)	230	butter tart	40
Bedtime Snack:		Bedtime Snack:	
pineapple juice	250	bread	40
bread	20	butter	10
butter	5	apple jelly	30
apple jelly	20	milk	240

weight of the subjects. For this reason, the subjects showed some initial dislike for the diet but were able to adapt satisfactorily.

Except for the lettuce, celery and cabbage, all foods were purchased in a quantity large enough for the duration of the study, and canned foods (with the same code number) were selected in order to minimize variation in the nutrient content of the foods. The bread, cookies, and butter tarts were prepared especially for the study. The round steak and turkey used in the high protein diet were frozen in aluminum foil in daily portions.

The meals were prepared, weighed, and served in the laboratory except for the evening snack which was prepared in the laboratory but eaten at home.

On the day following each experimental period, the subjects were given a 5.0 mg. oral test dose of riboflavin immediately upon arising. Then, for a period of four hours, during which the subjects fasted, complete urinary collections were made every 30 minutes. For the remainder of the day, the experimental diet was resumed although the day was not considered part of the 10-day experimental period.

METHODS OF ANALYSIS

Twenty-four hour urine samples were collected in brown bottles containing approximately 2.0 g of oxalic acid. The total urinary output was determined daily for each subject and

aliquots removed and frozen at -15°F until analyzed for riboflavin, nitrogen and creatinine. Aliquots for riboflavin analysis were frozen in brown bottles.

Feces marked with carmine and methyl-cellulose as suggested by Lutwak and Burton (34), in a ratio of 1 to 2, were collected in waxed containers and frozen at -15°F until analyzed for nitrogen. Prior to analysis, the collections were combined into 5-day composites, diluted to approximately 2 liters and brought to pH1 with concentrated hydrochloric acid. The composites were autoclaved for 1 to 1½ hours at 15 pounds pressure, weighed, and aliquots taken for nitrogen analysis. Riboflavin analyses were not performed on the fecal samples. Review of the information to date leads to the conclusion that fecal riboflavin is chiefly derived from bacterial synthesis in the large intestine.

Duplicate samples of the two experimental diets were collected and set aside for laboratory analysis. The foods were thoroughly mixed in a Waring Blendor, diluted to 2 liters and brought to pH3 with concentrated hydrochloric acid. Aliquots were taken from the weighed composite and frozen at -15°F until analyzed for riboflavin and nitrogen. Prior to analysis for riboflavin the aliquots were incubated with Clarase for 24 hours at 45°C (47).

A modification of the fluorometric method of Slater and Morell (29) was used for all riboflavin analyses. This method was found to give very clear solutions and recoveries of added

riboflavin ranged from 95 to 101 per cent. The A.O.A.C. (Association of Official Agricultural Chemists) method (27) was tested and found to be less satisfactory. The fluorescence of the riboflavin was obscured to some extent by the minute bubbles of oxygen in the aqueous medium. Recoveries, as a result, were lower ranging from 85 to 88 per cent.

Sodium fluorescein was used for the standardization of the photofluorometer (Model Coleman 12C). To correct for non-riboflavin fluorescence, the samples were exposed to light using a 150 watt bulb for approximately $1\frac{1}{2}$ hours, until 85 to 90 per cent of the riboflavin had been destroyed (29).

To ensure the reliability of the instrument and method, the following measures were taken. A standard curve was prepared to determine if the instrument gave a linear response to different concentrations of riboflavin solutions. Also, to determine if riboflavin could be adequately recovered from an unpurified sample, varying amounts of riboflavin were added to urine samples and the percentage recovery calculated. To ensure the reproducibility of the method, a standard urine sample, taken from a 6 hour urine collection, was included with the day's analyses on every fourth series. The mean value for riboflavin excretion of the standard urine sample was found to be 438 ± 38.8 µg.

Samples were analyzed in duplicate and repeat analyses were performed on all determinations that appeared to be out of range. When the repeat measurements were within 50 ug. of the original measurement, averages of the two readings were taken.

The nitrogen content of the food, urine and fecal samples was determined by the macro-Kjeldahl method (27). Daily creatinine excretion was determined by the Folin method (29) as a check on the completeness of the 24 hour urine collection.

DISCUSSION OF RESULTS

Dietary histories indicated that all the subjects were in a good state of nutrition. In all cases, the intake for protein and riboflavin met or exceeded the recommended intakes in the Canadian Dietary Standard (11). Three subjects were receiving slightly less iron than the recommended level, one slightly less calcium and one slightly less vitamin A. These deviations were small, however.

The caloric intake for all five subjects was below the level recommended for light activity (Category A). However, all subjects had been maintaining their weights. This would indicate that they were receiving sufficient calories for the degree of activity in which they were engaged.

The subjects appeared to divide into two groups according to age and body weight. Three subjects were between the ages of 45 and 50 years and weighed between 64 and 67 kilograms. The other two subjects were 23 and 32 years of age and weighed 50 and 53 kilograms respectively.

The weights of the subjects remained essentially constant while on the experimental diets. The only exceptions were the large weight losses reported by subjects HL and TW on day 2. The 1-3/4 lb. weight loss of subject HL was accompanied by an unusually large urinary volume which could account for the weight loss in this subject. For the remainder of the study, her weight stayed at this new level with only minor fluctuations. No

explanation, other than error in measurement, could account for the $2\frac{1}{2}$ lb. weight loss for subject TW. Her weight varied very little from the initial weight for the remainder of the study.

NITROGEN BALANCE

Table V shows the daily nitrogen balance for each of the five subjects during Periods I and II.

During Period I, on the 3 gm. nitrogen intake, all five subjects were in negative nitrogen balance. During the three day adjustment period the negative nitrogen balance was greater than during the following seven days of the experimental period. It is assumed that approximately three days are required for adjustment to a new level of nitrogen intake. All subjects had been on a high protein intake (approximately 13 gm. nitrogen) prior to Period I. The nitrogen balance ranged from a low of -2.167 to a high of -0.694 with a mean of -1.409 ± 0.5970.

Many factors are known to influence nitrogen metabolism. Among these are emotional state, endocrine secretion, fever, and other physiological abnormalities. Biochemical individuality must also be an important consideration when working with human subjects.

Subjects TW and JS did not show as strong a negative nitrogen balance as the other three subjects. These two individuals may have had a smaller protein requirement than the others. This could be the case for subject JS who had a smaller body

TABLE V

DAILY NITROGEN BALANCE OF SUBJECTS DURING TWO EXPERIMENTAL PERIODS

DAY	SUBJECT HL	TW	KY	RD	JS	Mean per Day
			PERIOD I			
1	-4.260	-2.091	-3.927	-4.587	-4.233	-3.733
2	-2.460	-2.333	-1.905	-2.407	-2.459	-2.313
3	-1.911	-1.693	-1.913	-2.847	-*	-2.091
4	-1.965	-1.196	-2.140	-0.761	-0.454	-1.303
5	-1.479	-1.625	-1.318	-1.274	-0.129	-1.165
6	-1.434	-0.393	-1.911	-1.295	-0.577	-1.122
7	-0.851	-0.579	-1.737	-1.099	-0.257	-0.905
8	-2.168	-1.293	-3.123	-2.451	-1.827	-2.173
9	-2.539	-0.915	-2.543	-2.297	-0.645	-1.787
10	-1.310	-0.959	-2.398	-1.405	-0.969	-1.408
MEAN (7 days)	-1.678	-0.995	-2.167	-1.511	-0.694	-1.409
S.D.**	±0.5745	±0.4189	±0.5888	±0.6253	±0.5690	±0.5790
			PERIOD II			
1	+3.455	+4.679	+0.432	+7.696	+4.456	+4.144
2	+1.043	+0.955	-2.469	+4.686	+1.444	+2.120
3	+3.317	+4.607	+1.983	+3.247	+2.274	+3.059
4	+1.147	+1.085	+1.733	+1.733	+2.635	+1.667
5	-1.396	+1.515	-0.219	+0.539	+1.425	+1.019
6	+1.007	+0.799	+0.177	+1.413	+2.423	+1.163
7	-1.393	+0.759	+2.279	+2.079	+2.047	+1.711
8	+2.898	+4.120	+1.705	+0.915	+2.664	+2.461
9	+2.183	+0.901	+0.762	+0.605	+2.315	+1.397
10	+1.335	+0.825	+2.208	+2.071	+1.596	+1.607
MEAN (7 days)	+0.825	+1.429	+1.235	+1.337	+2.157	+1.571
S.D.	±1.6525	±1.215	±0.9964	±0.6588	±0.4906	±0.5239

* No urine collection obtained

** Standard deviation

build and the lowest weight. Subject RD, however, was similar in body weight to JS but showed a stronger negative nitrogen balance. On the fifth day of Period I, subject RD developed a slight sore throat and head cold which may have had an effect on the nitrogen balance. Subject TW, however, was of similar body build and weight as HL and KY who were in strong negative nitrogen balance. Subject KY was in the strongest negative nitrogen balance throughout Period I and negativity appeared to increase as the period progressed. This may have been the result of a greater protein requirement than the other subjects or some form of stress during this period.

Except for HL and KY, all of the subjects were in positive nitrogen balance throughout Period II. Subject HL was in negative nitrogen balance on day 5 and day 7 of the experimental period. Subject KY was in strong negative nitrogen balance on the second day of the adjustment period and on the fifth day of the experimental period. The mean daily nitrogen balance for the subjects for this period was +1.571 $\pm$ 0.5239.

Subject RD showed fairly large fluctuations in daily nitrogen balance. This subject was ill on days 8 and 9 and this may account for the lower positive nitrogen balance observed on these days. Subject TW showed a very high positive nitrogen balance on day 8. It is uncertain what could have caused this deviation from the low positive nitrogen balances observed throughout this period. Subject JS was in strong positive nitrogen balance throughout the entire experimental period of

Period II. Her mean nitrogen balance was +2.157 with a standard deviation of ± 0.4906. This subject, also, showed the least negative nitrogen balance during Period I.

The total of the accumulated negative nitrogen balances for each subject for Period I as compared with the total of the positive nitrogen balances for Period II are shown in Table VI. The total of the negative nitrogen balances for subjects HL and KY were greater than the total of the positive nitrogen balances. Subject KY had a large nitrogen deficit of approximately 14 gm. and HL a lower one of 7 gm. For the remaining three subjects, the positive nitrogen balances were greater, to varying degrees, than the accumulated negative nitrogen balances. Subject JS showed the highest retention of approximately 12 gm. nitrogen. In the study by Oldham et al (46) retentions of approximately 19 and 28 gm. of nitrogen were found in the two subjects, who had been in a good nutritional state prior to the study, and a retention of approximately 87 gm. by the subject who had previously been on a nutritionally inadequate diet. These subjects received slightly higher nitrogen intakes in both the high and low protein periods than the subjects in this study.

URINARY RIBOFLAVIN EXCRETION

Daily urinary riboflavin excretion and percentage of the intake excreted daily for each subject may be found in Tables VII and VIII respectively.

During Period I, which was characterized by negative

TABLE VI

CUMULATIVE NITROGEN BALANCES DURING TWO EXPERIMENTAL PERIODS

PERIOD	SUBJECT				
	HL	TW	KY	RD	JS
	gm.	gm.	gm.	gm.	gm.
I	-20.3789	-13.0795	-22.9158	-20.4240	-11.5510 *
II	+13.5981	+20.2474	+ 8.5903	+24.9869	+23.2802
Difference	- 6.7808	+ 7.1679	-14.3255	+ 4.5629	+11.7292

* Urine collection not obtained for one day

TABLE VII

DAILY URINARY RIBOFLAVIN EXCRETION (MICROGRAMS) DURING TWO EXPERIMENTAL PERIODS

DAY	SUBJECT HL	TW	KY	RD	JS	Mean per Day
			PERIOD I			
1	1128	887	436	819	1159	886
2	415	806	174	792	839	605
3	590	408	403	1101	-	626
4	540	225	520	589	1067	588
5	344	386	371	555	704	472
6	331	222	374	500	788	443
7	365	323	431	610	829	512
8	355	347	533	710	700	529
9	359	346	431	628	717	496
10	287	202	293	599	724	421
MEAN (7 days)	369	293	421	599	790	494
S.D.	±79.9	±87.0	±84.6	±64.5	±131.6	±200.2
			PERIOD II			
1	85	119	130	300	358	198
2	74	165	38	290	388	191
3	16	61	25	259	214	115
4	38	120	90	221	256	145
5	79	156	99	288	151	155
6	26	164	83	217	248	147
7	82	219	100	185	370	191
8	100	146	76	217	376	183
9	106	217	80	342	435	236
10	79	248	137	340	512	263
MEAN (7 days)	73	181	95	259	335	189
S.D.	±30.0	±46.7	±20.7	±20.3	±123.8	±110.2

TABLE VIII

PERCENTAGE OF DAILY RIBOFLAVIN INTAKE EXCRETED DURING TWO EXPERIMENTAL PERIODS

DAY	SUBJECT HL	TW	KY	RD	JS	Mean per Day
			PERIOD I			
1	78	62	30	57	80	62
2	29	56	12	55	58	42
3	41	28	28	77	-	43
4	38	16	36	41	74	41
5	24	27	26	39	49	33
6	23	15	26	35	55	31
7	25	22	30	42	58	36
8	25	24	37	49	49	37
9	25	24	30	44	50	34
10	30	14	20	42	50	29
MEAN (7 days)	26	20	29	42	55	34
			PERIOD II			
1	6	9	10	22	26	15
2	5	12	3	21	28	14
3	1	4	2	19	16	8
4	3	9	7	16	19	11
5	6	11	2	21	11	11
6	2	12	6	16	18	11
7	6	16	7	14	27	14
8	7	11	6	16	28	13
9	8	16	6	25	32	17
10	6	18	10	25	38	19
MEAN (7 days)	5	13	7	19	27	14

nitrogen balance, the mean daily riboflavin excretion ranged from 293 to 790 µg. with a mean of 494 ± 200.2 µg. This represented 20 to 55 per cent of the intake. Although inter-individual variation was large, day-to-day values for each subject were fairly constant. Subject HL showed the least variation in daily urinary riboflavin excretion.

In Period II, after the daily intake of nitrogen was increased to 15 gm., the mean daily urinary excretion ranged from 73 to 335 µg. with a mean value of 189 ± 110.2 µg. This represented 5 to 27 per cent of the intake. Riboflavin excretion dropped promptly and nitrogen balance became positive for all subjects on the 15 gm. nitrogen intake. The largest drop in mean riboflavin excretion, approximately 80 per cent, occurred with subjects HL and KY. For subjects TW, RD and JS, the percentage drop was 38, 57 and 58 respectively. Subjects HL and KY had very low riboflavin excretions, 16 and 26 µg. respectively, for day 3 of the adjustment period. Otherwise, the daily excretion levels for each subject were quite constant. They tended to increase towards the end of the period for subjects TW, RD and JS. This might indicate that the flavoprotein reserve in these subjects had been replaced.

The subjects appeared to divide into two groups according to riboflavin excretion as they had for body weight and age. The heavier weight subjects HL, TW and KY, excreted less riboflavin per period than the lighter subjects RD and JS. The mean daily percentage of the riboflavin intake excreted by the heavier

subjects were 20, 26 and 29 during Period I. For the lighter subjects they were 42 and 55 per cent. For Period II, the mean percentage of the intake excreted daily were similar for subjects HL and KY, 5 and 7 per cent respectively, but that for TW was somewhat higher at 13 per cent. The lighter weight subjects excreted 19 and 27 per cent of the intake.

Unfortunately, it was not possible to observe what effect, if any, age had on urinary riboflavin excretion since all heavier weight subjects fell into the older age group and all lighter weight subjects in the younger age group. Some heavier, younger subjects or lighter, older subjects in the study might have revealed a difference. Other workers, however, have reported that age probably has no effect on riboflavin excretion.

The percentages of riboflavin excreted daily by the heavier subjects, in this study, are comparable to those obtained by Oldham et al (46) for three young women 26 to 27 years of age, weighing approximately 50, 55 and 60 kilograms. These subjects were on a daily intake of approximately 1.0 mg. riboflavin. While in negative nitrogen balance, on a 5 gram nitrogen intake, the daily excretion was 200 to 350 µg. representing 20 to 30 per cent of the intake. A prompt drop in riboflavin excretion was also noted by these workers when the intake of nitrogen was increased to 18 to 20 gm. During positive nitrogen balance the mean daily excretion of riboflavin was approximately 100 µg. or 7 per cent of the intake.

Similar results were obtained by Smith et al (52) for

young men on a daily intake of 1.6 mg. of riboflavin. The nitrogen intake of the subjects had been raised gradually from no nitrogen to 2 gm., to 4 gm., and then to 17 gm. for periods of 12, 10, 10 and 10 days duration. The mean daily percentage excretions were 46, 29, 27 and 17 per cent respectively. The differences between the last two periods were not as great as those observed for the subjects in the study by Oldham et al (46) or for the heavier subjects in this present study. It appears that flavoprotein reserves may have been exhausted by the time the subjects were placed on the 4 gm. nitrogen diet, leaving only the more stable protein reserves from which less riboflavin would be released.

Morley (39) reported a mean percentage excretion of 26 per cent of the intake for seven subjects on a diet adequate in protein and containing 1.4 mg. of riboflavin. The lighter subjects of the present study excreted 18 to 27 per cent of the intake during Period II, which is similar to the values reported by Morley. The heavier subjects excreted much lower levels. The weight of subjects in Morley's study ranged from 55 to 70 kg. with one obese subject weighing 110 kg. Individual weights of the subjects were not reported, therefore no comparison with urinary riboflavin excretion could be made with Morley's subjects.

Horwitt et al (25) reported a mean excretion of 434 µg. (25 to 30 per cent of the intake) for 42 subjects on adequate protein and 1.6 mg. riboflavin. Brewer (8) reported an excretion of 20 per cent for the same level of intake. These levels also

agree with those obtained for the two lighter subjects during Period II of this study but are much higher than those obtained for the three heavier subjects during the same high protein period.

Storvick, Wu and Warren (54) reported a mean excretion of approximately 33 per cent of a 1.2 mg. riboflavin intake in their subjects consuming adequate protein. They also observed that two subjects of larger body build excreted a lower percentage of the intake. This finding is in agreement with that of the present study.

TEST DOSE RETURNS

Among the many biochemical measurements which aid in the evaluation of nutritional status in man is the urinary excretion of a vitamin or its metabolite following an oral or parenteral test dose of the vitamin. It has been repeatedly shown that subjects whose tissues are depleted in respect to a given vitamin retain more of it than do healthy subjects.

The percentage of the test dose excreted by the subjects after the periods of negative nitrogen balance and positive nitrogen balance, fell within a similar range, 23 to 42 per cent and 25 to 49 per cent, respectively. The mean per cent excretion after Period I was 31 per cent and after Period II was 36 per cent. The maximum excretion of the test dose occurred 1 to $1\frac{1}{2}$ hours after ingestion and then decreased sharply to a level at which it stayed fairly constant for the remainder of the four

hour period. This response is illustrated in Figure I.

While the mean cumulative excretions of the test dose over a four hour period were similar for both trials, the values for individuals were quite varied. The amount of the test dose excreted and the cumulative percentages for 8 consecutive thirty minute periods are shown in Tables IX and X.

Subjects HL, JS and KY showed a higher total percentage excretion after the period of positive nitrogen balance than after negative nitrogen balance. The difference in total excretions were 10, 13 and 16 per cent respectively. The total percentage excretion for TW was similar in both periods and the percentage excretions for each 30 minute period were also similar. Subject RD, gave a different response than the other four subjects. Her reaction was closer to the response expected. She excreted a very high percentage of the test dose following the period of negative nitrogen balance, but about one half of this amount after the period of positive nitrogen balance. It had been assumed that, since sufficient protein was not available to combine with the riboflavin after the period of negative balance, a large percentage of the test dose might be excreted. After Period II, more of the test dose of riboflavin might have been retained to increase the flavoprotein reserves in the body. Oldham et al (46) reported this expected response in two of her subjects but not in the third. Following two periods of negative nitrogen balance, the percentage of the test doses excreted by the two subjects were 33 and 37 per cent for one subject and

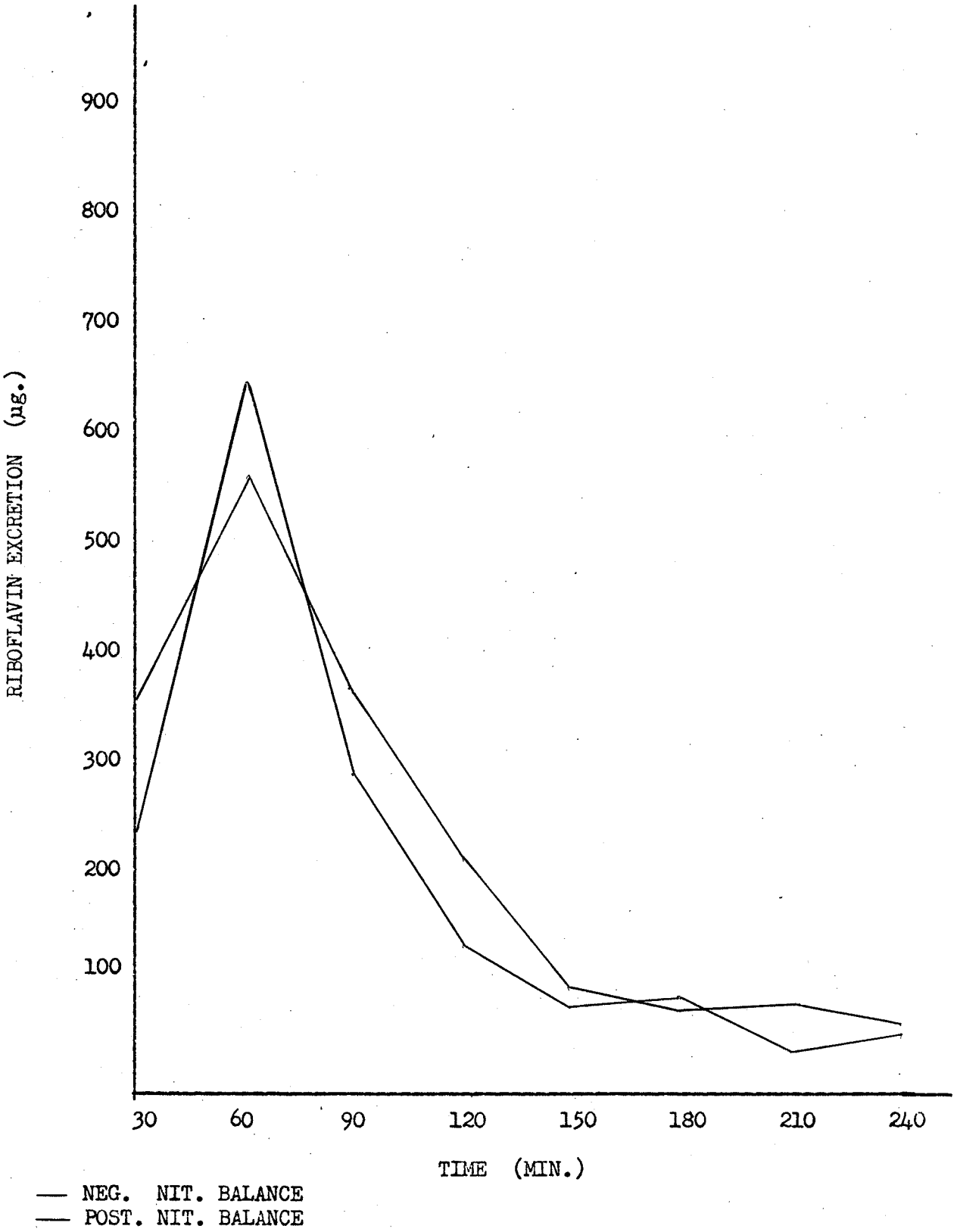


FIGURE I. MEAN TEST DOSE RETURNS FOR FIVE SUBJECTS FOLLOWING PERIODS OF POSITIVE AND NEGATIVE NITROGEN BALANCE.

TABLE IX

TEST DOSE RETURN IN MICROGRAMS PER THIRTY MINUTE PERIOD

30 min. period	SUBJECT HL	TW	KY	RD	JS	Mean per 30 min.
			PERIOD I			
1	482	188	275	93	126	233
2	725	583	507	1042	397	651
3	331	283	99	561	157	286
4	115	63	157	175	176	137
5	99	51	15	164	50	76
6	62	133	21	-*	113	82
7	64	49	45	54	64	35
8	41	35	64	48	66	51
TOTAL	1919	1385	1183	2137	1149	1551

* Loss due to breakage of sample bottle

			PERIOD II			
1	415	262	386	159	549	354
2	1035	224	571	258	706	559
3	417	451	444	346	132	358
4	184	207	305	192	174	212
5	156	63	105	60	80	93
6	148	74	39	73	32	73
7	53	123	72	75	77	80
8	41	91	62	63	27	57
TOTAL	2449	1495	1984	1226	1777	1786

TABLE X

CUMULATIVE PERCENTAGE OF TEST DOSE EXCRETED FOR A FOUR HOUR PERIOD

Time (min.)	SUBJECT					Mean
	HL	TW	KY	RD	JS	
			PERIOD I			
30	10	4	6	2	3	5
60	24	15	16	22	11	17
90	31	21	18	33	14	23
120	33	22	21	37	17	26
150	35	23	21	40	18	27
180	36	25	22	-	20	29
210	38	27	22	41	22	30
240	38	27	24	42	23	31
			PERIOD II			
30	8	5	8	3	11	7
60	29	10	11	8	25	18
90	37	19	28	15	28	25
120	41	23	34	19	31	30
150	44	24	36	20	33	32
180	47	26	37	22	33	33
210	48	28	38	23	35	35
240	49	30	40	25	36	36

4 and 9 per cent for the second. After the intermediate period of positive nitrogen balance, they excreted 22 and 5 per cent respectively. Sarett and Perlzweig (49) also noted this inverse relationship in dogs and rats.

Lossy and Goldsmith (33) reported that the response to a test dose is affected by the size, route of administration and duration of the urine collection. They suggested the use of a 5 mg. test dose since it was large enough so that the total excretion in four hours was not markedly affected by the basal level of excretion. They also suggested the use of a four hour collection period since it represents 50 per cent of the excretion for a 24 hour test period. Excretion during a 2 hour period was found to be small and appeared to be influenced by variations in rate of absorption, renal function and urine volume.

Morrison and Campbell (40) also obtained more satisfactory results with a large test dose, 5 - 10 mg., than a small one. They suggest that with low doses, the fasting riboflavin excretion may obscure the true test dose returns.

Oldham et al (46) used a test dose of 20 µg. per kg. body weight for her subjects which represented approximately 1 mg. for each subject. The use of a smaller test dose in this present study may have produced different results.

PROTEIN-RIBOFLAVIN INTERRELATIONSHIP

An inverse relationship between nitrogen intake and

riboflavin excretion was observed in all five subjects, as shown in Table XI and Figure II. The urinary riboflavin excretion decreased sharply when the subjects changed from a low nitrogen to a high nitrogen intake and from a negative to positive nitrogen balance. Both riboflavin and nitrogen were being lost by the body during Period I and retained in Period II.

For subjects TW and JS, nitrogen and riboflavin appeared to be retained in about the same proportion. Subject TW showed only a slightly negative balance during Period I and during Period II retained about the same amount of nitrogen that had been lost. Riboflavin was also retained to a small extent and for a shorter period of time for TW than for the other subjects. Subject JS retained large amounts of both nitrogen and riboflavin during Period II. This subject, however, was not in strong negative nitrogen balance during Period I.

Application of the t test showed the differences in means for both nitrogen balance and urinary riboflavin excretion to be highly significant at the 99 per cent level, for each subject. Correlation coefficients for daily nitrogen balance and riboflavin excretion were high for all five subjects and reflected the inverse relationship. They were -.81, -.70, -.66, -.88, and -.93 for subjects JS, HL, TW, KY and RD respectively.

Pollack and Bookman (48) postulated that labile proteins, which include the flavoproteins, increase or decrease rapidly as the body shifts from a negative to a positive nitrogen balance or, conversely, from a positive to a negative balance. The

TABLE XI

SUBJECT MEANS FOR RIBOFLAVIN EXCRETION AND NITROGEN BALANCE DURING THE TWO EXPERIMENTAL PERIODS

SUBJ.	RIBOFLAVIN (µg.)		NITROGEN (gm.)	
	PERIOD		PERIOD	
	I	II	I	II
HL	369	73	-1.678	+0.825
TW	293	181	-0.995	+1.429
KY	421	95	-2.167	+1.235
RD	599	259	-1.511	+1.337
JS	790	335	-0.694	+2.157
MEAN	494	189	-1.409	+1.397
S.D.	±200.2	±110.2	±0.5790	±0.5239

FIGURE 2. MEAN DAILY NITROGEN BALANCE AND URINARY RIBOFLAVIN OF FIVE SUBJECTS DURING TWO EXPERIMENTAL PERIODS.

amount of riboflavin stored in conjunction with these proteins likewise changes rapidly. Oldham et al (46) and Smith et al (52) obtained evidence in their studies with human subjects to support this hypothesis and Sarett and Perlzweig (49) in animals.

Unna et al (57) and Czaczkes and Guggenheim (14) also demonstrated that the riboflavin content of the liver of animals was dependent on protein intake rather than on riboflavin intake. Czaczkes and Guggenheim (49) showed that rats on high riboflavin intakes died of ariboflavinosis when on a diet deficient in protein.

According to the hypothesis of Pollack and Bookman (48) it may be suggested that, while the subjects in this present study were in negative nitrogen balance, flavoproteins in the labile protein reserve were being used to supply the amino acids which were not being provided by the diet. The high urinary riboflavin excretions would be due to the release of the vitamin from the flavoprotein, in addition to the inability of the liver to store dietary riboflavin without a significant amount of nitrogen.

Although this hypothesis explains the observations of this study and similar studies, the human organism is a complex structure and individuals vary in their biochemical and physiological responses. Further studies from varied approaches are necessary to better understand the apparent protein-riboflavin relationship.

SUMMARY AND CONCLUSIONS

Urinary riboflavin excretion and nitrogen balance were studied in five healthy women to obtain further information on the protein-riboflavin interrelationship. The study consisted of two consecutive 10-day periods during which the riboflavin intake was kept constant at approximately 1.4 mg. During the first period nitrogen intake was 3.5 gm. and during the second 15.4 gm. All subjects were in negative nitrogen balance while on the 3.5 gm. nitrogen intake and positive balance while on the 15.4 gm. intake.

Daily riboflavin excretions were fairly constant within individual subjects but inter-individual variation was large. Three heavier-weight, older subjects excreted less riboflavin than two lighter-weight, younger subjects. The mean riboflavin excretion during negative nitrogen balance was 494 $\pm$ 200.2 µg. and 189 $\pm$ 110.2 µg. during positive nitrogen balance. This represented 34 and 14 per cent of the intake respectively.

All five subjects showed an inverse relationship between nitrogen balance and riboflavin excretion. Riboflavin excretion dropped considerably immediately upon change to the 15.4 gm. nitrogen intake. The t test showed a high degree of significance at the 99 per cent level between the periods and high negative correlation coefficients reflected the inverse relationship.

A 5 mg. oral test dose of riboflavin was administered on the morning following each experimental period. The mean

percentage return following both periods was very similar, 31 and 36 per cent; however, individual response was varied. Only one subject showed an inverse relationship with the nitrogen balance. Three showed lower returns following the period of negative nitrogen balance than after positive balance and one showed no change. Thus, no conclusions could be drawn as to the effect of nitrogen balance on test dose returns.

The results obtained in this study would tend to support the suggestion that protein intake be considered in any evaluation of riboflavin nutriture through the use of urinary excretion levels. For underprivileged groups, among whom nutrition surveys are frequently conducted, protein is likely to be either insufficient or of poor quality. It is important, therefore, that the protein-riboflavin interrelationship not be ignored when evaluating survey data.

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APPENDIX A

INSTRUCTIONS TO SUBJECTS

The objective of this study is to determine what effect the protein intake has upon the quantity of riboflavin excreted in the urine. The exact protein and riboflavin intake and excretion must be known, therefore, you will be on weighed diets throughout the study and will be required to make a complete collection of both urine and feces during the study. The study will be as follows:

Period I (Sept. 8 - 17)

A low protein diet will be given for ten days. The same diet will be given for each of the ten days. This diet is adequate in all nutrients except protein.

Period II (Sept.19 - 28)

A high protein diet will be given for ten days. Again, the same diet will be given for each day in the ten day period.

Sept.18 and 29

On each of these two days you will be required to go without breakfast. You will be given a test dose of riboflavin and will be required to collect the urine every half hour for four hours.

The riboflavin intake will be kept at a constant level throughout the whole study. The caloric intake will be sufficient to maintain your weight. No weight must be lost or gained during the study as this will interfere with the results of the experiment.

1. FOOD INTAKE

All foods will be weighed and prepared for you so that the exact food intake is known. All meals except the evening snack will be eaten in Room 417. The weighed foods may be taken home the night before. It is very important that all foods prescribed in the diet be eaten each day and that no other foods be eaten - except those listed in the free food list. Record the weight of all free foods eaten using the dietetic scale and also record the number of cups of coffee and tea consumed. Record this information in the booklets provided. Since it is important that the caloric intake be adequate each day, sufficient amounts of the free foods must be eaten to make up the required number of calories.

FREE FOODS

- carbonated beverages - 80 Calories per 6 oz. bottle
- white sugar - 18 Cal. per 5 gm.
- butter - 36 Cal. per 5 gm.
- hard candy, fondant, gum drops, jelly beans, mints - 50 Cal. per 15 gm.

  (NO caramels, chocolate bars, or chocolate covered candies)
- apple jelly - 25 Cal. per 10 gm.
- vinegar
- tea - three cups (8 oz. cup) per day
- coffee - three cups (8 oz. cup) per day

2. COLLECTION OF URINE

Empty the bladder immediately after the usual rising time (before breakfast). Only on the first day of the study is the rising sample discarded. All the following collections plus the collection upon rising the day after the study is finished are to be saved. The urine collected upon rising is considered a part of the urine for the preceding day.

The urine must be kept in a cool place until it is brought to the laboratory from home. Label the container - name, date, time of collection.

If the urine is transferred to a large container, rinse the small container used for collecting the urine with only a small amount of distilled water.

The sample must be protected from the light at all times as riboflavin is destroyed by light.

3. COLLECTION OF FECES

Collect all feces in the waxed containers provided by the laboratory. Use one carton per bowel movement. To hold the plastic container under the toilet seat, a double layer of brown paper may be placed between the toilet seat and the china bowl. Place the container on top of the paper and place a plastic bag in the container. This should allow normal posture for the bowel movement. Discard toilet paper. Indicate the hour and date when each sample is collected on

the lid of the waxed container in which the plastic bag containing the sample is placed.

Collect all feces from the time of the first meal of the study until the carmine appears after the end of the study.

Keep the samples in a cool place until they are brought to the laboratory and placed in the freezer.

One carmine capsule (a harmless dye used for coloring feces) is taken one-half hour before breakfast on the following days:

September 8th

September 19th

September 29th

4. TEST DOSE OF RIBOFLAVIN (Sept.18 and Sept.29)

Breakfast will not be eaten on these two days. Empty the bladder immediately upon rising, then take the test dose. Collect the urine every half hour for a period of four hours. On Sept. 8th you will then be given the low protein diet again. The completion of this four hour collection period ends this study on Sept.29th. Normal eating habits may then be resumed.

FIRST DAY OF THE STUDY (Sept. 8th)

1. Record your weight upon rising today and for each of the following days of the study. Record the weight in the

booklet provided.

2. The urine sample upon rising, <u>for this day only</u>, does not have to be collected. All the following collections, however, must be saved.
3. Take one carmine capsule one-half hour before breakfast.
4. Collect all feces after the first meal is eaten.

TEST DOSE OF RIBOFLAVIN

INSTRUCTIONS TO SUBJECTS

1. Immediately upon rising Saturday morning empty the bladder completely and save this sample as it is a part of yesterday's collection.
2. Drink the ginger ale containing 5 mg. of riboflavin.
3. Rinse the container with water and drink this so that all the riboflavin is taken.
4. At exactly 30 minute intervals empty the bladder completely and pour the sample into the small brown bottles provided. Continue for a period of four hours.
5. Be sure to label the bottles. Put the name and time of collection on each bottle. It is very important that you put the time of collection on each bottle.
6. Keep the bottles in a cool place until they are placed in the refrigerator in Room 400.

APPENDIX B

URINARY RIBOFLAVIN BY A MODIFICATION OF THE METHOD BY SLATER AND MORELL

REAGENTS

Acetic acid, glacial

Riboflavin, dry, USP reference standard: 20 mg pure riboflavin dissolved in 1 liter of distilled water containing a few drops of acetic acid. Stored in the dark under toluene. For use, diluted 1 to 20 with distilled water.

5 per cent aqueous $KMnO_4$:prepared daily.

3 per cent aqueous H_2O_2 solution.

Na_2SO_4, granular anhydrous, reagent grade.

Butanol-pyridine mixture: 8 volumes of redistilled pyridine, added to 92 volumes of redistilled n-butanol.

Fluorescein standard: about 10 mg of sodium fluorescein dissolved in 1 liter of distilled water. For use, diluted 1 to 200 or to another appropriate volume.

PROCEDURE

Exactly 0.5 ml of glacial acetic acid was pipetted into glass-stoppered test tubes. Each sample required the tubes, A and B.

To tube A and tube B, 1.0 ml of urine was added (if urine contained a small quantity of riboflavin 2.0 ml was used). A reagent blank was prepared by substituting distilled water for the urine.

0.5 ml of distilled water was added to tube A and 0.5 ml of the riboflavin standard to tube B. The reagent blank was treated similarly.

0.5 ml of 5 per cent $KMnO_4$ was added to each tube, mixed and allowed to stand for exactly 1 minute.

0.5 ml of 3 per cent H_2O_2 was added to each tube and agitated gently.

10 ml of the butanol-pyridine solution was added.

Solution shaken vigorously for 30 seconds and allowed to stand in the dark for 10 minutes.

The bottom (aqueous) layer was removed.

Anhydrous granular Na_2SO_4 (ca. 1.5 gm) was added with the aid of a small funnel.

Solution shaken gently and placed in the dark for 10 minutes. The crystal-clear supernatant was decanted into Coleman cuvettes (19 x 150 mm).

The photofluorometer was set at 70 or 80 units with the fluorescein standard and each sample read.

The cuvettes were placed in the irradiation rack and irradiated until 80 to 90 per cent of the riboflavin was destroyed. The reagent blank containing riboflavin was checked at 30-minute intervals to determine the rate of destruction.

All tubes were re-read.

CALCULATIONS

$A-A^1$ (after irradiation) - deflection due to riboflavin destruction in unknown

$B-B^1$ (after irradiation) - deflection due to riboflavin destruction in recovery

$C-C^1$ (after irradiation) - change in reagent fluorescence induced by irradiation

$$\frac{(A-A^1)-(C-C^1)}{(B-B^1)-(C-C^1) \; - \; (A-A^1)-(C-C^1)} \times 0.5 = \mu g \text{ of riboflavin per tube}$$

APPENDIX C

STATISTICAL EQUATIONS

1) STANDARD DEVIATION

$$s = \sqrt{\frac{\sum x_i^2 - \frac{\sum x_i^2}{n}}{n - 1}}$$

2) CORRELATION COEFFICIENT

$$r = \frac{\sum (x - \bar{x}) \; (y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

3) STUDENT'S t TEST

$$t = \frac{(\bar{x} - \bar{y}) - (\mu_1 - \mu_2)}{\sqrt{\frac{(n_1 - 1) s_1^2 + (n_2 - 1) s_2^2}{n_1 + n_2 - 2} (\frac{1}{n_1} + \frac{1}{n_2})}}$$