A COMPARISON OF THREE METHODS OF ASSAY FOR THE RIBOFLAVIN CONTENT OF THREE MIXED DIETS

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A COMPARISON OF THREE METHODS OF ASSAY FOR THE RIBOFLAVIN CONTENT OF THREE MIXED DIETS

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INTRODUCTION

INTRODUCTION

That the animal body needs riboflavin for its most efficient functioning has been recognized for many years, and to riboflavin have been attributed many functions now known to be performed by other factors of the Vitamin B complex. The distinction between riboflavin and the other members of the Vitamin B family has been impeded greatly by the lack of methods for its measurement and extraction.

A short time after fat-soluble A and water-soluble B vitamins were postulated, experimenters discovered that they were working with at least two water-soluble factors, one thermolabile and the other thermostable. The thermostable portion became known as B_2.

Among the early workers who attempted to assay this material were Chick and Roscoe (1928), and Sherman and his co-workers (1931-1935) whose efforts led to the establishment of the Bourquin-Sherman rat unit. Those who have worked to improve this technique are Wagner, Axelrod, Lipton and Elvejhem (1940), who studied the basal diet; Morgan, Cook and Davison (1938), who worked with the effect of the kind of carbohydrate in the riboflavin-depletion diet; Clark, Lechycka and Cook (1940), who studied the value of the various B-complex supplements and who greatly simplified the diet and clarified the symptoms of ariboflavinosis; Shaw and Phillips (1941), and Mannering, Lipton and Elvejhem (1941),

who determined the effect of fat in riboflavin-deficient diets; and Street (1941), who has reviewed the rat assay methods in current use in an effort to standardize and improve them.

Other animals than rats have been used in the biological assay. Chicks, monkeys and mice were used by Day (1934), and Jukes (1937) outlined the present assay methods using chicks. Dogs were used as the experimental animals by Street and Cowgill (1939). In 1940, Koch suggested using the larval stage of development of the beetles Tribolium confusum, dropsophila and sitodrepa in the assay of flours for riboflavin, and in 1941 Fraenkel and Blewett and Barton-Wright made improvements in Koch's method with Tribolium confusum.

Human studies also have been conducted in an effort to learn the human requirement.

In vitro experiments have paralleled the biological. Much of this early work was done in Europe. Warburg and Christian (1933) first isolated crystalline riboflavin, or "flavine" as they called it. Kuhn and Wagner-Jauregg (1933) are credited with first noting its fluorescence. The same authors with Kaltschmitt (1934) studied its distribution in plants as "lumniflavin" by means of a photometer. Charite and Khaustov (1934) and Murthy (1937) measured the flavine content of foodstuffs by a colorimetric comparison with potassium dichromate. Cohen (1935) measured the fluorescence of unknown solutions with a selenium cell. Weisberg and

Levin (1937) compared the fluorescence of the unknown and standards using fluorescein as the standard. Von Euler and Adler (1934). Supplee, Ansbacher and Bender (1935), and Whitnah, Kunnerth and Kramer (1937) made similar visual comparisons using lactoflavin as the standard. Von Euler, Adler and Schlotzer (1934) used a carbon arc lamp and blue glass filters. Josephy and Cohen (both 1934) used filters also, Josephy comparing his samples with fluorescein, and Cohen with lactoflavin. Hand (1939) used a uranium glass cube calibrated with standard riboflavin solutions, and estimated that with it, riboflavin present in the amounts of one-tenth to four milligrams per liter of solution could be detected, whereas the colorimetric procedure was accurate on solutions containing from four to forty milligrams per liter. In 1939, Hodson and Norris described a method for use with foods by which the riboflavin content was indirectly measured, and Sullivan and Norris (1939) sought to prevent the interference of impurities with light absorption readings by reading the samples on the photometer, reducing them with sodium hyposulfite and reading again, the difference in readings being taken as the riboflavin value. Supplee, Bender and Jensen (1939) outlined a similar procedure and checked it by rat assay of the adsorbates of samples. These methods have since been modified by Ferrebee (1930) and Najjar (1941) for use with

biological fluids. Conner and Straub (1941) have simplified these methods and their procedure is in current use.

Much of the work done on the extraction of flavins from samples was done by György, Kuhn and Wagner-Jauregg (1934). Von Euler, Adler and Karrer (1934) are responsible for the beginning of work which showed all flavins to be riboflavin.

Experimentation on the microbiological assay of riboflavin has been done for a large part in the United States, but a search of the literature indicates that the conception of the need of microorganisms for riboflavin is due to Warburg and Christian (1933), who discovered it in large amounts in yeasts, lactic-acid and acetic acid bacteria, and believed that it was a cell oxidant for these organisms. Little was done with this information until Snell and Strong (1939, 1939) and Krauskopf, Snell and McCoy (1939) experimented to find which of these groups of organisms needed riboflavin for growth. Four of the lactic-acid producers and one Streptococcus exhibited poor growth in the absence of riboflavin. Further research produced the method of Snell and Strong (1939) now used.

Revisions of the method of extracting the samples and removing of interfering, growth-stimulating substances followed, the first being suggested by Feeney and Strong (1940), Scott, Randall and Hessel (1941), and Wegner, Kemmerer and Frans (1942). Andrews, Boyd and Terry (1942) found a

material present in flour after digestion and extraction which did not contain riboflavin and which did not stimulate growth unless it was in the presence of riboflavin. Bauernfeind, Sotier and Boruff (1942) showed that some of the stimulating materials were fatty acids, salts of fatty acids and alcohols. Strong and Carpenter (1942) revised the original method of preparation of samples, but there still is need for improvement as their results with mixed food samples varied as much as eight-tenths of a microgram per gram of dry sample.

Before the microbiological method of assay was suggested the biological assay was used as a standard of comparison for chemical or fluorometric determination. Since that time, and due to a feeling that the biological assay is not quantitative, the fluorometric assay has become the accepted standard. Whether or not the microbiological assay will replace the fluorometric assay as a standard procedure will depend on the development of a more complete means of removing other growth-stimulants from the sample.

Emmett and his co-workers (1941) have compared the existing standard procedures and have reported "similar" results on dried milk samples. Strong and Carpenter (1942) consider the results of a like comparison of method to give only a "fair degree of correlation". Andrews (1943) in his report of the committee on methods of analysis for 1942-1943 believes the microbiological assay gives the most accurate

analysis of the riboflavin content of foods, the fluorometric assay to give values of 87 per cent of the microbiological value, and the biological assay to give values of 81 per cent of the microbiological value in the assay of flours and cereals.

As far as is known to the author, a comparison of assay methods on a mixed human diet has never been attempted. This study has been undertaken for the purpose of comparing the three standard methods of assay on a mixed human diet.

EXPERIMENTAL PROCEDURE

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The samples assayed by these three methods were the three diets fed by Brewer and Ingalls (1944)* in the study of human requirement for riboflavin. The diets were similar in composition, but fluorometric analysis by Brewer and Ingalls revealed that the three diets varied somewhat in riboflavin content.

The sampling and preparation of food for analysis was conducted as follows: as the food prepared for consumption was weighed, an additional portion was weighed for assay purposes. The food for each meal was combined into a single sample, mixed in a Waring blendor, acidified to a pH of 4.5 with glacial acetic acid, dried to constant weight at 40°C., ground, placed in amber bottles and stored until assayed. In this study the food samples were mixed in the original proportions of breakfast, lunch and dinner as calculated from the moisture loss in the drying of each meal, and equal amounts of all samples of the same diet mixed and stored in amber bottles. Samples were protected from strong light at all times, and all weighing and other procedures were carried out in semi-darkness.

The composition of the three diets may be found in the Appendix in Table I. Calculations of food values were made by Miss Brewer and Miss Ingalls.

* Unpublished data from this laboratory.

Biological Assay

The method of Clark, Lechycka and Cook (1940) was followed for the biological assay, using twenty-four day old albino rats of the Sprague-Dawley strain. The rats weighed thirty to forty-five grams when they were placed on the basal diet. The diet was similar to those recommended by Sherman, and by Clark, Lechycka and Cook (1940), but had an additional amount of fat as suggested by Shaw and Phillips (1941), and Mannering, Lipton and Elvehjem (1941). The amount of fat used was the amount suggested by the latter authors.

Basal Diet

Cornstarch	50 %
Alcohol-extracted casein	18
Crisco	26
Osborne and Mendel salt mixture	4
Cod liver oil	2
Yeast Supplement	6.5

All of the animals were given 50 micrograms of thiamine hydrochloride daily throughout the experiment. It was planned to give 150 milligrams of Borden's rice polish factor a day, but as that company could not supply the rice polish factor, the amount on hand was fed for the first five days, during which time a yeast supplement was prepared for the remainder of the feeding period. The yeast supplement was the same as that used in the microbiological assay method of Snell and Strong (1939), and was given in amounts equivalent to 150 milligrams of whole yeast a day. The rats ate very little

of the yeast supplement even when it was given on sugar, so it was mixed with the basal diet and baked in a hot oven, with the heat turned off, for ten minutes. The supplement was concentrated so that one milliliter of the solution mixed with ten grams of the basal diet supplied 6.5 per cent of whole yeast to the diet.

The rats were placed in individual cages with raised, large-mesh screen floors, and given the basal diet and distilled water, ad libitum. They were weighed twice weekly until a weight plateau was reached and maintained for a week, or until they lost weight on two successive weighing days. This depletion period was seventeen and eighteen days in length. At this time they were separated according to weight, and placed on the experimental diets with four males and four females in each of nine dietary groups. Each of the three human diets was fed in amounts to supply 2.5, 3.75 and 5.0 micrograms of riboflavin daily as calculated from the fluorometric analysis. (Table I) Negative and positive control rats paralleled the experimental rats with four males and four females in each group. Negative controls received no further supplement. Positive controls were divided into three groups of eight animals and received 2.5, 3.75 and 5.0 micrograms of pure riboflavin daily as a standard solution prepared by dissolving pure riboflavin (Merck) in 0.02 N acetic acid to contain 100 micrograms per milliliter, and diluted to contain 10 micrograms per milliliter as needed.

The experimental period lasted twenty-eight days. At the end of that time the rats were chloroformed and one half of each group autopsied. All of the negative controls were autopsied.

Chemical Assay Using the Fluoroscope

The chemical assay method of Hodson and Norris (1939) with modifications by Connor and Straub (1941) was followed. Samples of the three experimental diets were weighed in duplicate on the analytical balance and transferred quantitatively to amber glass flasks. Fifty milliliters of 0.04 N sulfuric acid were added and the flasks were weighed. Food samples were hydrolyzed on the water bath for one hour, cooled, and the weight adjusted to the weight before hydrolysis. Ten milliliters of a solution containing 0.01 grams of taka-diastase and 0.01 grams of papain per milliliter were added, and the flasks incubated over night at 37°C. The enzymes were inactivated by heating the samples on the steam bath, after which the cooled samples were filtered through No. 12 Whatman pleated filter paper. Adsorption columns of pyridine-treated Florosil were prepared and kept wet until the samples had filtered. Then a twenty milliliter aliquot was adsorbed on the Florosil, washed several times with warm, distilled water, and eluted with thirty milliliters of twenty per cent pyridine in two per cent acetic acid. The eluate was collected in fifty milliliter volumetric

flasks and made to volume. A fifteen milliliter aliquot was pipetted into twenty-five milliliter volumetric flasks, and one milliliter of four per cent potassium permanganate solution added. This was mixed and allowed to stand for one minute, then three milliliters of three per cent hydrogen peroxide were added, the whole mixed and made to volume. A reagent blank was prepared during this process, starting with the adsorption column. The standard riboflavin solution containing 100 micrograms per milliliter was diluted to contain 0.1 micrograms per milliliter. The fluoroscope (Lumetron) was standardized with this solution, corrected with the reagent blank, and the samples read as per cent of 0.1 micrograms per milliliter.

This procedure was repeated on a second set of samples except that these were autoclaved for fifteen minutes at fifteen pounds of pressure in place of the hydrolysis period. This was done to determine the effect of autoclaving of samples on the fluorescence of the resulting solutions.

Microbiological Assay

The microbiological assay for riboflavin was that of Snell and Strong (1939), except that the samples were digested over night prior to assay to release protein and carbohydrate-bound riboflavin.

A pure culture of Lactobacillus caseii was obtained from the Experiment Station, Department of Agricultural

Chemistry, Michigan State College, East Lansing, Michigan. This organism was carried as a stab culture in yeast agar, and transferred every forty-eight hours for eight days, at which time it was used for the assay.

The various ingredients were prepared according to Snell and Strong's method (1939) except the cystine and the yeast supplement. The method for preparing the latter is from Strong and Carpenter (1942).

Cystine Solution

Cystine was suspended in a small amount of distilled water and heated gently. Concentrated hydrochloric acid was added dropwise until the cystine was in solution. This was cooled and made to volume with distilled water, and stored under toluene and chloroform in the refrigerator. One milliliter of this solution contained one milligram of cystine. Snell and Strong (1939) recommended using cystine hydrochloride, but this is not readily soluble in water. The method of preparation outlined above results in the same solution, and is more easily prepared.

The medium for fifty assay tubes contains:

50 ml. of photolyzed, NaOH-treated peptone
50 ml. of Cystine solution
10 ml. of yeast supplement
5 grams of anhydrous, C.P. glucose
2.5 ml. of salt solution A
2.5 ml. of salt solution B

These were mixed and the pH adjusted to 6.6 - 6.8 with sodium hydroxide. After the solution was diluted to 250 milliliters

five milliliters were pipetted into each of fifty standard bore test tubes. This medium is of double strength to allow for dilution by the addition of samples. An aliquot of riboflavin-containing extract was added, and distilled water to make each tube to a volume of ten milliliters. The tubes were plugged with cotton and sterilized in the autoclave at fifteen pounds of pressure for fifteen minutes. When they had cooled, they were innoculated and incubated.

The innoculum was prepared by the method of Snell and Strong (1939), and one drop in a sterile standard four millimeter innoculating loop was used to seed each tube.

Samples of the three experimental diets were prepared for assay as follows: 0.04 N sulfuric acid was added so that each milliliter of solution would contain approximately 0.1 microgram of riboflavin as calculated from the results of the fluorometric assay. The samples were autoclaved for fifteen minutes at fifteen pounds of pressure. When they had cooled, ten milliliters of a solution containing 0.1 grams of each of takadiastase and papain were added, and the samples incubated over night. Since riboflavin is found in foods combined with cereals and protein, and since all of these diets contained cereals, egg and meat, this digestion period was considered necessary. Enzyme action was stopped by heating the samples on the steam bath for five minutes, and the pH adjusted to 6.6 - 6.8 with N NaOH solution. After filtering through No. 12 Whatman pleated

filter paper, the samples were restored to their original volume with distilled water. Aliquots then were pipetted into tubes containing the basal medium, made to a volume of ten milliliters, and sterilized in the autoclave. Nine sets of triplicate tubes were prepared, and each of the three diets was samples in amounts giving an estimated 0.05, 0.15 and 0.25 micrograms of riboflavin. With each assay, duplicate standard tubes were set up containing 0.0, 0.05, 0.075, 0.1, 0.15, 0.2, 0.3 and 0.5 micrograms of pure riboflavin per tube. All of the tubes were innoculated as described above, and incubated for 24, 48 and 72 hours at 37°C. (Table III).

When the incubation time was finished, the contents of the tubes were transferred to Erlenmeyer flasks, the tubes washed with ten to twenty milliliters of distilled water, and titrated with 0.1 N. sodium hydroxide (approximately) to a pH of 7.0, using Brom Thymol Blue as an indicator and a color comparison flask. The indicator was added to the flasks before the samples were transferred.

RESULTS AND DISCUSSION

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The results of the biological assay of the three experimental diets are recorded in Tables I and II. A standard curve was constructed from the total average weight gains of the control animals, but the rise of this curve was not great enough to allow for calculating the riboflavin content of the experimental diets from the gains in weight made by the animals on these diets. It was not considered logical to extend the standard curve because all of the weight gains of the animals on the experimental diets were above the highest weight gain on a pure riboflavin supplement. Thus the experimental diets seemed to contain much more riboflavin than was found by fluorometric assay. It is unlikely that the fluorometric analysis was this inaccurate, and the discrepancy in weight gains must be explained as due to other substances in the experimental diets which were growth-promoting in the rat, or to an inadequacy in the basal diet. Since the rise of the standard curve was less than that reported in the literature, the latter seems the more likely explanation although this same diet has been used successfully by Shaw and Phillips (1941) and by Mannering, Lipton and Elvehjem (1941). However even if a standard curve of the usual rise were used, the weight gains of the animals on the experimental diets far exceed those of control animals except on the highest concentration of riboflavin. The smallest gain in weight on the

experimental diets, the 2.5 microgram level of Diet III, would then correspond to the highest gain in weight on the control diet.

Plate I shows the total average gains in weight for each group. It will be noted that the gains in weight on each diet were well separated for each amount of riboflavin fed.

On autopsy five of the negative control animals had yellow edged livers, and all had mottled livers. There was no body fat on these animals, and they were less mature sexually than any of the other groups; the testes in the males were smaller and there was no follicular development in the females. The three groups of positive controls were much the same, the amount of body fat corresponding to the riboflavin intake. All of the positive controls had mottled livers, but none showed yellowing. Testes in male rats were larger, and the ovaries were faintly pink. The animals on the three experimental diets were comparable. These rats also had stores of body fat corresponding to the supplement intake. There were no yellow livers, and mottling appeared only on the lowest amount of diet fed. Testes were correspondingly larger, and all of the females on the five microgram level of riboflavin intake in the diets showed mature follicles. The external appearance of all of the animals was much the same, except that the negative control rats had less hair and this was matted with oil.

TABLE I

Results of the Biological Assay

Diet	Gamma of riboflavin/gram	Amount of diet fed			Total Average Weight Gains on the Experimental Diets		
		gamma riboflavin			gamma of riboflavin		
		2.5 grams	3.75 grams	5.0 grams	2.5 grams	3.75 grams	5.0 grams
I	1.51*	1.65	2.5	3.3	17.5	21.0	22.0
II	1.34	1.85	2.8	3.7	17.0	19.0	22.0
III	1.83	1.4	2.05	2.7	15.0	18.0	23.0
Controls	10/ml.	.25ml.	.375	.50	7.5	10.0	13.5

* Values calculated from Brewer and Ingalls (1944)

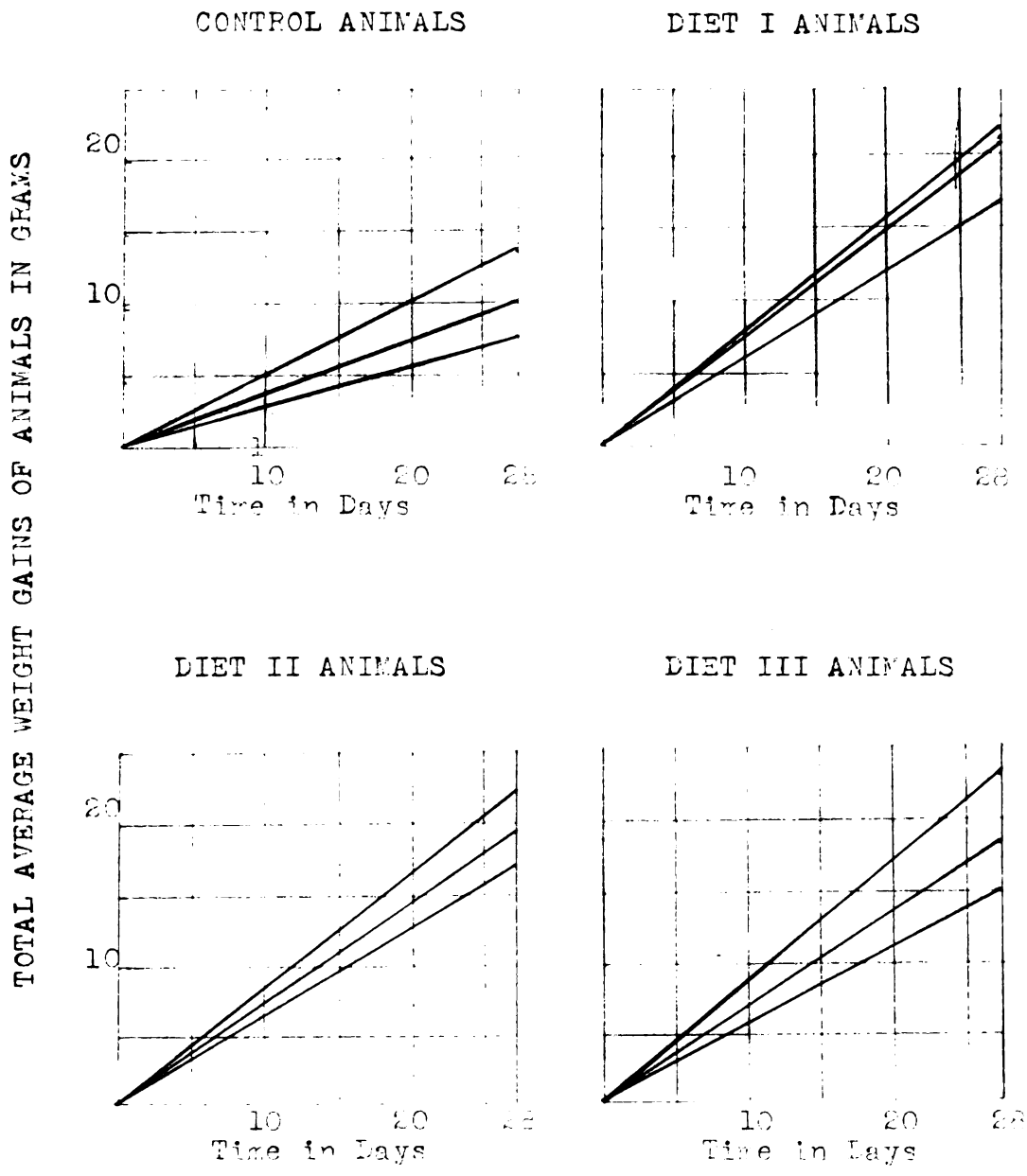
TABLE II

Results of the Biological Assay Calculated from the Standard Curve

Diet	Values calculated from Standard Curve						Average gamma/gm.
	gamma of riboflavin fed						
	2.5		3.75		5.0		
	per cent	gamma/ gram	per cent	gamma/ gram	per cent	gamma/ gram	
I	233	4.18	210	3.36	163	2.66	3.4
II	226	3.62	190	2.67	163	2.37	2.8
III	200	4.14	180	3.46	165	3.4	3.66

PLATE I

TOTAL AVERAGE WEIGHT GAINS
OF
THE EXPERIMENTAL DIETS



These results are not quantitative, but they do corroborate the findings of Shaw and Phillips (1941) with regard to the effect of ariboflavinosis on the reproductive organs. They would seem to indicate that riboflavin is intimately concerned in fat metabolism and storage, as Mannering, Lipton and Elvejhem (1941) affirmed.

Chemical Assay Using the Fluoroscope

There were two sets of samples for this assay, one was hydrolyzed to release the riboflavin and the other was autoclaved. The results are found in Table III. Autoclaving of samples in place of hydrolyzing them had little effect on the results of the fluorometric assay. The difference was slight in all but Diet III, in which autoclaved samples gave readings of 117 per cent of the hydrolyzed samples. Since autoclaving apparently made only a small difference, the values of the hydrolyzed samples were taken as a basis of comparison of fluorometric values with the values of the microbiological method.

Microbiological Assay

Diet samples assayed by the microbiological method of Snell and Strong (1939) and Strong and Carpenter (1942) indicated that the twenty-four hour incubation period was inadequate and gave misleading results. (Table IV) Forty-eight and seventy-two hours of incubation gave results more nearly like the standard curves, and compared more closely

with the fluorometric assay. The correlation also was closer between the forty-eight hour standards and samples, than between samples which had been incubated for seventy-two hours. Strong and Carpenter (1942) believe that the seventy-two hour incubation period gives the most accurate results, but as this did not appear to be true of these samples, all comparisons were made with the riboflavin values of samples which had been incubated for forty-eight hours. (Table V).

Standard curves were constructed for each of the incubation periods, and the riboflavin values of the samples were calculated from them. (Plates II, III, IV).

The results of the three methods of assay are compiled in Tables V and VI. If the microbiological assay results are taken as the true riboflavin values of the samples, the fluorometric assay values are thirty-four to one hundred and five per cent higher, and the biological assay values would be two hundred and thirty-one to three hundred and sixty-seven per cent higher.

The estimated riboflavin values calculated from the microbiological assay of each of the three diets indicate that there is little basis for comparing the three methods of assay when a food mixture is tested. The present methods of assay apparently are not accurate enough for use when a large number of foodstuffs are present.

TABLE III

Results of Fluorometric Assay

Diet	Weight of sample	Lumetron readings		Gamma Riboflavin/gram		Difference
	Grams	Untreated	Autoclaved	Untreated	Autoclaved	Per Cent
I	5	32	33	1.6	1.65	+3.1
II	5	28	27.5	1.4	1.375	-1.8
III	5	38	45.5	1.9	2.225	+17.0

TABLE IV

Results of the Microbiological Assay

Standard tubes gamma/ tube	NaOH used in titration Hours 24 ml.	48 ml.	72 ml.	Diet	Weight of Sample grams	.04 N H_2SO_4 ml.	Estimated riboflavin in sample gamma	NaOH used in titration Hours 24 ml.	48 ml.	72 ml.	Riboflavin in sample calculated from standard tubes for assay Hours 24	48	72
0.00	2.25	3.0	3.2				0.05	3.40	4.25	4.30	.15	.06	.03
0.05	2.6	4.0	5.1	I	5	82.5	0.15	4.25	5.75	6.10	--	.105	.095
0.075	2.75	4.8	5.5				0.25	5.10	6.80	7.10	--	.135	.135
0.10	3.0	5.7	6.2				0.05	3.20	4.00	4.60	.13	.05	.035
0.15	3.3	7.2	7.5	II	5	68.7	0.15	4.25	5.60	6.10	--	.10	.095
0.2	3.65	8.3	9.5				0.25	6.00	8.00	8.30	--	.185	.17
0.3	4.0	9.0	10.8				0.05	3.20	4.25	4.35	.13	.06	.03
0.5	4.15	9.5	10.5	III	5	111.3	0.15	4.60	6.80	6.80	--	.12	.12
							0.25	5.60	7.95	8.75	--	.185	.18

PLATE II

Standard Curves for Microbiological Assay

with

Diet I Values Plotted Upon Them

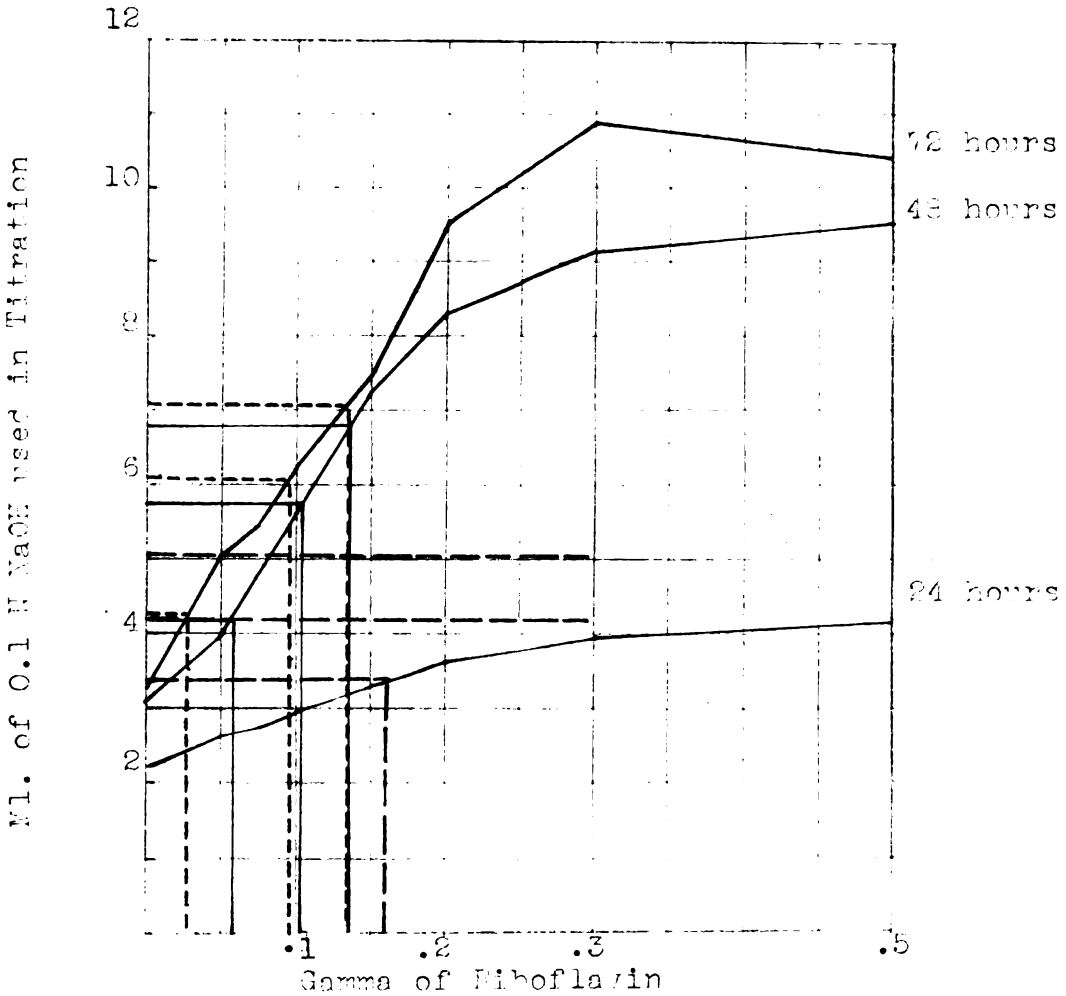


PLATE III

Standard Curves for Microbiological Assay

with

Diet II Values Plotted Upon Them

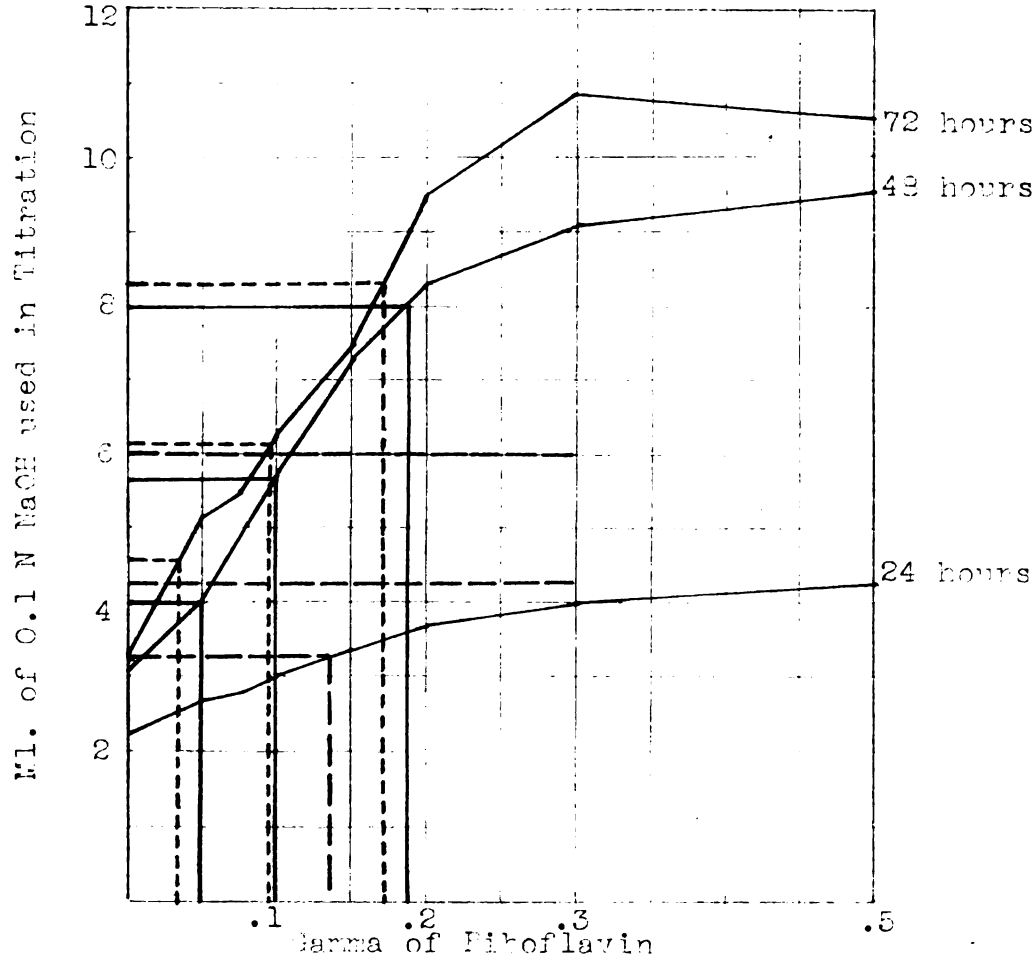


PLATE IV

Standard Curves for Microbiological Assay

with

Diet III Values Plotted Upon Them

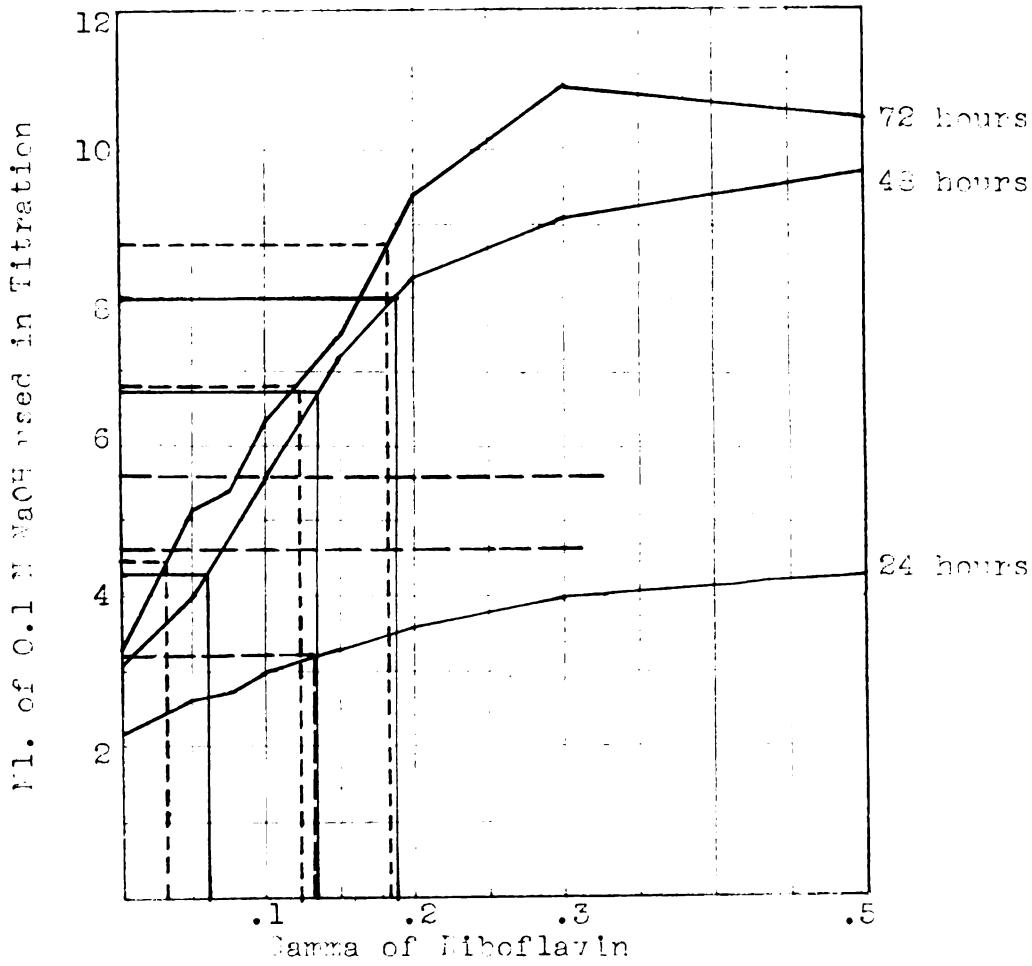


TABLE V

Comparison of Values for Riboflavin
in Diets from
Microbiological, Fluorometric and Biological
Results

Diet	Microbiological assay value	Fluorometric assay value	Biological assay value
	Gamma/gram	Gamma/gram	Gamma/gram
I	0.775	1.60	3.4
II	0.89	1.40	2.8
III	1.41	1.00	3.66

TABLE VI

Results of
Microbiological, Fluorometric and Biological
Assays

Diet	Riboflavin calculated from microbiological assay	Microbiological assay*	Fluorometric assay*	Biological assay*
I	0.775	100 %	205 %	467 %
II	0.89	100	156	331
III	1.41	100	134	354

* In per cent of Microbiological assay results.

SUMMARY AND CONCLUSIONS

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When albino rats, which had been depleted of their riboflavin stores as indicated by weight plateaus, were fed the three experimental mixed human diets, their weight gains far exceeded those of rats on the basal diet plus pure riboflavin. It is felt that growth-stimulating substances other than riboflavin were present in the experimental diets which prevented an accurate determination of the riboflavin value of the diets, and also that the basal diet did not permit normal growth and development.

Autopsy revealed that the animals on the experimental diets had better developed reproductive organs than the control animals, and the positive controls showed better development than the negative controls. The amount of body fat was directly related to the amount of riboflavin fed, but all of the animals given the experimental diets had larger fat deposits than the control animals. Most of the negative controls had yellow-edged livers, and all had mottled livers, as did all of the positive controls and the experimental animals fed the smallest amounts of the three diets.

Autoclaving the food samples before determining the riboflavin value fluorometrically gave values of -1.79 to + 17.0 per cent of the hydrolized samples. From these

results it is indicated that the substances causing interference with readings of fluorescence are not removed by autoclaving.

A forty-eight hour incubation period for food samples by the microbiological assay resulted in values for riboflavin which were more closely related to the standard curve and to the fluorometric assay values than the results obtained by seventy-two hours of incubation.

The results of the fluorometric assay were thirty-four to one hundred and five per cent higher than the microbiological assay values, and the biological assay values were two hundred and thirty-one to three hundred and sixty-seven per cent higher. Since it is unlikely that any of the methods is as inaccurate as this comparison would indicate, a detailed study of the materials which cause variation in fluorescence and a means of removing growth-stimulants for the biological and microbiological assays are necessary before any comparison of these methods of assay of a mixed diet for riboflavin can be useful. Due to a large number of unknowns in a mixed diet, and to ignorance of the manner in which they may operate as stimulants or interferents, the present assay methods do not give comparable results.

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APPENDIX

COMPOSITION OF FOODS IN DIET I

Meal	Food	Measure	Weight Gms.	Calories	Calcium Mgs.	Phosphorus Mgs.	Iron Mgs.	Vitamin A I.U.	Thiamin Mgs.	Riboflavin Mgs.	Niacin Mgs.	Ascorbic Acid Mgs.
Breakfast	Grapefruit	1/3 c.	79	37	8	0.013	.16	16	.059	.008	--	31
	Farina (cooked wt.	½ c.	135	79.69	4.76	.028	.17	--	0.113	.034	--	--
	Dates	4	29	91.6	20	0.002	0.61	50	0.023	0.013	--	--
	Cream 18.5%	¼ c.	51	105.9	50	0.039	0.10	640	0.018	0.089	--	1
	Sugar	1 T.	13	52	--	--	--	--	--	--	--	--
	Bread	1 slice	20	52.6	5	0.02	0.18	--	.014	.014	0.13	--
	Butter	1 T.	13	95.3	2	.002	.03	550	0	0	0	0
	Jelly	½ T.	18	51.6	1.5	0	.05	0	0	.067	0	0
Lunch	Rice	3/4 c.	100	97.5	2.8	.031	.25	0	.009	0	.3	0
	Tomatoes	½ c.	80	18.6	8	.022	.35	680	.043	.04	.46	18
	Egg	1	47	75.1	29	.105	1.36	720	.072	0.168	.04	0
	Bread	2 slices	40	105.3	10	.04	.36	--	.028	.028	.26	--
	Butter	1 T.	13	95.3	2	.002	.03	550	--	--	--	--
	Jelly	½ T.	18	51.8	1.5	0	.05	0	0	.067	0	0
	Peaches	2 halves 3 T.Juice	170	108.4	21	.038	.76	50	.045	.045	2.02	9
	Cashew nuts	2 T.	17	102.8	8	.082	0	†	†	.032	--	--
	Gelatin	1 T.	14	16	0	0	0	0	0	0	0	0

COMPOSITION OF FOODS IN DIET I (CONT.)

Meal	Food	Measure	Weight Gms.	Calories	Calcium Mgs.	Phosphorus Mgs.	Iron Mgs.	Vitamin A I.U.	Thiamin Mgs.	Riboflavin Mgs.	Niacin Mgs.	Ascorbic Acid Mgs.
Dinner	Ground beef	1 patty	88	145.8	12	0.179	3.15	30	.12	.220	6.6	--
	Potato	1 large	120	103.1	14	.064	1.10	45	.131	.072	1.42	7
	Green beans	½ c.	67	28.2	37	.034	.80	800	.042	.072	.43	10
	Bread	2 slices	40	105.3	10	.040	.36	--	.028	.028	.26	--
	Butter	2 T.	26	190.6	4	.004	.06	1100	0	0	0	0
	Jelly	1 T.	36	103.6	3	0	.1	0	0	.067	0	0
	Apple-sauce	½ c.	126	158.2	10	.014	.45	90	.034	.038	.63	5
	Sugar Cookies	3	28	109.8	4	.022	.10	200	.012	.018	.55	--
	Totals			2,181.4	267.1	.781	10.58	5,505	.862	1.116	13.10	81

-- none present
0 not known
† present but only in traces

COMPOSITION OF FOODS IN DIET II (CONT.)

Meal	Food	Measure	Weight Gms.	Calories	Calcium Mgs.	Phosphorus Mgs.	Iron Mgs.	Vitamin A I.U.	Thiamin Mgs.	Riboflavin Mgs.	Niacin Mgs.	Ascorbic Acid Mgs.
	Egg	1	47	75.1	29	.105	1.36	720	.072	.168	.038	0
	Cashew Nuts	2 T.	17	102.8	8	.082	0	+	+	.032	--	--
	Gelatin	½ t.	2	16	0	0	0	0	0	0	0	0
Supper	Ground Beef	1 cake	88	145.8	12	.179	3.15	30	.120	.22	6.6	--
	Canned Asparagus	6 stalks	60	13.1	10	.026	.5	250	.082	.05	--	10
	Potato	1 large	120	103.1	14	.064	1.1	45	.131	.072	1.42	7
	Bread	2 slices	40	105.3	10	.04	.36	--	.028	.028	.26	--
	Butter	1 T.	13	95.5	2	.002	.03	550	0	0	0	0
	Jelly	½ T.	18	51.8	1.5	0	.05	0	0	.067	0	0
	Applesauce cake	3X3X2	50	177.6	3	.023	0	50	.012	.009	--	0
	Dates	3	29	91.6	20	.002	.61	50	.023	.013	--	0
	Totals			2,130.4	257.7	.972	10.86	4,494	.779	1.01	11.3	82

-- None Present
0 Not known
+ Present but only in traces

COMPOSITION OF FOODS IN DIET II

Meal	Food	Measure	Weight Gms.	Calories	Calcium Mgs.	Phosphorus Mgs.	Iron Mgs.	Vitamin A I.U.	Thiamin Mgs.	Riboflavin Mgs.	Niacin Mgs.	Ascorbic Acid Mgs.
Breakfast	Grapefruit Juice	½ c.	137	49	10	.017	.21	21	.079	.010	--	41
	Shredded Wheat	1 biscuit	27	101.7	11	.099	1.23	--	.084	.028	0	--
	Bread	1 slice	20	52.64	5	0.02	.18	--	.014	.014	.13	--
	Butter	1 T.	13	95.4	2	.002	.03	550	0	0	0	0
	Cream 18.5%	¼ c.	51	105	50	.039	.10	640	.018	.089	0	1
	Sugar	1 T.	13		--	--	--	--	--	--	--	--
	Jelly	½ T.	18	51.8	1.5	0	.05	0	0	.067	0	0
Lunch	Spaghetti	3/4 C.	100	130	8	.052	. 43	0	0	0	2.25	0
	Tomato Juice	½ c.	62	14	3.7	.009	.37	528	.057	.031	.34	14.4
	Celery, raw	1 stalk	20	4.4	16.5	.01	.14	5	.007	.008	0	1.2
	Bread	2 slices	40	105.3	10	.04	.36	--	.028	.028	.26	--
	Butter	1 T.	13	95.4	2	.002	.05	550	0	0	0	0
	Jelly	½ T.	18	51.8	1.5	.05	0	0	0	.067	0	0
	Brownies	2	38	150.4	14	.052	.40	270	.024	.018	0	0
	Royal Ame Cherries	½ c. 3 T.Juice	122	95.2	13	.027	0.27	235	†	†	--	7

COMPOSITION OF FOODS IN THE DIET

Meal	Food	Measure	Weight Gms.	Calories	Calcium Mgs.	Phosphorus Mgs.	Iron Mgs.	Vitamin A I.U.	Thiamin Mgs.	Riboflavin Mgs.	Niacin Mgs.	Ascorbic Acid Mgs.
Breakfast	Grapefruit Juice	½ c.	137	.64	14	.023	0.28	29	0.102	0.015	--	55
	Grapenuts	1/3 c.	40	152	16.4	.134	1.91	0	0	0	2.699	0
	Cream 18.5%	¼ c.	51	105	50.0	.039	0.10	640	0.018	0.089	0	1
	Sugar	1 T.	13	52	--	--	--	--	--	--	--	--
	Bread	1 slice	20	52.6	5	.02	0.18	--	0.014	0.014	0.13	--
	Butter	1 T.	13	95.3	2	.002	0.03	550	0	0	0	0
	Jelly	½ T.	18	51.8	1.5	.0	0.05	0	0	.067	0	0
Lunch	Bacon	1 slice	8	41	0.98	.0145	0.24	--	0.008	0.008	--	--
	Egg	1	47	73.9	29.0	0.105	1.36	720	0.072	0.168	0.04	--
	Carrot strips	1/3 med.	20	8.7	9.1	0.008	0.12	622	0.02	0.018	0.29	0.8
	Bread	2 slices	40	105.2	10.0	0.040	0.36	--	0.028	0.028	.026	--
	Butter	1 T.	13	95.3	2.0	0.02	0.03	550	0	0	0	0
	Jelly	½ T.	18	51.8	1.5	0	0.05	0	0	0.067	0	0
	Pears Canned	2 T.juice 2 halves	100	67.6	11	0.016	0.29	--	0.3	†	0.14	2
	Date Bars	2	50	167.3	23.08	0.0397	0.962	173.07	0.046	0.035	0	0
	Cashew nuts	2 T.	17	102.8	8.00	0.082	0	†	†	0.032	—	--
	Gelatin	½ t.	2	8.0	-0	0	0	0	0	0	0	0

COMPOSITION OF FOODS IN DIET III (cont.)

Meal	Food	Measure	Weight	Calories	Calcium	Phosphorus	Iron	Vitamin A	Thiamin	Riboflavin	Niacin	Ascorbic Acid
			gms		mgs	mgs	mgs	I.U.	mgs	mgs	mgs	mgs
Dinner	Prune juice	½ c.	120	95	12	0	.905	0	0	0	0	0
	Ground beef	1 patty	88	145.3	12	0.179	3.15	30	.12	.22	6.6	0
	Potato	1 lg.	120	103.1	14	.064	1.1	45	.131	.072	1.42	7
	Canned corn	½ c.	97	97	6	.116	.46	500	.131	.06	1.51	9
	Bread	2 slice	40	105.3	10	.04	.36	--	.023	.022	.26	--
	Butter	1 T.	13	95.3	2	.002	.03	550	0	0	0	0
	Jelly	1 T.	18	51.3	2	0	.05	0	0	0	0	0
	Fruit Cocktail	½ c., 3 T juice	30	75	11	0	.32	85	.066	.027	0	23
	Sugar Cookies	3	28	109	4	.013	.1	200	.012	.013	0	0
	TOTAL			1975	252	.959	12.4	5294	1.09	.966	13.12	99