# THE RIBOFLAVIN OF MILK SAMPLES COLLECTED UNDER TWO FEEDING CONDITIONS FROM THREE BREEDS OF COWS

by

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# INTRODUCTION

Previous studies reported from this laboratory on the vitamin G (riboflavin) content of milk indicated the desirability of a further and a more carefully controlled investigation of this factor in milk. The vitamin A and D values of milk are known to be influenced by the ration the cow receives. While a similar tendency has been inferred to exist with reference to vitamin G, the evidence on this point is not conclusive and requires further investigation. The present study deals with the riboflavin content of milk collected from three breeds of cows with special reference to ration, breed, and stage of lactation.

# REVIEW OF LITERATURE

Since the meeting of the American Society of Biological Chemists in 1929, at which it was decided to retain the term vitamin B for the heat-labile, antineuritic fraction and adopt the term vitamin G for the heat-stable, growth-promoting fraction, continued investigation of this heat-stable factor has proved it to be multiple in nature.

There has been much confusion regarding nomenclature because the different phases now known have been separated and described within a short period in a number of different laboratories. Elvehjem (13) suggested that the term vitamin $B_2$ be used for the antipellagra factor and that the flavins be reclassified. Gyorgy (15) suggested a further division of vitamin $B_2$ into flavins, the growth factor, and vitamin $B_6$, the pellagra-preventing factor. Chick, Copping, and Edgar (7) found lactoflavin to have no connection with the pellagra-like dermatitis in rats. Jansen (20) proposed to omit letters in the description of vitamins and designate vitamin G as flavin. As the term flavin represents a group of substances with similar structure but includes many compounds which do not have the vitamin activity, the term riboflavin, indicating a ribose derivative of isoalloxazine, was suggested by the committee of vitamin standards of the American Society of Biological Chemists in 1937 (25) to replace the terms vitamin G and lactoflavin. Lactoflavin had been used to indicate this factor as isolated from milk, and in a similar way heptoflavin and ovoflavin have been used. As lactoflavin, heptoflavin, and ovoflavin have been found to be identical with riboflavin, the latter term can be used to replace them all.

In addition to the flavins, the vitamin $B_2$ complex is now known to contain a growth-promoting factor, vitamin H or $B_6$, recently concentrated from whey by Booher (4) and a pellagra-preventive factor now thought to be nicotinic acid (8) (14).

The growing complexity of the vitamin B factors has raised the question as to the validity of the biological method for measuring the vitamin G developed by Bourquin and Sherman (6). However, Booher, Blodgett, and Page (5) and Bisbey and Sherman (3) have confirmed the accuracy of this method for measuring vitamin G (riboflavin) values of food-stuffs. More recently Bessey (2) has presented evidence that the Bourquin-Sherman method (6) actually measures riboflavin values.

Various workers (24) have demonstrated the presence of riboflavin in a wide variety of foods, including milk of different species, liver, kidney, fish eyes, malt, grass, dandelion flowers, fruits, and vegetables. Milk in its various forms has been tested probably more than any other one food for riboflavin content. Sherman (26) says that milk is and will continue to be our most valuable source of riboflavin because of its prominence in the diet and its other nutritional values. Todhunter (29) made a

comparative study of vitamin G content of diluted evaporated milk and pasteurized milk and concluded that they are equally valuable sources.

Some investigations have been made concerning the factors which affect the riboflavin value of milk. Hunt and Krauss (18) in a study of the influence of the ration of cows upon the vitamin G values of milk found that fresh green grass was a better source of vitamin G than overmature grass. In 1935, Hunt, Record, and Bethke (19) reported further that, as the alfalfa and timothy hays matured, the vitamin G content decreased. MacLeod, Brodie, and Maclean (23) found little difference in the vitamin G content of milk collected at different seasons from stall-fed cows. Studies made by Dickman (10) on the riboflavin content of milk at the end of the abnormally hot, dry summer of 1936 when no pasture was available gave riboflavin values approximately one-fourth lower than those collected from the same cows after they had access to good pastures as a result of fall rains. From this work it would appear that pasture supplement might be responsible for increasing the riboflavin content of milk. Sherman and Lanford (27) in reviewing the information relative to the effect of the ration of cows on the riboflavin content state that

the vitamin value of milk as ordinarily produced under present day dairy conditions probably is little affected by seasonal variations.

Kramer and associates (21) found colostrum to have a higher vitamin G value than milk collected from the same cows after one month of lactation. Whitnah, Kunerth, and Kramer (32) report that milk from cows between 15 days and 10 months after freshening, showed no significant difference in flavin content.

Riboflavin values have been frequently expressed in Bourquin-Sherman units, or that amount of test food which, when fed to a standard test animal, will result in a gain of 3 grams per week during the test period. Day and Darby (9) have reported that the feeding of 4 micrograms of riboflavin to a test animal daily for a period of eight weeks produced weight gains equal to those of animals receiving a Bourquin-Sherman unit daily. More recently, Bessey (2) indicated that a Bourquin-Sherman unit is 2$\gamma$ or 2.5$\gamma$ (micrograms) riboflavin or that there are 400,000 to 500,000 Bourquin units per gram of riboflavin.

# PROCEDURE

The samples of milk used in this experiment were obtained from cows in the college dairy herd. The milk from each cow was collected and a portion of each milking, based on the weight, was saved and pooled for bioassay. Composite samples for the different breed groups were made by pooling proportional aliquot quantities of milk from 4 individual cows of each of the Guernsey, Holstein, and Jersey breeds. The average stage of lactation for the Guernseys at the time of collecting the first sample was 4 months, with a range of 3 to 5 months. For the Holsteins it was 4.5, with a range of 2.5 to 7 months; and for the Jerseys, 4 months with a range of 4 to 5 months. Samples were first collected at the close of the winter feeding period when the cows were on the regular winter ration of alfalfa hay (No. 2 leafy grade), Atlas Sorgo silage, and a grain mixture consisting of yellow corn, bran, oats, cottonseed meal, and linseed meal. Similar samples were collected from the same cows after they had been on good pasture for about three weeks.

All composite samples were frozen and held below 0° C.

until ready to use. Grayson (16) found that no deterioration of the vitamin G (riboflavin) occurs when samples are kept in a frozen condition. Amounts sufficient for a feeding were thawed as needed.

Albino rats from the Wistar stock were used in this study. The stock rats were fed the usual stock diet suggested by Sherman and Crocker:

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

When the young, growing rats reached 38 to 40 grams, groups of five were placed in cages with raised wire bottoms and given the Bourquin-Sherman vitamin G-free diet and water ad libitum.

| | grams |
|---|---|
| Extracted casein | 18 |
| Osborne and Mendel salt | 4 |
| Cod liver oil | 1 (or 2) |
| Butter fat | 9 (or 8) |
| Cornstarch (wheat extract) | 68 |

The starch containing the alcoholic extract of whole wheat was made according to the method outlined by Bourquin. This extract supplies adequate amounts of vitamin B when 50 grams of whole wheat is incorporated into each 100 grams

of diet. The method suggested by Ellis (12) was used to extract the vitamin G-free casein. Osborne and Mendel salt mixture was prepared by the short cut method proposed by Wesson (30). Water and curd were removed from the butterfat by filtering at 45 to 50° C. in a hot water filter.

After the animals had been on this diet for one week, they were weighed and placed in separate cages. They were checked daily, and if any animal was suspected of coprophagy, it was discarded. The rats were weighed every day until the weight remained stationary for three days or showed a decrease after two days of constant weight. This was taken to indicate that the body store of vitamin G was depleted and that the animal was ready for experiment. The average weight at the end of the depletion period was 49 grams with a range from 43 to 58 grams.

At the end of this fore-period, which averaged 15 days, the animals were carefully divided into six comparable groups according to their weight, sex, and litter. At least 10 rats to each group, 5 males and 5 females, were then placed on the experimental diet for 8 weeks. Each group received a different milk supplement. The portions of milk supplements were fed at the rate of 3 grams of milk daily, 6 days per week during the 8-week test period. A pipette was used to measure the milk, as this method was

more convenient than weighing and sufficiently accurate. Double portions, or 5.8 ml. (6 grams) of milk were given regularly three times per week.

The rats were weighed each week and the weights recorded. The amount of vitamin G-free diet consumed was weighed and recorded. Food which fell on the floor of the cage, but was uncontaminated, was recovered by passing it through a fine wire sieve and returning it to the cup. Animals were observed daily and notation made concerning their appearance or activity. Unusual weather conditions were also recorded.

Positive and negative control groups provided checks for the experiment. Each of the control groups contained a rat from each of the litters used in the experiment. The negative group received no other food than the vitamin G-free diet. In addition to the basal diet the positive controls received a supplement 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 redistilled water. It was fed by having animals lick drops from the tip of a one ml. syringe graduated in hundredths.

The supplement was administered at 2 levels, 2.5 micrograms and 5 micrograms, 6 days per week.

The milk samples were also assayed fluorimetrically, using the rapid method for determination of lactoflavin in milk developed by Whitnah, Kunerth, and Kramer (31). This method is based upon the fluorescent properties of riboflavin described by Supplee and associates (28). Fifteen ml. of 10 per cent trichloracetic acid was added to 10 ml. of milk. After standing for thirty to sixty minutes, the mixture was centrifuged five minutes at about 2000 r.c.f. per minute. A 10 ml. portion of the resulting serum was neutralized, with methyl orange as an indicator, and the diluted samples were matched in the light of an Eveready Fluoray Lamp with standard flavin solutions (Labco PX grade) containing 0.12 to 0.06 microgram of flavin per ml. Usually three people estimated the riboflavin content of the milk samples as judged by the fluorescence of the serum. Samples containing less than 4 $\gamma$ per ml. produced fluorescence which was too weak to be measured accurately and concentration of 12 micrograms or more gave intensities too great. Readings from 7 to 11 $\gamma$ were most satisfactory for comparison with standards. Solutions were diluted until readings were within the best range. Duplicate samples were always prepared, and the riboflavin content was calculated according to the dilutions necessary.

Table 1. Data concerning milk yields of cows used in experiment

| Source of Sample | Milk Yields Collected on 4-14-'37 | | | Amount Retained | Milk Yields Collected on 5-12-'37 | | | Amount Retained |
|---|---|---|---|---|---|---|---|---|
| | A.M. | M. | P.M. | Total | A.M. | M. | P.M. | Total |
| | Pounds | Pounds | Pounds | Pounds | Pounds | Pounds | Pounds | Pounds |
| Guernsey cows | | | | | | | | |
| Carnation | 5.8 | - | 7.3 | 13.1 | 7.8 | - | 8.9 | 16.7 |
| Cherry Blossom | 8.3 | - | 9.4 | 17.7 | 9.9 | - | 9.6 | 19.5 |
| Elderberry | 10.4 | - | 10.9 | 21.3 | 10.8 | - | 12.2 | 23.0 |
| Morning Glory | 10.5 | - | 10.8 | 21.3 | 11.3 | - | 8.9 | 20.2 |
| Average | | | | 18.4 | | | | 19.9 |
| Holstein cows | | | | | | | | |
| Coral | 25.6 | - | 26.8 | 52.4 | 24.1 | - | 22.0 | 46.1 |
| Ella | 23.8 | - | 25.5 | 49.3 | 22.3 | - | 24.8 | 47.1 |
| Iola | 8.5 | 10.7 | 10.2 | 29.4 | 8.0 | 12.7 | 10.7 | 31.4 |
| Isabel | 23.5 | - | 26.9 | 50.4 | 21.3 | - | 24.6 | 45.9 |
| Average | | | | 45.4 | | | | 37.6 |
| Jersey cows | | | | | | | | |
| Tesse | 8.3 | - | 13.7 | 22.0 | 8.0 | - | 11.3 | 19.3 |
| Tempest | 10.4 | - | 10.6 | 21.0 | 9.2 | - | 10.5 | 19.7 |
| Tickler | 7.6 | 7.1 | 6.2 | 20.9 | 6.6 | 6.7 | 6.0 | 19.3 |
| Tint | 9.3 | 10.6 | 9.4 | 29.3 | 10.2 | 10.4 | 9.7 | 30.3 |
| Average | | | | 23.3 | | | | 22.2 |

Table 2. Data concerning composite milk samples

| Source of Sample | Date of Collection | | | | |
|---|---|---|---|---|---|
| | 4-14-1937 | | | 5-12-1937 | |
| | Average Stage of Lactation | Fat | Total Solids | Fat | Total Solids |
| | Month | Per Cent | Per Cent | Per Cent | Per Cent |
| Guernsey composite | 4 | 4.99 | 14.21 | 5.17 | 14.50 |
| Holstein composite | 4.5 | 3.21 | 11.54 | 3.30 | 11.80 |
| Jersey composite | 4 | 5.20 | 14.67 | 5.09 | 14.53 |

Table 3. Summary of data from rat feeding experiment

| Milk Sample | Daily Portion Fed | Number of Rats | Average Depletion Period | Average Initial Weight | Average Weekly Weights During Test Period | | | | | | | | Average for 8-Week Period | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Gain in Weight | Diet Consumed |
| | Grams | | Days | Grams | | | | | | | | | Grams | Grams |
| Guernsey Herd ration | 3.0 | 10 | 15.0 | 49 | 57 | 64 | 69 | 77 | 81 | 85 | 89 | 93 | 44 | 248.7 |
| Guernsey Herd ration + pasture | 3.0 | 10 | 14.5 | 50 | 57 | 64 | 69 | 76 | 80 | 83 | 84 | 89 | 40 | 258.8 |
| Holstein Herd ration | 3.0 | 10 | 15.7 | 49 | 55 | 60 | 64 | 68 | 69 | 72 | 75 | 80 | 31 | 227.6 |
| Holstein Herd ration + pasture | 3.0 | 11 | 15.0 | 49 | 55 | 60 | 65 | 69 | 73 | 76 | 78 | 80 | 32 | 233.6 |
| Jersey Herd ration | 3.0 | 10 | 15.4 | 48 | 54 | 61 | 67 | 74 | 77 | 80 | 84 | 88 | 40 | 252.5 |
| Jersey Herd ration + pasture | 3.0 | 10 | 15.0 | 50 | 59 | 66 | 72 | 77 | 83 | 86 | 90 | 95 | 45 | 266.6 |
| Negative controls | - | 16 | 14.8 | 50 | 50 | 50 | 51 | 51 | 50 | 48 | 48 | 48 | -1 | 181.6 |
| | Micrograms | | | | | | | | | | | | | |
| Lactoflavin | 2.5 | 10 | 13.0 | 46 | 50 | 52 | 55 | 57 | 60 | 62 | 63 | 63 | 17 | 235.0 |
| Lactoflavin | 5.0 | 9 | 14.0 | 49 | 53 | 60 | 65 | 67 | 72 | 75 | 80 | 82 | 33 | 343.0 |

Table 4. Riboflavin of milk tested

| Milk Sample | Biological Method | | | | | Fluorimetric Method | | | Average Milk Yield per Cow per Day | Average Riboflavin Yield per Cow per Day |
|---|---|---|---|---|---|---|---|---|---|---|
| | Daily Portion Fed | Number of Rats | Average Gain for 8 Weeks | Estimated Riboflavin Per Portion | Riboflavin Per Gram Milk | Riboflavin per Gram Milk Fluoray Lamp | Differences from Biological Value per Gram of Milk | | | |
| | Gram | | Gram | Microgram | Microgram | Microgram | Microgram | Per Cent | Gram | 1000 Micrograms |
| Guernsey Herd ration | 3.0 | 10 | 44 | 6.6 | 2.2 | 1.9 | -.3 | 13.6 | 8,346 | 18 |
| Guernsey Herd ration + pasture | 3.0 | 10 | 40 | 6.0 | 2.0 | 1.9 | -.1 | 5.0 | 9,027 | 18 |
| Holstein Herd ration | 3.0 | 10 | 31 | 4.8 | 1.6 | 1.9 | +.3 | 18.75 | 20,593 | 33 |
| Holstein Herd ration + pasture | 3.0 | 11 | 32 | 4.8 | 1.6 | 1.5 | -.1 | 6.25 | 17,055 | 27 |
| Jersey Herd ration | 3.0 | 10 | 40 | 6.0 | 2.0 | 2.2 | +.2 | 10.0 | 10,569 | 21 |
| Jersey Herd ration + pasture | 3.0 | 10 | 45 | 6.8 | 2.3 | 2.1 | -.2 | 8.7 | 10,070 | 23 |
| | Microgram | | | | | | | | | |
| Lactoflavin | 2.5 | 10 | 17 | - | - | - | - | - | - | - |
| Lactoflavin | 5.0 | 9 | 33 | - | - | - | - | - | - | - |

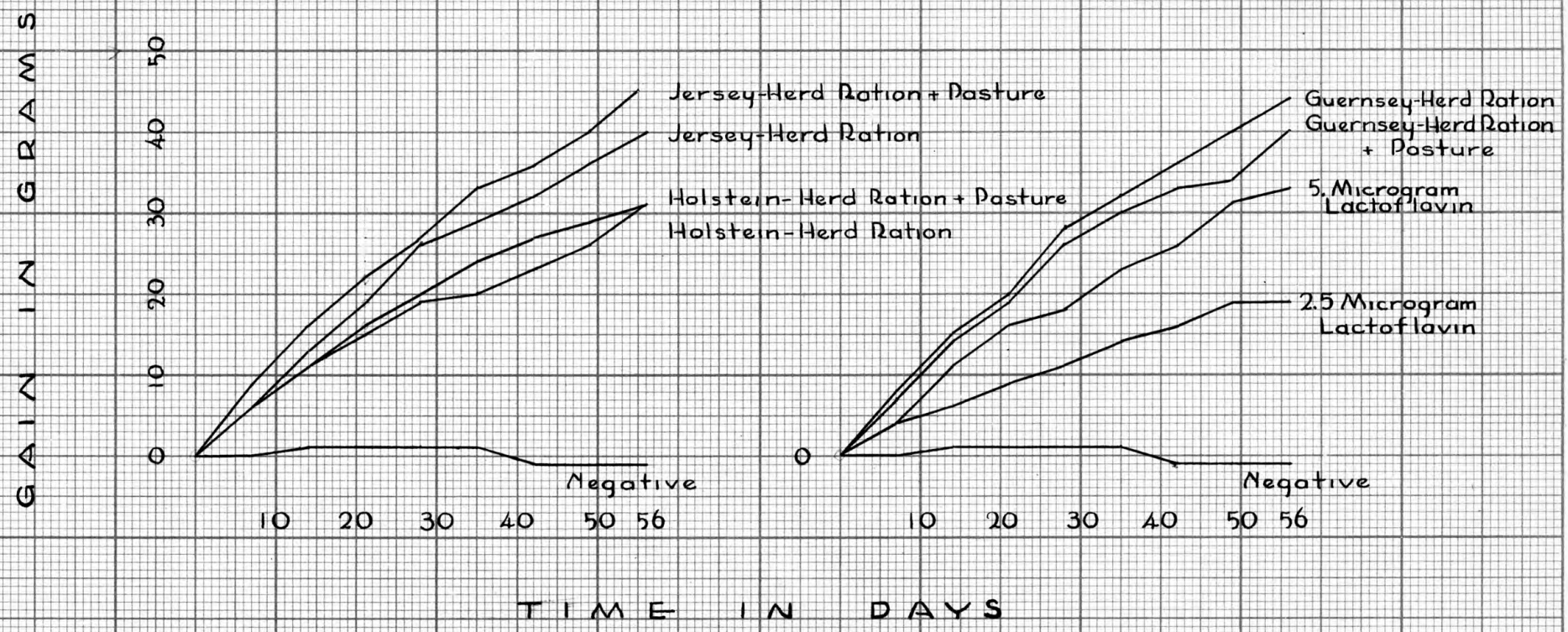


FIG. 1 – AVERAGE GAIN CURVES FOR EXPERIMENTAL RATS

# DISCUSSION

Tables 1 and 2 present the data concerning the samples of milk used in this experiment. Some of the cows were milked more than twice daily as indicated in Table 1. The yield per milking in pounds is given for the individual cows in each collection period. The average fat percentage and total solids content of the different samples are reported in Table 2. A summary of the data from the rat feeding experiment is given in Table 3. The rats were placed on the Bourquin-Sherman vitamin G-free diet when they reached an approximate weight of 38 to 40 grams.

The previous use of 4-week old rats in this laboratory has been thought less satisfactory than the use of younger, smaller animals. Todhunter (29) used rats 28 to 29 days old having an average weight of 49.5 grams because it is during the early growing period that deficiencies in the diet of the rats become most apparent. Lassen (22), in discussing the suitability of rats of various ages, preferred those aged 3 weeks because variation in size at this age seemed to cause less variation in subsequent response. For this reason rats weighing 38 to 40 grams were employed.

There was satisfactory uniformity in development among the groups receiving the supplement of the different milks. The average depletion period was 15 days and ranged from 11 to 21 days. The average weight at the time of beginning the milk supplements was 49 grams and ranged from 43 to 58 grams. The animals receiving the supplement of the different milks showed good gains throughout the experiment, as indicated in Figure 1.

The average gains for the two groups of rats receiving the Holstein milk composite were the same at the end of the 8-week experimental period. However, for the 6-week period beginning at the end of the 2nd week, the milk collected after the cows had received pasture supplements, appeared to favor better growth, as indicated by the growth curves. Toward the end of the experimental period the difference in the weights became less.

The greatest difference that might be attributed to the effects of pasture on the riboflavin content of milk was noticed in the rats receiving the Jersey sample. The rats receiving the milk from the cows (Jersey) on pasture grew better consistently throughout the entire length of the experiment than the animals receiving the milk collected one month previously when the cows were on the

winter ration.

The growth records of the 2 groups of rats receiving the Guernsey milk composites were similar during the first half of the experimental period. Later, the group receiving the milk collected from the cows on the winter herd ration continued to grow at about the same rate. The other group of rats receiving the milk from the same Guernsey cows collected after the cattle had access to pasture gained at a slower and more erratic rate and at the end of the experiment were noticeably smaller. In other respects they appeared to be normal. This group may, perhaps, have had some significant abnormality, for in a previous study by Whitnah (31) the group of Guernsey cows showed unusual behavior in regard to riboflavin values as well as other constituents of milk collected in different months in the spring of 1937. No explanation was given for the variations observed with this breed.

The positive control groups of rats receiving the 2 levels, 2.5 micrograms and 5 micrograms of riboflavin (lactoflavin, PX grade) showed total gains comparable to the proportion of riboflavin received, or 17 and 33 grams respectively, for the 8-week period. The curve of the lower lactoflavin level showed a tendency to flatten during

the last 2 weeks of the experimental period. However, the lactoflavin curves appear reliable for reference because the growth response was very nearly in direct proportion to the amount fed. Also, these results are in agreement with those of Ansbacher, Supplee, and Bender (1) who reported that growth response diminished as the supplements became larger or more potent. The negative control group shows a smooth curve with an average weight variance of 2 grams during the 8-week experimental period. The average weight of the group at the end of the experiment was only slightly less than the weight at the end of the fore-period.

Since only those animals fed on milk from Jersey cows on pasture showed consistently larger gains, while the Holsteins showed no difference and the Guernseys showed actually lower weights, the conclusions of this study do not confirm the results reported by Dickman in the study of cows transferred from stall-feeding to fall pasture in 1936. It is difficult to explain the inconsistent results reported in this study when compared to those of Dickman (10) in which a consistent rise of riboflavin content of milk was found in all groups following the supplement of the herd ration with pasture feeding. Possibly the marked change in climatic conditions in the fall of

1936, when the samples used in Dickman's study were collected, might have been responsible for some part of the rise in riboflavin content. The following spring, when the samples used in this study were collected, no marked climatic changes intervened in the month between collections.

Hunt and Krauss (18) found that milk from cows on pasture had a higher vitamin G content than milk from cows on dry feed. Perhaps the quality of the hay used in dry feeding might have been a determining factor. However, as a result of other research, some workers show that some changes in ration are not reflected in riboflavin content of milk. As a result of feeding summer and winter milk to rats, Henry and Kon (17) found no difference in the growth responses which would indicate the superiority of summer milk.

The Jersey composite from cows on spring pasture gave a riboflavin content of 2.3 micrograms per gram of milk, which was .3 micrograms higher than that of the Jersey samples collected from the cows before pasture. The riboflavin value of milk from the Guernsey cows before pasture was 2.2 micrograms per gram. The riboflavin content of Guernsey milk decreased .2 microgram after pasture. The riboflavin content of the Holstein samples was 1.6 micrograms before and after pasture feeding.

These riboflavin values are in reasonable agreement with those reported by other workers. Todhunter (29) found 2.64 micrograms of vitamin G per gram of diluted evaporated milk and 2.96 micrograms of vitamin G per gram of pastuerized milk. MacLeod, Brodie, and Maclean (23) found milk from stall-fed cows to contain 1.2 micrograms of riboflavin per gram of milk and in 1933 Dutcher, Guerrant, and McKelvey (11) demonstrated that 3 millliliters of milk were sufficient to furnish at least one Sherman unit of vitamin G.

It is doubtful if the stage of lactation affected these results as all groups of cows averaged in approximately the same stage of lactation (Table 1). Kramer (21) and Whitnah (32) have shown that following the first month of lactation there is practically no effect attributable to change in stage of lactation on the riboflavin content of milk. The group of Holstein cows showed the highest total average yield of milk and because of this fact produced the largest average yields of riboflavin, although the riboflavin content per gram of milk was estimated to be less than that for either the Guernsey or Jersey groups.(Table 4).

There was not perfect agreement in measuring the riboflavin content of milk by the 2 methods employed. The

fluorimetric measurements tended to show less riboflavin content than the biological findings; but they are useful in determining the general evaluation. The maximum difference between the biological findings and fluorimetric estimations was ± 18.75 per cent.

## SUMMARY AND CONCLUSIONS

Samples of milk used in this experiment were secured from the college dairy herd in the spring of 1937 before and after the cows had access to pasture. Aliquot composite samples were prepared from twenty-four-hour collections of milk from well-matched groups of Guernsey, Holstein, and Jersey cows, 4 cows of each breed. The composite samples were frozen and kept below 0° C. until studied.

Biological assay according to the Bourquin-Sherman method was employed for riboflavin determinations. Pure riboflavin (lactoflavin, PX grade) was procured and fed in addition to the basal diet to a group of rats that served as positive controls. The same samples of milk were assayed fluorimetrically by the rapid method developed by Whitnah and associates in this laboratory. The riboflavin values of the different milk samples studied were similar

to values previously reported from this laboratory for milk produced after one month of lactation.

The biological assay of milk samples collected from the group of Guernsey cows on the winter herd ration indicated values of 2.2 micrograms per gram of milk. After approximately one month of spring pasture the riboflavin content was 2.0 micrograms per gram. Both composite samples of Holstein milk were estimated biologically to contain 1.6 micrograms of riboflavin per gram of milk. The sample of milk from the group of Jersey cows on the winter herd ration yielded riboflavin values of 2.0 micrograms per gram and after spring pasture supplement, 2.3 micrograms per gram. Of the 3 breeds studied, the Holstein cows, because of their higher daily milk yield, produced greater amounts of riboflavin per cow per day, both before and after receiving pasture supplement.

The rapid fluorimetric method for riboflavin determination, although yielding somewhat lower results in most instances, compares fairly well with the biological method of Bourquin and Sherman. The maximum range of difference between the 2 methods was $\pm$ 18.75 per cent.

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