THE CONCENTRATION OF RIBOFLAVIN AND ITS PHOSPHORYLATED DERIVATIVES IN THE TISSUES OF RATS FED VARYING QUANTITIES OF VITAMIN B_2

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THE CONCENTRATION OF RIBOFLAVIN AND ITS PHOSPHORYLATED DERIVATIVES IN THE TISSUES OF RATS FED VARYING QUANTITIES OF VITAMIN B_2

By

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INTRODUCTION

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Flavins as a class of biological pigments have been known since about 1879 (1). However, their importance was not fully recognized until the work in 1932-33 by Szent-Györgyi (2) and Warburg and Christian (3) on "cytoflav" or the "yellow enzyme". Following this, the active components of the flavins were found to be phosphorylated derivatives of riboflavin, either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). In 1933 (4) riboflavin was isolated, crystallized and shown to have vitamin activity for the rat.

The importance of riboflavin in regard to its redox properties was then recognized. In addition to these properties, it was found to stimulate growth and to prevent loss of hair (alopecia), inflammation of the conjunctiva and certain other pathological conditions (5).

It has been shown that the concentration of certain of the enzyme systems requiring the participation of riboflavin or a derivative are diminished in the riboflavin deficient animal. In addition, several authors have measured the FAD concentration in tissues of normal and deficient rats. However, most of the work on the nutritional requirement of vitamin B_2 has been concerned with growth measurements. It would seem of interest to see whether the tissue levels of the coenzymes FAD and FMN might not be used as a more critical requirement index and also to compare a requirement for vitamin B_2 based on these levels with the growth criterion.

The purpose of the present study, therefore, was (a) to measure the concentrations of riboflavin and its phosphorylated derivatives in tissues of rats fed synthetic diets with varying riboflavin concentrations and (b) to compare the dietary vitamin B_2 requirement based on tissue levels with that based on growth.

HISTORICAL

HISTORICAL

The biochemical studies on riboflavin first began in the enzyme field, rather than the nutrition field. A role for riboflavin-containing enzymes as hydrogen carriers was first shown when Warburg and Christian (3) isolated their "Old Yellow Enzyme" from yeast. Isolation of the pure coenzyme from this enzyme system showed it to be flavin mononucleotide (6). (This system is not specific since FAD may be substituted for FMN as the coenzyme with, however, less activity.) L-amino acid oxidase is another enzyme which requires specifically FMN and it has been isolated from rat kidney and liver (7,8). This system catalyzes the oxidation of about thirteen amino acids and also catalyzes dehydrogenation of L-alpha hydroxy acids having structures related to the amino acids (9). An enzyme which specifically catalyzes the reaction between reduced TPN and cytochrome c has been isolated by Haas, Horecker and Hogness (10) and called TPN-cytochrome c-reductase. Its prosthetic group is also flavin mononucleotide.

FAD acts as the coenzyme or prosthetic group of several enzymes of biological interest. (A) D-amino acid oxidase which catalyzes the oxidation of all the common alpha-D-amino acids except lysine, via imino acids to alpha-keto acids can use only FAD as its coenzyme (11). The biological function of this enzyme is unknown since although it occurs in most tissues, its substrates are the unnatural isomers.

(B) Glycine is deaminated in the presence of glycine oxidase which occurs in the liver and kidneys of most mammalian species, however, rat kidney appears to contain none (12). (C) In 1902, Schardinger (13) discovered an enzyme in milk which catalyzed the oxidation of formaldehyde to formic acid. Morgan, Stewart and Hopkins, twenty years later (14), found that certain animal tissues and milk contained a catalytic system which led to the oxidation of either xanthine or hypoxanthine under aerobic or anaerobic conditions which may be the same enzyme as that discovered by Schardinger. Ball in 1939 (15) isolated a flavoprotein from milk with the property of a coenzyme for xanthine oxidase. One of the two prosthetic groups obtained in this preparation seems to be FAD. Corran and Green (16) using a different method of preparation reported the isolation of a flavoprotein from milk which catalyzed the oxidation of aldehydes and which was not identical with xanthine oxidase. Morgan (17) investigated the distribution of xanthine oxidase in different animal tissues and found that xanthine oxidase and aldehyde oxidase activity were invariably present together. Because of these observations, the question of the possible identity of xanthine oxidase with aldehyde oxidase has been brought up by many. Dixon and Thurlow (18) and Booth (19) have concluded that xanthine and aldehyde oxidase are the same enzyme. Booth (19) has suggested that the enzyme be called xanthine oxidase, even when speaking of its participation in aldehyde reactions. However, the question of possible identity has not been definitely settled.

The presence in liver of an aldehyde oxidase which may be separated from liver xanthine oxidase has been reported by Gordon, Green and

Subrahmanyan (20). The prosthetic group for the aldehyde oxidase is flavin adenine dinucleotide. The fact that the milk flavoprotein apparently catalyzes the oxidation of three different substrates, purines, aldehydes and dihydro coenzyme I while liver aldehyde oxidase catalyzes only one of these reactions has led these authors to suggest that the milk enzyme may be a close association of three enzymes which have not before been resolved.

(D) The idea that a special enzyme was required for the transport of electrons between Coenzyme I-reduced (DPN·H_2) and the cytochromes was arrived at independently by Adler (21) and Dewan and Green (22). An enzyme which could mediate in the oxidation of DPN·H_2 has been described variously as "coenzyme factor" by Dewan and Green and "diaphorase" by Euler and Hellstrom (23). In the reduction of cytochrome c by reduced DPN in animal tissues, it is not known whether the reaction is catalyzed by a single enzyme or the joint action of two systems. The enzyme which does catalyze it may be specifically designated as DPN-cytochrome c-reductase (24) even though the relation between it and the diaphorases remains to be settled.

(E) Straub (25) has isolated a soluble flavoprotein from heart muscle which Corran, Green and Straub (26) describe as a soluble "diaphorase". (F) Haas (27) has isolated a yellow enzyme from yeast in which the protein portion differs from that of Warburg and Christian since FAD is the specific coenzyme. (G) An enzyme having FAD as the coenzyme which catalyzes the hydrogenation of fumaric acid to succinic acid has been discovered by

Fischer (28), but whether this reaction is the physiological function of the enzyme has not been established.

Since riboflavin is part of the coenzyme in the catalyst for the reactions described above it would be logical to presume that a deficiency of it might lead to a decreased enzyme concentration in animal tissues. That this is true has been shown for the xanthine oxidase content of rat liver (29) and the D-amino acid oxidase content of liver and kidney (30).

When rats were maintained on a vitamin B_2 deficient diet, Ochoa and Rossiter (31) found that the FAD content was significantly less in the liver and heart of those animals than in the same organs of normal rats. Bessey, Lowry and Love (32) have found that a deficient diet decreased considerably the tissue concentration of riboflavin in all forms, namely: free, FMN and FAD especially in liver, kidney and heart.

The most obvious effect of a severe riboflavin deficiency is the failure of animals to grow. The Bourquin-Sherman procedure involving measurement of rat growth using a standard diet containing different quantities of riboflavin has been used successfully as a bioassay method for the riboflavin content of various materials (33). Within certain limits of growth this diet elicits a quantitative response to graded doses of the vitamin.

In addition, the growth method has also been used as a criterion for establishing the vitamin dietary requirement.

10 μg riboflavin/day has been suggested as a minimum requirement by Supplee (34) for raising young weanling rats to maturity. Using

purified diets, Ellis (35) found that a diet containing 3 μg/gram diet was adequate to support normal activity in adult rats and enabled them to live a normal life span. However, 10 μg/gram diet appeared to confer additional benefits on the offspring, and to give increased reproduction. Mannering (36) has suggested a minimum requirement of 18 μg per day on a low fat diet. Mayfield and Hedrich (37) place the daily riboflavin requirement for maximum efficiency of protein utilization at about 5 μg. Using the criterion of maintenance of tissue level concentrations for a period of 4 to 12 weeks, Czaches and Guggenheim (38) found that on a synthetic diet 7.5 μg/day was sufficient for growth of fifty gram rats, although optimal growth was not obtained with this amount of B_2.

It has also been of interest to see how the composition of the diet influences the requirement. Czaches and Guggenheim (38) showed that the level of fat, protein or carbohydrate in diets isocaloric with the normal influenced the requirement. On a low protein diet, tissue levels were not maintained with 20 μg/day and even then secondary deficiency effects were present. On a high protein diet, the vitamin requirement was 15 μg/day, the high fat diet required 20 μg/day while the low fat diet maintained tissue levels on only 3.5 μg/day. According to these authors, the variance in requirement may be due to either metabolic factors or differences in the amount of intestinal synthesis of riboflavin, or both. The small amount required by the low fat diet could be attributed to increased intestinal synthesis and the amount required by the high fat and the high protein diets due to the possibility of B_2 entrance in both protein and fat metabolism.

Schweigert, et al., (39) fed various diets consisting of either high carbohydrate, high fat or high protein, each with high (60 μg/day) and low (8 μg/day) intakes of vitamin B_2. They state that the fat, carbohydrate, or protein content did not affect the tissue concentrations when the same level of B_2 was fed. This does not agree with results given by Czaches and Guggenheim for the diets containing 8 μg/day, however, the high B_2 concentration was much higher than any which Czaches and Guggenheim fed and might, therefore, give optimal amounts in the tissues. It should be noted that the carbohydrate used by Czaches and Guggenheim was rice flour, as compared with the use of sucrose by Schweigert which might account for the differences on the low B_2 diets.

In addition to the effects shown by various protein, carbohydrate and fat levels, the type of each used is important. Mannering (40) has shown that dextrin, markedly, and cornstarch, moderately, stimulate bacterial synthesis of B_2 in the rat intestine, probably by providing a more favorable medium for bacteria and, in this way, their use in diets reduces the dietary requirement. Sucrose which is absorbed more rapidly than the other two does not promote this synthesis.

Riesen, Schweigert and Elvehjem (41) showed that on ingestion of 30 μg/day the total riboflavin level of the liver was a function not only of protein intake, but also methionine and cystine content of the diet. About one third of the increased riboflavin content of the liver caused by greater protein intake could be accounted for by ingestion of increased amounts of methionine, but none of the increase could be accounted for by ingestion of increased cystine.

The intake of vitamin C has been shown to control total riboflavin storage in liver, kidney and adrenals of the guinea pig (42). However, since rats are able to synthesize their own vitamin C and guinea pigs can not, this may be of no significance in the rat.

Sure and Ford (43) found an interrelationship between thiamine and riboflavin indicating that the efficiency of riboflavin utilization was decreased in the absence of thiamine, but that the reverse was not true.

It may be noted then that the requirement of the rat for vitamin B_2 varies depending on the diet composition, age of the animals and other factors.

In view of the importance of the riboflavin containing enzyme systems it was of interest, first, to measure the tissue concentration of the riboflavin coenzymes and, second, to compare the dietary vitamin requirement for their optimal concentrations with the requirement for growth.

EXPERIMENTAL

EXPERIMENTAL

Animals and Diets

Male and female albino rats of a strain developed in the Michigan State College Chemistry Department were 28 to 35 days old when selected for these experiments. The animals which were divided into groups of eight with each group having an equal number of males and females weighed between 45 and 50 grams. The average initial and final group weights are listed in table II.

Purified diets which differed only in the vitamin B_2 content were fed to the animals ad libitum for approximately 31 days. The basal diet, a modification of the Bourquin-Sherman diet (33) contained the following constituents by weight: casein (vitamin free)* 18%, sucrose 68%, corn oil (Mazola) 7%, salt mixture (44) 4% and Roughex 3%. A vitamin supplement consisted of the following amounts per 100 grams of diet: thiamin hydrochloride 500 μg, nicotinic acid 2,000 μg, pyridoxine hydrochloride 250 μg, P.G.A. (folic acid) 100 μg, and choline chloride 100 mg. These were dissolved in 50% ethanol and added to the casein by means of a pipette. After the casein had dried the other constituents were added. The vitamin B_2 content of the diets was varied by addition of the proper aliquot of a solution of crystalline riboflavin** in 40% ethanol-water to the casein as in the case of the other vitamin supplements. The

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* Vitamin-free test casein (14.9% N, 0.14 μg riboflavin per gram) manufactured by General Biochemicals Inc., Chagrin Falls, Ohio.

** Crystalline riboflavin obtained from Eastman Kodak Company, C.P. grade.

riboflavin content of the diets is shown in table I. The diets were then stored in dark bottles in a refrigerator. Vitamins A and D were fed as three drops of cod liver oil per rat every other day. Distilled water was supplied for drinking.

A careful record of food consumption was made. Special food cups were weighed every two days and food spilled was collected on paper towels and also weighed. The animals were kept in individual, raised cages and the room temperature maintained between 75 and 78° F.

Analytical Method for Riboflavin, FMN and FAD

After sacrificing the animals using ether, the tissues were removed as quickly as possible and immediately frozen between sheets of solid carbon dioxide. Until they were used for the analyses, the tissues were kept frozen at -10° C.

The method used for analysis was essentially that of Bessey, Lowry and Love (32). It depends on the fact that when FAD is split to flavin phosphate or free riboflavin there is an increase in fluorescence. Also, FMN can be distinguished from free riboflavin on the basis of its distribution coefficient between water and benzyl alcohol.

Since riboflavin solutions are sensitive to light, amber glassware was used and an Eastman safety lamp with Wrattan Filter OA used for illumination as much as possible. All reagents were made up with glass redistilled water and redistilled trichloroacetic acid was used throughout.

In the cold room, the tissue sample was extracted with a large volume of ice water, using a Waring Blendor. After the addition of an equal

TABLE I

RIBOFLAVIN CONTENT OF EXPERIMENTAL DIETS

Diet Number	Riboflavin μg/100 g. diet
1	0
2	25
3	50
4	100
5	200
6	300
7	400

TABLE II

AVERAGE DIETARY INTAKE AND AVERAGE WEIGHT GAINS

Diet Number	Average Daily Riboflavin Intake*	Average Daily Food Intake	Average Initial Weight	Average Total Weight Gained	Average Daily Weight Gained
	γ	g.	g.	g.	g.
1	0.0	4.4	46.6	2.8	0.1
2	1.0	3.9	44.5	4.6	0.1
3	2.9	5.8	46.6	19.8	0.6
4	6.6	6.6	45.6	29.5	1.0
5	18.6	9.3	49.9	77.6	2.5
6	30.0	10.0	47.4	101.5	3.2
7	42.8	10.7	50.4	121.7	3.9

* Calculated from actual food consumption.

volume of 20% trichloroacetic acid, the solution was centrifuged and an aliquot of the supernatant neutralized with one fourth its volume of 4 M K_2HPO_4. Another aliquot was incubated overnight at 38° C. and then neutralized. The fluorescence of the suspensions were measured as soon as possible after neutralization, using the Coleman Electronic Photofluorometer (Models 12 and 12 B).

The concentration of riboflavin in the aliquot was read from a standard curve prepared by using various concentrations of riboflavin dissolved in a water solution containing trichloroacetic acid and K_2HPO_4 in the same proportions as in the final tissue extract. The pH of this mixture which is about 6.6 is important since the fluorescence of riboflavin changes with pH, being at a maximum between pH 5.9 and 7.7 (45).

The validity of using the standard curve was checked by taking readings on a tissue extract aliquot before and after a known amount of riboflavin had been added. These analyses using the standard curve agreed within the experimental error with the amount of riboflavin added.

Calculation of Results

In a highly concentrated salt solution FAD (calculated as riboflavin) has a fluorescence equal to 15% of that of riboflavin. FMN (calculated as riboflavin) and free riboflavin have equal fluorescence. If the apparent riboflavin of the initial sample = R_i and the apparent riboflavin in the hydrolyzed sample = R_t, then FAD = $\frac{R_t - R_i}{0.85}$. The total riboflavin R_t = FAD + non FAD (FMN + free riboflavin).

An attempt was made to determine the free riboflavin by the benzyl alcohol extraction method (32). This was unsuccessful since the amount of free riboflavin was too small to be read accurately with the photofluorometer used. Therefore, the FMN + free riboflavin was calculated as the difference between the total riboflavin and the FAD.

In order to test the validity of using frozen tissue samples, riboflavin analyses were made on tissues before freezing and after freezing and storing for a few weeks. There was no appreciable effect on the tissue concentration either due to freezing or to storing while frozen.

RESULTS

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Growth Rate of Rats

At the end of the experimental period, the rats on diets 1, 2 and 3 showed typical symptoms of a vitamin B_2 deficiency. The fur coat was oily looking and uneven and there was a considerable reddish accumulation of porphyrin on the paws, nose and whiskers. Two animals on diet 1 died before the experimental period was completed, the remaining animals on this diet and diet 2 were small and either had gained very little weight or had lost weight as shown in table II, page 13. Animals on diet 3 gained more weight but resembled the above animals in appearance. Several animals on diet 4 also showed these symptoms but to a lesser degree.

The animals on diets 5, 6 and 7 were all large healthy looking animals with good fur coats at the end of the feeding period. The gain in weight of these animals increased as the ingested B_2 increased; the largest gain, 3.9 g./day, was on diet 7 (43 μg riboflavin consumed per day). The growth rate for males is generally greater than that for females. This may be equalized either by selecting all of one sex for the experiment or, as in the present study, by having an equal number of both sexes. On the lower diets there was no noticeable growth difference between the sexes, however, on diets 5, 6 and 7 the growth of the male rats was above that of the females. The average weight gains are shown in figure 1.

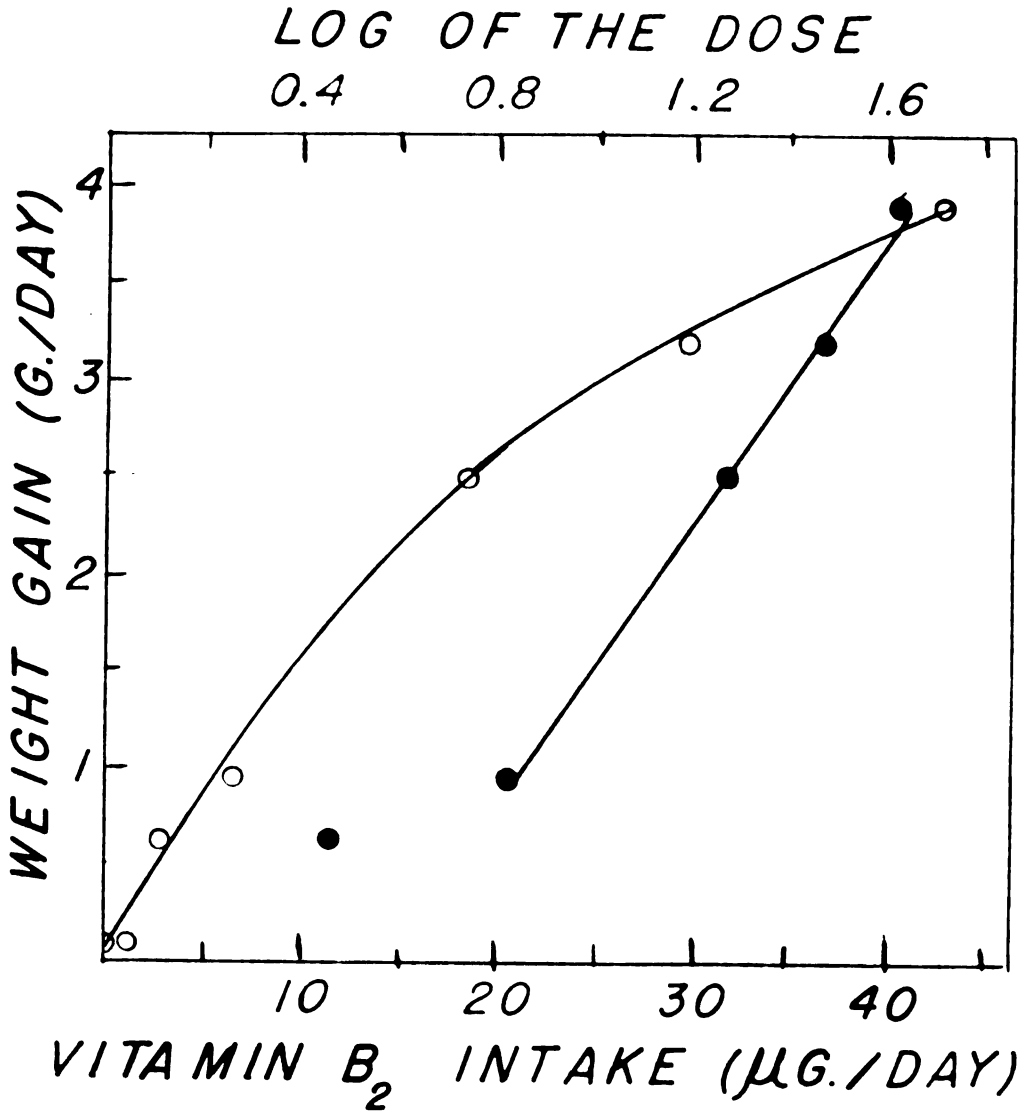

Figure 1

The relation between growth response and the riboflavin intake. Weight gain is expressed as the average group gain in grams/day; riboflavin intake is expressed as μg vitamin B_2/day and logarithm of the dose in micrograms.

○ = vitamin B_2 intake μg/day ● = logarithm of the dose

The vitamin B_2 intake which was calculated from the food consumption is given in table II. It will be noted that the amount of vitamin B_2 consumed on diet 2 was very small and these rats and those receiving no B_2 were almost the same as far as growth and appearance were concerned.

FAD, FMN and Free Riboflavin Concentration in Tissues

The FAD and total riboflavin were determined as described before. Since the free riboflavin was too small to be accurately measured the values of FMN + free are equal to the difference between the total riboflavin and the FAD. However, it was apparent that the largest part of the difference between total riboflavin and FAD is FMN.

The results of the tissue analyses are given in tables III, IV and V and shown graphically in figures 2 and 3. The coenzyme concentrations were calculated as riboflavin and all were expressed as μg of riboflavin per gram of wet tissue weight since previous experiments of this type have shown that calculations based on either μg/gram of dry weight or μg/gram of nitrogen give practically the same picture as those based on wet tissue weight. Tissues from diet 2 animals were not analyzed as the amount of vitamin B_2 ingested was so low and these animals were much the same as diet 1 animals.

If one examines the effect of dietary B_2 concentration on the FAD and FMN + free riboflavin concentrations in each tissue it is noted that the level of these constituents in brain is nearly independent of dietary B_2 concentration. There are small differences noted but because of the low levels of riboflavin and its phosphorylated derivatives the experimental

TABLE III

AVERAGE FAD CONTENT OF TISSUES

Diet Number	Brain μg/g fresh* tissue	Heart μg/g fresh* tissue	Kidney μg/g fresh* tissue	Liver μg/g fresh* tissue
1	1.93 ± 0.06	9.47 ± 0.65	12.68 ± 0.52	10.61 ± 0.39
3	2.16 ± 0.06	10.43 ± 0.46	16.22 ± 0.74	12.38 ± 0.57
4	2.25 ± 0.06	12.06 ± 0.35	14.46 ± 0.71	11.71 ± 0.42
5	2.39 ± 0.05	13.96 ± 0.39	15.89 ± 2.21	17.14 ± 1.32
6	2.08 ± 0.16	13.19 ± 0.57	15.84 ± 1.03	22.07 ± 0.82
7	2.27 ± 0.06	13.75 ± 0.41	16.94 ± 0.99	19.37 ± 0.35

*μg FAD (calculated as riboflavin)

** The ± is one standard deviation from a mean of 6, 7 or 8 values (would be expected to include 67% of all values).

TABLE IV

AVERAGE FMN + FREE RIBOFLAVIN CONTENT OF TISSUES

Diet Number	Brain µg/g fresh* tissue	Heart µg/g fresh* tissue	Kidney µg/g fresh* tissue	Liver µg/g fresh* tissue
1	1.01	2.54	5.43	2.23
3	0.94	2.52	7.19	2.99
4	1.08	3.03	11.21	3.77
5	1.24	4.38	14.77	7.49
6	1.12	3.93	15.20	6.05
7	1.09	3.18	14.74	5.57

* FMN (calculated as riboflavin) + free riboflavin.

TABLE V

AVERAGE TOTAL RIBOFLAVIN CONTENT OF TISSUES

Diet Number	Brain μg/g fresh* tissue	Heart μg/g fresh* tissue	Kidney μg/g fresh* tissue	Liver μg/g fresh* tissue
1	2.94 ± 0.05	12.01 ± 0.36	18.11 ± 0.43	12.84 ± 0.46
3	3.10 ± 0.04	12.95 ± 0.33	23.41 ± 0.73	15.37 ± 0.64
4	3.33 ± 0.08	15.09 ± 0.50	25.67 ± 0.99	15.48 ± 0.52
5	3.63 ± 0.06	18.34 ± 0.41	30.66 ± 0.62	24.53 ± 1.99
6	3.20 ± 0.04	17.12 ± 0.46	31.05 ± 1.01	28.12 ± 0.91
7	3.36 ± 0.02	16.93 ± 0.46	31.68 ± 1.51	24.94 ± 0.36

* Total riboflavin (calculated as riboflavin).

** The ± is one standard deviation from a mean of 6, 7 or 8 values (would be expected to include 67% of all values).

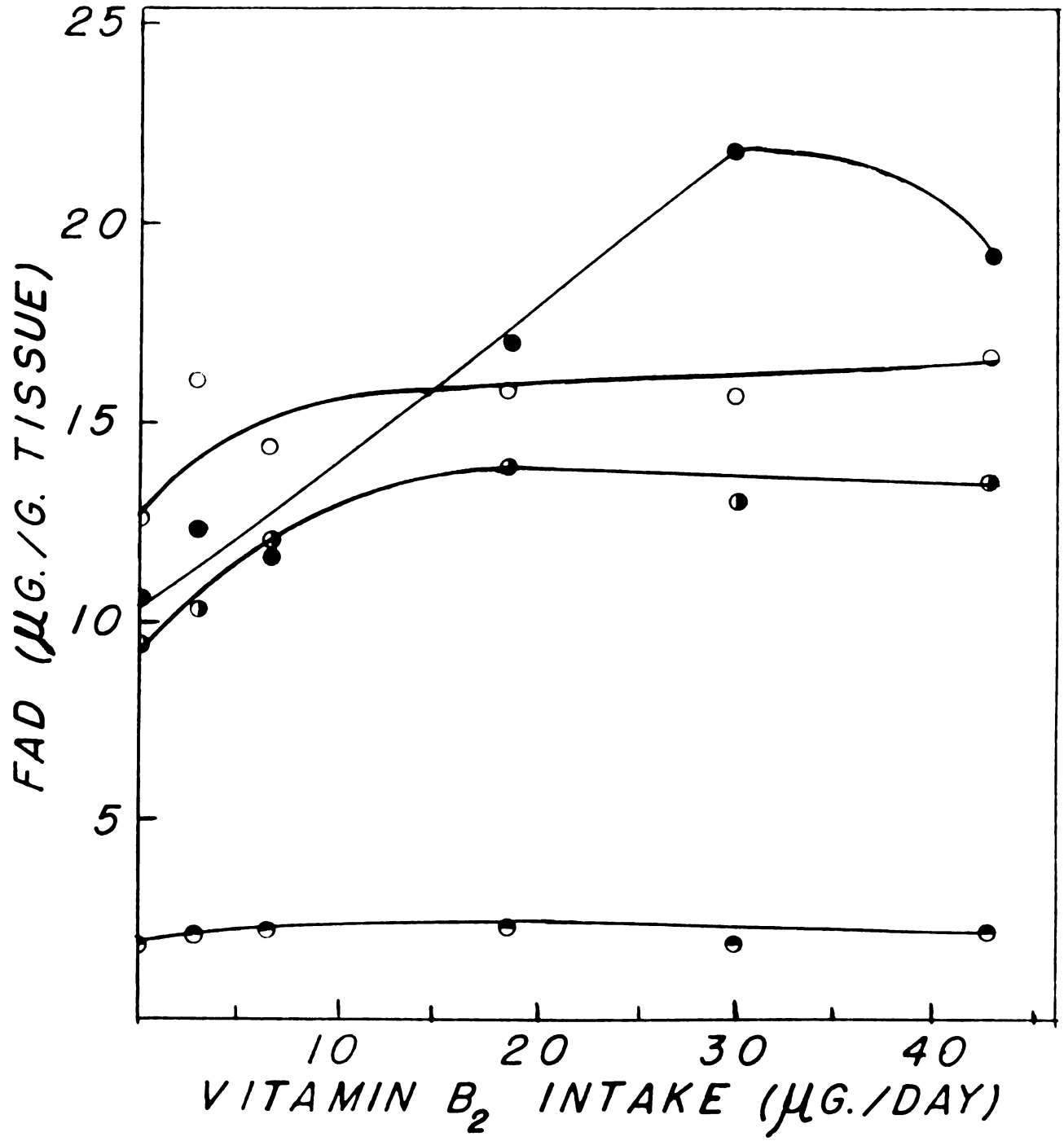

Figure 2

The relation between the FAD content of tissues and the dietary vitamin B_2. FAD (calculated as riboflavin) is expressed as µg FAD/g. fresh tissue weight (average) and riboflavin intake as µg vitamin B_2/day (average).

● = brain, ◑ = heart, ○ = kidney, ◒ = liver

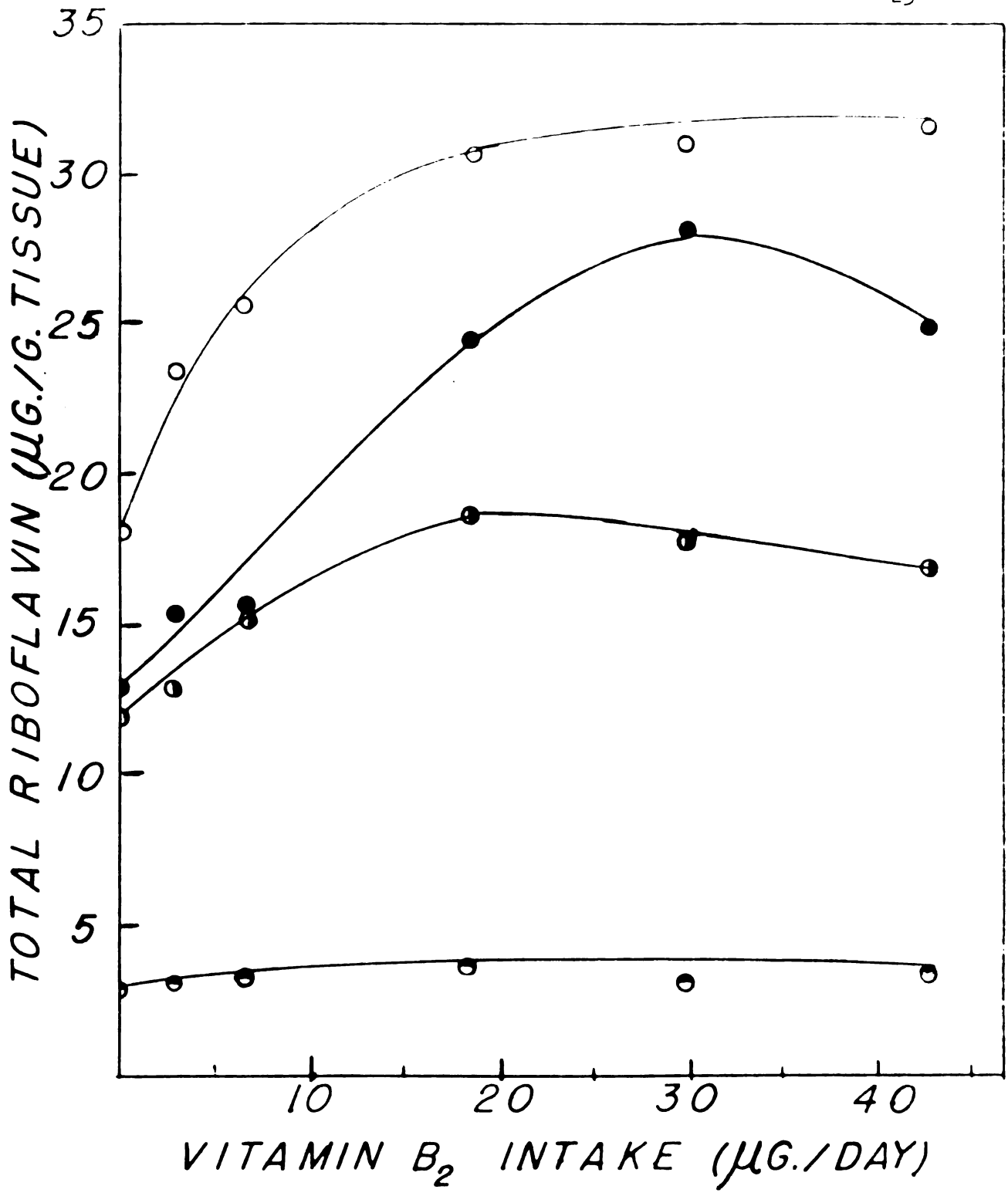

Figure 3

The relation between the total riboflavin content of tissues and the dietary vitamin B_2. Total riboflavin is expressed as µg riboflavin/g. fresh tissue weight (average) and riboflavin intake as µg vitamin B_2/day (average).

● = brain, ◗ = heart, ◑ = kidney, ◉ = liver

error of determination is probably large enough to account for these. In liver, however, the tissue level of FAD increased with increasing amounts of dietary B_2 up to an intake of about 30 μg/day. Larger intakes of vitamin B_2 did not increase the FAD further, in fact, a small drop in its tissue level was noted. Approximately the same findings were observed for FMN + free riboflavin concentration in liver.

In heart the pattern was similar to that of liver where both the FAD and the FMN + free increased up to an intake of 19 μg/day (diet 5), but on diets 6 and 7 the FAD remained the same while the FMN free concentration dropped slightly.

The FAD concentration in kidney increased considerably on a daily intake of approximately 3 μg (diet 3) dropped slightly on diet 4 and then gradually leveled off. The FMN + free riboflavin increased more gradually with the B_2 intake up to a daily intake of 19 μg (diet 5) and then larger amounts did not increase the FMN in the kidneys.

It is interesting that a deficiency greatly affected the FMN + free riboflavin concentration in kidney and liver, but not to such an extent in heart. It may also be noted that that particular coenzyme concentration in kidney and liver tissues of deficient animals was about half of that in the tissues of animals fed diet 7 (43 μg/day).

These results obtained for tissue concentrations of riboflavin and its phosphorylated derivatives after feeding rats a diet adequate in vitamin B_2 agree essentially with those reported by Bessey, Lowry and Love (32), but are lower than those given by Ochoa and Rossiter (31).

The method of analysis used in this study is the same as that used by the former authors. Ochoa and Rossiter used an enzymatic method of analysis which may help to account for the great differences.

The growth rates obtained were somewhat lower than those which have been reported for the same dietary B_2 intake by Burch, et al. (46), but in agreement with those given by Edgar and Macrae (47). In the present study intestinal synthesis was kept at a minimum by the use of sucrose as the dietary carbohydrate. The use of a different diet by Bessey, et al. which would allow increased synthesis of riboflavin in the intestine may account for the higher growth rate which they obtained with the same dietary intake of vitamin B_2.

DISCUSSION OF RESULTS

DISCUSSION OF RESULTS

Vitamin B_2 Intake and Growth Rate

It was shown that when other dietary components are held at constant concentration increasing the amount of vitamin B_2 ingested increases the rate of growth. Part of the differences in growth of rats on diets 4 and 3 as compared with those on diets 1 and 2 might be explained by the increased food consumption on the two former diets. This could not be the case with the animals on diets 5, 6 and 7 since average food consumption was almost the same for these three groups.

The inability to grow when animals are fed the vitamin B_2 deficient diets may be due either to the inability to properly utilize the food eaten or simply to a lowered food intake resulting from anorexia. Rats receiving a B_2 deficient diet have been shown to exhibit a real depression of food utilization as compared with pair-fed rats supplemented with 20 μg B_2 per day, according to Sure (48). The differences in food utilization result in part from differences in completeness of food oxidation as riboflavin is an essential component of about a dozen flavoproteins. This may explain the differences in growth found on diets 5, 6 and 7, where, although the rats ate almost the same amount of food, the growth increased as dietary vitamin B_2 increased.

It is difficult to set a definite figure for normal growth rate of rats but a growth approaching 4.0 grams per day is considered very good. Edgar and Macrae obtained growth gains of 4.3 to 4.6 grams per day for

males on a purified ration with 50 μg vitamin B_2 given as an additional supplement (47). However, in a group composed of equal numbers of males and females, it is thought that 3.9 grams per day, the average group gain on diet 7, is close to the maximum obtainable with this synthetic diet.

FAD and FMN + Free Riboflavin Concentration in Tissues

Of the four tissues studied, brain tissue was affected least by a Vitamin B_2 deficiency, heart somewhat more and kidney and liver were affected most. If one compares the decrease in coenzyme concentration caused by a deficiency of vitamin B_2 (i.e. comparing tissue levels of animals receiving diet 1 with those receiving diet 7) it will be seen that in heart the FAD concentration is lowered relatively more than the FMN, whereas in liver and kidney the reverse is true. This might suggest that the animal body attempts to maintain the concentration of that particular riboflavin containing coenzyme which is more important to the functions carried out by each organ, or in the case of the heart, that the ability to synthesise FAD from FMN, which is an ability of most cells of normal animals (49), is impaired.

The intake of vitamin B_2 necessary to maintain the tissue levels varies with the organ and with the particular coenzyme; the amount needed to maintain kidney FAD is relatively small, a larger intake is required for both FMN and FAD in heart, while kidney FMN and liver FAD and FMN all require about 30 μg per day.

The tissue coenzyme concentrations, especially in the latter groups exhibit deviations which may be explained in part by the influence of

body stores at the beginning of the experiment, to seasonal variation (the experimental period extended from October to April), or to other unknown variables.

Since the liver is concerned to a great extent with metabolism and the coenzyme concentrations in liver do not reach the optimal range until the amount of vitamin B_2 ingested (19-30 μg/day) is enough to cause normal growth and "well being" the liver coenzyme concentrations might be used to give an indication of the nutritional status of the animal.

A Suggested Dietary Requirement

An attempt was made to compare the dietary requirement for vitamin B_2 based on (a) that necessary for growth and (b) that necessary for maintenance of coenzyme tissue levels. On this synthetic diet, which was selected to eliminate as much intestinal bacterial synthesis of riboflavin as possible maximum growth required an intake of somewhat more than 43 μg per day. It should be noted that the increased growth was not attributed to a larger food consumption, but to increased utilization of ingested food.

For an optimal tissue level of both FAD and FMN the necessary intake would seem to be around 30 μg per day (the amount received by animals on diet 6) which is lower than that necessary for maximum growth. However, taking into consideration both criteria it is suggested that an adequate dietary intake of vitamin B_2 would be 30 to 40 μg per day, or 3 to 4 μg per gram of diet.

SUMMARY

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1. When rats are fed a diet containing no added vitamin B_2 growth ceases. Between an intake of 6.6 and 43 μg B_2 per day the growth response with respect to the log of the dose is linear.

2. The FAD and the FMN + free riboflavin concentration in brain is almost independent of the amount of B_2 in the diet. However, in kidney and liver coenzyme concentrations were almost doubled as the concentration of the intake was increased from no added B_2 to 30 μg B_2 per day. In the heart muscle of deficient animals the FAD is decreased relatively more than the FMN, whereas in kidney and liver tissues the reverse is true.

3. The vitamin intake necessary to maintain tissue levels varies with the organ and the coenzyme. In general, an intake of 30 μg per day is sufficient to maintain optimal concentrations in all tissues.

4. A dietary requirement for growing rats on a synthetic diet of the type employed in this study is suggested. For optimal coenzyme tissue levels the intake should be around 30 μg B_2 per day and for maximum growth the intake should be somewhat higher than 43 μg per day. For both optimal tissue levels and growth it would seem that 30 to 40 μg B_2 per day or 3 or 4 μg per gram of diet would be adequate.

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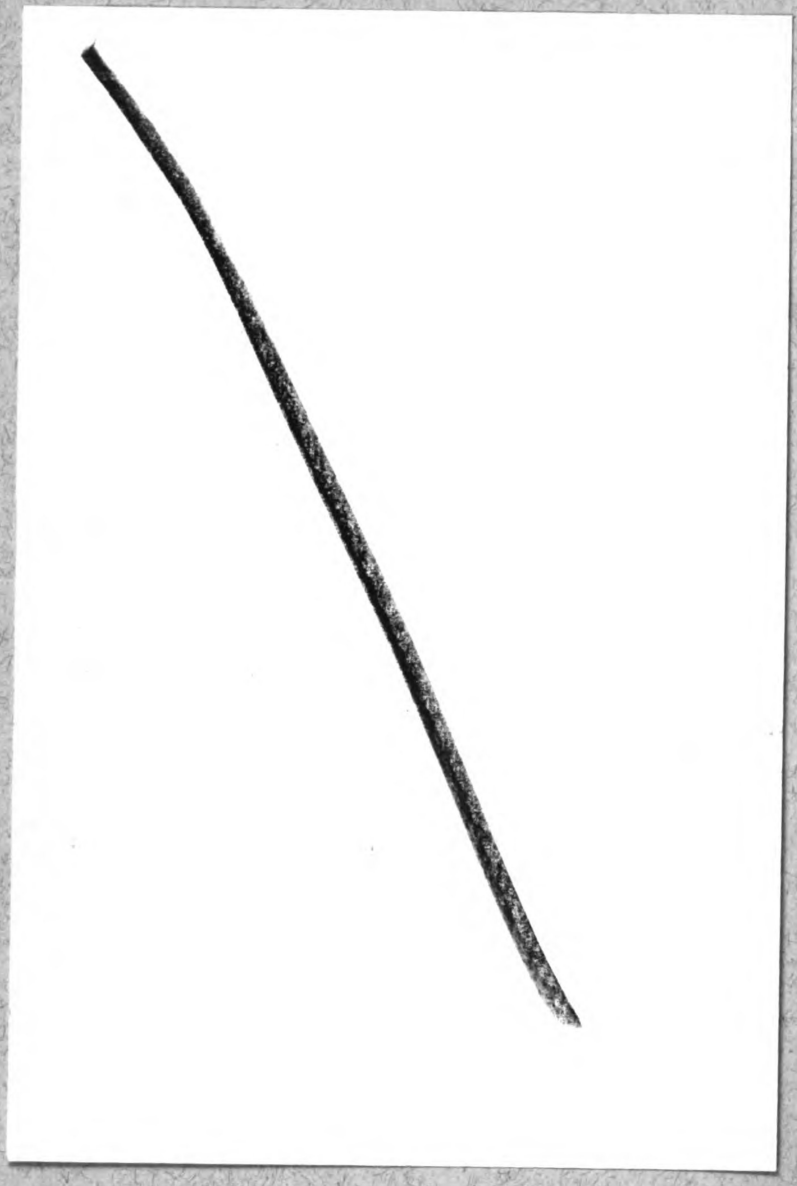

THE CONCENTRATION OF RIBOFLAVIN AND ITS PHOSPHORYLATED DERIVATIVES IN THE TISSUES OF RATS FED VARYING QUANTITIES OF VITAMIN B_2

By

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AN ABSTRACT

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In the tissues of the rat the vitamin, riboflavin, is found in the free form and also as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). These flavin nucleotides act as coenzymes in various enzyme systems. It has been shown that the tissue concentrations of certain specific enzyme systems, i.e., D-amino acid oxidase and xanthine oxidase and of the coenzyme FAD in general were diminished during a vitamin B_2 dietary deficiency.

Various criteria have been set up to determine the dietary requirement for vitamin B_2, such as the amount necessary for maintenance of total riboflavin tissue levels or that necessary for minimum growth and life span. There has not been an attempt to correlate the dietary requirement with the optimal coenzyme tissue level. It was thought to be of interest to see how the FMN and the FAD concentrations in tissues were affected by feeding varying amounts of vitamin B_2, and to ascertain the dietary intake of vitamin B_2 necessary for an optimal tissue coenzyme concentration.

Therefore, the purpose of the present study was (a) to measure the concentrations of riboflavin and its phosphorylated derivatives in tissues of rats fed synthetic diets with varying riboflavin concentrations and (b) to compare the dietary vitamin B_2 requirement based on tissue levels with that based on growth.

The composition of the diet affects the dietary requirement for riboflavin. A synthetic diet which was a modification of the Bourquin-Sherman assay diet for vitamin B_2 was chosen to minimize as much as possible extraneous sources of riboflavin, such as that from intestinal synthesis.

Rats were fed the synthetic diets for approximately four weeks. After sacrificing the animals, the brain, kidney, liver and heart tissues were quickly frozen and later analyzed for FAD and FMN + free riboflavin. The method of analysis depends on the fact that in a highly concentrated salt solution FAD has a fluorescence equal to 15% of that of riboflavin and FMN and free riboflavin have equal fluorescence. It was not possible to determine accurately the very small amount of free riboflavin present, thus the FMN + free were calculated by difference.

Of the tissues analyzed, brain coenzyme concentrations were almost independent of the dietary vitamin B_2. In the heart of deficient animals the FAD is decreased relatively more than the FMN, whereas in kidney and liver the reverse is true. An intake of approximately 3 μg B_2/day is sufficient to maintain kidney FAD concentration on the same level as those of the animals receiving the highest intake (43 μg B_2/day); FMN + free is not maintained until an intake of 19 μg B_2/day is attained. In liver an intake of around 30 μg B_2/day is necessary for optimal concentration of both coenzymes, while in heart 19 μg B_2/day is sufficient for both.

Using maximum growth as a criterion the requirement is different. The highest intake gave growth which, it was felt, closely approached the maximum attainable with this synthetic diet. Therefore, the dietary

requirement for maximum growth would be somewhat higher than 43 μg B_2/ day. There was an increase in growth obtained with each increase in vitamin B_2 intake over 19 μg/day which could not be attributed to an increased food consumption.