BIOLOGICAL ASSAY OF THE RIBOFLAVIN CONTENT OF BEEF, CALF, LAMB, MUTTON, AND PORK LIVER

by

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INTRODUCTION

Previous studies have indicated that liver is high in riboflavin value. Other animal products such as milk and eggs, and green leaves are known to contain riboflavin. Very little work has been done on the comparative riboflavin value of different kinds of livers and most of that was done before the development of the Sherman-Bourquin unit of measurement. This study, based on the new technique, was undertaken to obtain additional information on the riboflavin value of various livers, and to compare their riboflavin value with that of other foods.

REVIEW OF LITERATURE

Riboflavin has been separated from the vitamin G (B_2) complex (20) since 1933. It is a water-soluble, yellow-green, fluorescent pigment found abundantly in egg white, whey, liver, and many other products. Its chemical and physical properties have now been studied. It has been variously designated as lactoflavin, hepatoflavin, ovoflavin and other similar names indicating the sources

from which it was isolated. The flavins obtained from egg white, milk, and liver are considered identical in structure and all have the properties of vitamin G (Sherman's terminology) (23). Euler et al. (12) showed that a synthetic flavin prepared by Karrer is identical with the lactoflavin isolated from milk (14).

To avoid further confusion due to the use of several terms, the Committee on Nomenclature of the American Society of Biological Chemists at a meeting in 1937 (21) agreed that the term riboflavin should be used to designate this factor and that such terms as lactoflavin, vitamin G, and vitamin B_2 be dropped. Riboflavin is a suitable name for this factor as it is a ribose derivative of iso-alloxazine described in chemical terms as 6, 7-dimethyl-9-(d, l'ribityl)iso-alloxazine. The chemical and physical properties of riboflavin were reviewed by Booher (5) as one of a series of reviews on the nature, properties, dietary standards, and physiological action of riboflavin prepared under the general auspices of the Council on Pharmacy and Chemistry and the Council on Foods of the American Medical Association.

Although the chemically pure riboflavin has been available for study only within the past few years, it has been

recognized as an important dietary factor and its presence has been measured in foods since 1931. Further investigations by Booher, Blodgett and Page (6), Bisbey and Sherman (4), Bessey (3), Kunerth et al. (17) and Kramer et al. (15) showed that the Bourquin-Sherman method (7) for determining the vitamin G content of foods measures the riboflavin fractions of the vitamin G complex. Riboflavin is found rather abundantly in a variety of foodstuffs including milk, lean meat, and the green growing parts of plants. Daniel and Munsell (10) reported 800-1200 Sherman-Bourquin units of this factor in beef and veal livers.

Hoagland and Snider (13) found that beef liver was higher in riboflavin content than pork liver. Of the 17 foods that Aykroyd and Roscoe tested (2), beef liver was highest in riboflavin.

Kuhn et al. (16) stated that muscles contained about one-thirteenth as much riboflavin as liver; also that the riboflavin values of the livers of negative controls decreased with the length of experiment, whereas those of the positive controls increased. Carlsson and Sherman (8) found the concentration in the liver 10 to 20 times greater than in the skeletal muscles. Information upon the riboflavin content of other kinds of liver than beef and pork

seems to be lacking.

As to measurements of riboflavin values, they are usually expressed in Sherman-Bourquin units (9, 10). A Sherman-Bourquin unit may be defined as that amount of test food which when fed to a standard test animal previously depleted according to the prescribed technique, will promote over a period of 8 weeks an average gain of 3 grams per week (7). The amount of riboflavin which the Sherman-Bourquin unit represents has been estimated to be from 2 to 5 micrograms (25). Bessey (3) more recently indicated that a Sherman-Bourquin unit is 2γ or 2.5γ (micrograms) of riboflavin or that there are 400,000 to 500,000 Sherman-Bourquin units per gram of riboflavin.

The chief causes of this variation, probably, so far as present knowledge suggests, are differences in purity of previously available preparations of riboflavin, variations in riboflavin requirements with the size of the test animal, completeness of prevention of coprophagy, and possibly the influence of some less well defined difference in the nutritional background of the test animal (25).

PROCEDURE

Livers for these experiments were purchased in August, September, October, and again in December, 1936 at local retail markets which had secured their meats from packing plants in Kansas City.

Nine samples of liver were purchased. The beef, calf, pork, and lamb livers for the first series of analyses were bought in the early fall months. The same kinds of livers were used in the second series of experiments, and mutton was also included. These samples were purchased in the early winter. In each case, the liver samples represented two to six animals.

The livers were prepared for sampling by removal of the gristle and as much of the blood vessel walls as possible. They were then ground through a food chopper with a fine blade, next carefully mixed in a large container and again ground and mixed. The prepared liver was packed into fruit jars with glass covers, frozen, and stored below 0° C. in the freezing room of a local packing plant. Small amounts of liver were removed (from time to time) as needed for feeding experiments. These were kept frozen in the

freezing compartment in a refrigerator in the laboratory until needed.

The Sherman-Bourquin (7) biological method was used for the riboflavin determinations. Booher, Blodgett, and Page (6) and also Bisbey and Sherman (4), and Bessey (3) have validated this method of assay as a reliable one for testing quantitatively the flavin factor in food. Albino rats of the Wistar Institute strain were used for the tests. The stock rats were fed ad libitum the usual stock diet suggested by Sherman and Crocker (24) as follows:

Dried whole milk . .	1 part
Ground whole wheat .	2 parts
Sodium chloride . .	2 per cent of weight of wheat

Five young normally growing rats 21 days old and weighing 38 to 40 grams each were placed in each cage, which had a raised wire bottom. They were given water ad libitum and the Bourquin-Sherman (7) vitamin G-free diet as follows:

	per cent
Extracted casein .	18
Cornstarch . .	68
Butterfat . .	8
Cod liver oil .	2
Osborne and Mendel salt mixture .	4
	100

Adding the extract of 50 grams of whole wheat to each 100

grams of diet assured adequate amounts of B_1. The whole wheat extract which supplied B_1 was prepared by washing whole wheat with 80 per cent alcohol for 3 hours, filtering, distilling the filtrate and air drying it over cornstarch according to the Sherman and Bourquin procedure (7). The method suggested by Ellis (11) was used to prepare the riboflavin-free casein. This was made by soaking casein in 60 per cent alcohol for 24 hours, washing, filtering, and drying the casein. The Osborne and Mendel salt was made by the short-cut method suggested by Wesson (27). The butterfat was melted and filtered at 45° to 55° C.

The animals were kept on the Sherman-Bourquin vitamin G-free diet for one week to deplete them of body stores of riboflavin. They were then weighed and placed in separate cages with raised bottoms. The rats were weighed daily until the weight remained stationary for three days or showed a decrease after two days of constant weight. This indicated that the body store of riboflavin was depleted and that the animal was ready for experimentation. The average weight was 48 grams at the end of the depletion period with a range of 40 to 56 grams. Fourteen days was the average period required for depletion.

Having been depleted of riboflavin, the animals were

divided into 12 experimental groups; nine of them to be fed the test livers, one to be used for a negative control group, and the remaining two for positive controls. At least five males and five females were included in each group. During the eight-week experimental period, each animal continued to receive the riboflavin-free diet and water ad libitum.

The samples of the nine different kinds of liver to be tested were weighed on an analytical balance and fed to the rats in small individual containers. Each animal received two feedings of 200 milligrams each, twice weekly. The animals liked the liver and consumed it within a few seconds with no perceptible waste.

Positive and negative control groups included a rat from each of the litters used in the experiment. The animals of the negative control group received only the G-free diet and water. The positive control groups received in addition to the basal diet supplements of purified riboflavin (lactoflavin, PX grade). This crystalline material was secured from the Biological and Chemical Laboratories of the Borden Company Research Division. A solution was made containing 5 mg. of the material dissolved in 500 ml. of distilled water. It was fed by having the animals lick

drops from the tip of a one ml. syringe graduated in hundredths. The riboflavin was administered to the positive controls at two levels, 2.5 micrograms and 5 micrograms, daily, seven days a week.

During the test period, the animals were weighed each week and records were made of the amount of food consumed. Food which fell on the floor of the cage but was not contaminated was returned to the cup. Observations were made upon the activity and appearance of an animal at the end of the experimental period and any unusual behavior during the test period was recorded. Any animal suspected of coprophagy was removed from the experiment.

Composite growth curves were plotted from the average weekly weight records of the animals in any one group and estimates of the riboflavin content of the liver were made by comparison of the growth curves of the animals receiving the liver with those of the animals receiving the purified riboflavin (lactoflavin, PX grade).

Table 1. Summary of data from riboflavin rat feeding experiments.

Experimental groups	Daily portion fed (mg.)	Number rats	Average depletion period (days)	Average initial weight (gm.)	Average weight in grams during test period week 1	2	3	4	5	6	7	8	Average for 8-week period: Gain in weight (gm.)	Diet consumed (gm.)
Beef liver fall	66.7	12	14	46	49	52	54	54	55	56	57	59	13	301
Beef liver winter	66.7	8	14	47	50	53	56	58	61	62	63	63	16	209
Calf liver fall	66.7	10	14	46	50	54	55	57	58	58	58	61	15	262
Calf liver winter	66.7	10	16	50	54	59	62	66	67	69	70	71	20	255
Pork liver fall	66.7	15	14	47	50	53	55	56	57	58	59	58	12	283
Pork liver winter	66.7	11	15	48	50	53	54	56	56	57	59	61	12	213
Lamb liver fall	66.7	15	14	45	50	55	58	60	63	63	65	67	22	287
Lamb liver winter	66.7	10	13	47	51	53	55	58	60	65	69	71	24	219
Mutton winter	66.7	10	16	49	52	56	58	61	62	64	67	69	20	225
Negative controls	0 micrograms	16	15	50	50	50	51	51	50	48	48	49	-1	182
Riboflavin	2.5	10	13	46	50	52	55	57	60	62	63	63	17	235
Riboflavin	5.0	9	14	49	53	60	65	67	72	75	80	82	33	343

Table 2. Comparison in grams of gain in weight with amount of food consumed.

Experimental group	Average gain in weight	Average diet consumed
Pork liver, winter	12	213
Pork liver, fall	12	283
Beef liver, fall	13	301
Calf liver, fall	15	262
Beef liver, winter	16	209
Mutton liver	20	225
Calf liver, winter	20	255
Lamb liver, fall	22	287
Lamb liver, winter	24	219
Negative controls	-1	182
Riboflavin 2.5 mg.	17	235
Riboflavin 5.0 mg.	33	343

Table 3. Riboflavin value of other foods compared with livers used in this study.

	Riboflavin values in Sherman-Bourquin units per 100 gm.
Fruits and vegetables	
Peas, fresh	50- 100
Pecans	100
Beans, lima, fresh	100
Peaches, dried	90- 140
Spinach	100- 150
Peanuts	200
Kale	140- 220
Chickpea, dried	250
Prunes, dried	260
Broccoli	275
Turnip greens	300
Squash	325
Soybean	900
Food of animal origin	
Milk, average	60
Salmon	80
Milk, whole	30- 100
Egg, white	60- 120
Pork, lean	125
Beef, lean	60- 130
Egg, whole	100- 150
Cheese cheddar	140- 280
Egg, yolk	150- 300
Milk, whole, dried	500
Milk, skim, dried	630
Liver	800-1200
Test livers, average	
Pork	620-1560
Beef	750-1880
Calf	915-2270
Mutton	1030-2590
Lamb	1195-2985
Mean for all test livers	900-2255

Table 4. Riboflavin value of foods tested.

	Sherman-Bourquin units per 100 gm.
Pork liver, winter	620-1560
Pork liver, fall	620-1560
Beef liver, fall	665-1685
Calf liver, fall	800-1945
Beef liver, winter	830-2080
Mutton liver	1030-2590
Calf liver, winter	1030-2590
Lamb liver, fall	1140-2850
Lamb liver, winter	1250-3120
Mean for all test livers	900-2255
Riboflavin 2.5 mg.	50,000,000
Riboflavin 5.0 mg.	48,910,700

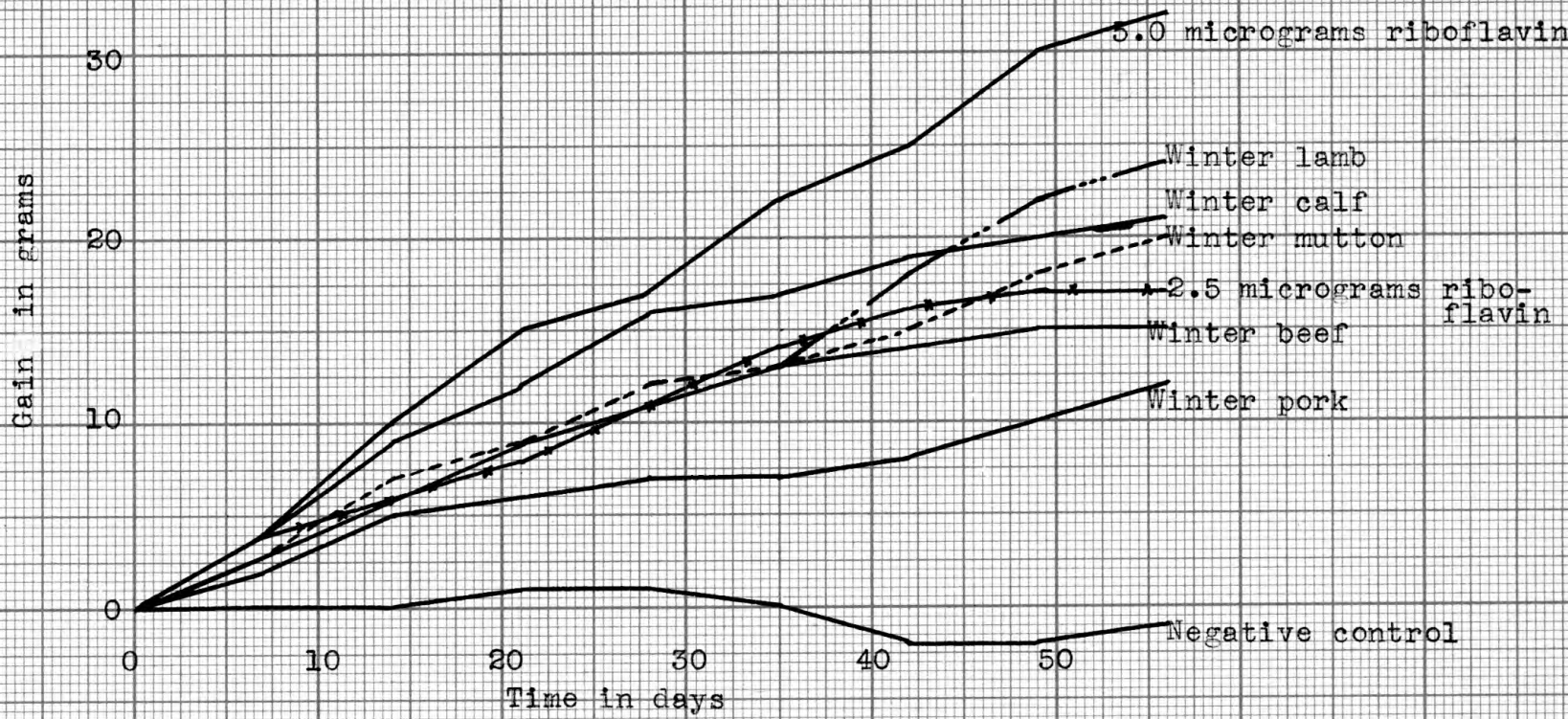

Fig. 1. Average gain curves for rats on riboflavin experiments.

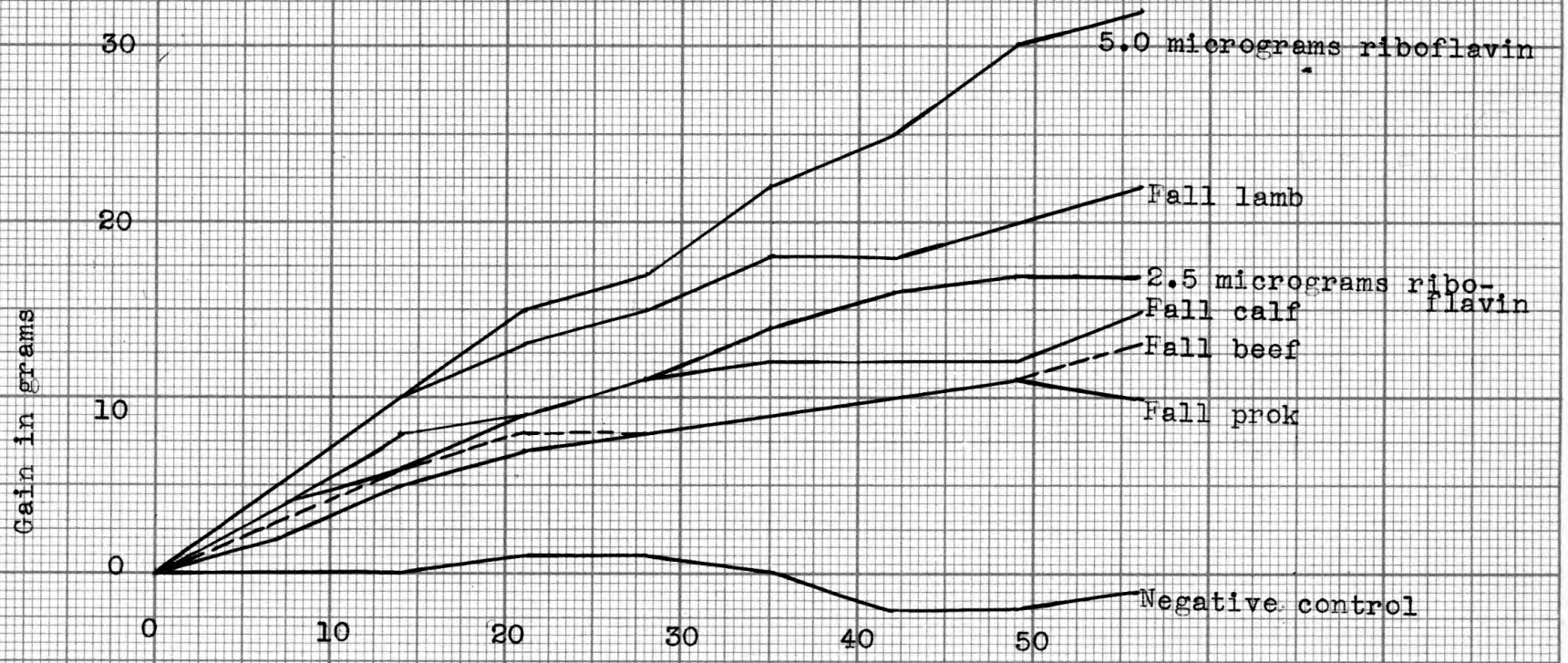

Fig. 2. Average gain curves for rats on riboflavin experiments.

FINDINGS

A summary of the data from the riboflavin rat feeding experiments is given in Table 1. All but two of the groups of rats were composed of ten or more animals. The winter beef groups included eight, and the riboflavin group fed at the 5 mg. level numbered nine. The animals were placed on the Sherman-Bourquin (7) vitamin G-free diet when they reached an approximate weight of 38 to 40 grams at 21 days of age which was most satisfactory weight and age to use. Rats 28 to 29 days' old, having an average weight of 49.5 grams were used by Todhunter (26) and Roscoe (22) because dietary deficiencies become the most apparent during the early growing period. Rats 3 weeks old have been found more satisfactory than 4-week ones by workers in the Food Economics and Nutrition Laboratory of the Kansas State College of Agriculture and Applied Science. These findings are in accord with those of Lassen (18). The average length of time required for the depletion period was 14 days. At the end of the depletion period, the average weight was 48 grams, with a range of 40 to 56 grams.

At the end of the 8-week period, the test animals showed a gain of 12 to 24 grams of weight. The negative control animals lost 1 gram of weight. The control group fed on the level of 2.5 micrograms riboflavin gained 17 grams whereas the control group fed 5.0 micrograms of riboflavin daily gained 33 grams. These are the levels of riboflavin that have been used for positive control groups by other workers: Kunerth et al. (17), Kramer et al. (15), and Ansbacker et al. (1). The gains of these control groups compared favorably with those of Ansbacker's experimental animals (1) in that the more lactoflavin fed the greater was the weight of the animals. The negative controls were satisfactory in that they maintained approximately the same average weight throughout the experiment.

The food consumption of the test animals was considerably greater than that of the negative controls. It approximated that of the positive controls fed on the 2.5 mg. level of riboflavin, and was noticeably lower than that of the 5 mg. riboflavin level.

There is a question as to whether the increased food consumption in the test animals and in those of the positive control groups is due to the increased riboflavin in the diet or to the higher state of nutrition of the animals.

The positive control groups and the test animals fed livers containing highest riboflavin content had bright eyes, glossy fur, good muscular tone, and a normal appetite. They were vigorous in their activity, and seemed to be satisfied. The test animals fed pork liver as a source of riboflavin were vicious, restless, and lighter in weight, with shaggy fur and dull eyes. The negative control group lacked appetite and good muscular tone, and were light in weight; their general appearance was extremely poor, as the fur was rough, the eyes listless, the posture extremely hump-backed, and movements very sluggish.

The rats fed on the liver samples collected in the winter showed slightly larger gains, averaging 2.5 grams more than those fed on livers collected in the fall. This finding, however, is of slight importance as the type of food consumed by the animals from which the livers were taken is unknown; and between collection of samples there was only a three-month interval, which could almost, in this case, be considered the beginning and close of the fall season. The lamb liver showed the greatest potentiality for gain while mutton was second; calf was third; beef was fourth; and pork lowest as shown in Table 1 and Figs. 1 and 2.

The conversion factor of 2 to 5 micrograms to 1 Sherman-Bourquin unit as suggested by Bessey (3) was used in converting the Sherman-Bourquin units to riboflavin given in Table 2. Daniel and Munsell (10) reported calf liver as containing 800-1000 units and beef liver as 1000 units. These determinations are not as low as our lower values, 665 Sherman-Bourquin units, and are much below our highest ones, 2590 Sherman-Bourquin units (Table 4). Lunde, Kringstad, and Olsen (19) reported 400-1200 units of riboflavin per 100 grams fish liver, which is lower than any of the livers tested in this experiment (Table 4). Chaney and Ahlborn (9) gave liver riboflavin values as 400-1200 Sherman-Bourquin units per 100 grams liver (the kind of liver unknown). In this experiment, the test livers averaged 900-2255 Sherman-Bourquin units for 100 grams, a figure much higher than that given by Chaney and Ahlborn (9).

The riboflavin content of liver of this study is approximately 10 to 20 times the riboflavin content of most of the other food with which it was compared (Table 3). Most of the foods showed a riboflavin value of 50 to 300 Sherman-Bourquin units per 100 grams while the mean for the test livers was 900 to 2255. Soybeans ranked highest of the

foods compared with liver with a value of 900 Sherman-Bourquin units. Whole milk had a riboflavin value of 30 to 100 which is much lower than the mean of the test livers. However, milk will no doubt be considered our best source of riboflavin since the amount produced can be increased without increased cost to the consumer. This would not be true with liver.

SUMMARY AND CONCLUSIONS

1. Samples of beef, calf, lamb, and pork livers were purchased in the early fall, then again in early winter with the addition of mutton livers. Each of the five liver samples represented two to six animals. The livers were finely ground and mixed, reground and then again mixed. The samples were frozen and kept below 0° C., then removed as needed for experiment and kept in a refrigerator freezing compartment until fed.

2. The biological assay for riboflavin was made according to the procedure of Bourquin and Sherman. Pure riboflavin (lactoflavin, PX grade) was fed in addition to the basal diet to a group of rats that served as positive controls at two levels: 2.5 and 5.0 micrograms daily. The

G-free diet only was fed to a group of rats that served as negative controls. The riboflavin content of the livers was figured on the basis of 2 to 5 micrograms per Bourquin-Sherman unit.

3. The riboflavin content of the liver samples purchased in the winter season was slightly higher than that of the samples purchased in the fall. The lamb liver was highest in riboflavin value while mutton ranked second; calf was third; beef was fourth; and pork was lowest. The average riboflavin content for all livers was 900-2255 Sherman-Bourquin units, which is much higher than the highest range (400-1200 Sherman-Bourquin units) of previous studies.

4. The mean for the test livers in this experiment was approximately 10 to 20 times higher in riboflavin value than the fruits and vegetables, milk, legumes, eggs, cheese, and salmon with which it was compared.

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