There is considerable information indicating that the requirement for the B-complex vitamins may be met by microbiological synthesis in the rumen of the bovine species, once the rumen has reached full functional activity (7, 8, 11, 22). There is growing evidence, however, that prior to the full functional development of the rumen a dietary supply of certain of the B-complex vitamins, including riboflavin, is required for adequate nutrition.

The need for a dietary source of riboflavin by the very young calf has been demonstrated by Wiese et al. (23) by feeding a synthetic milk in which riboflavin was lacking and by Warner and Sutton (21) who fed whole milk in which about 96 per cent of the riboflavin had been destroyed by photolysis.

The experiment reported herein was undertaken in order to determine the riboflavin requirement of the dairy calf up to 8 wk. of age.

**EXPERIMENTAL PROCEDURE**

The deficient basal ration used in this study consisted exclusively of photolyzed whole milk supplemented with vitamin A. The general procedure followed for the preparation of the photolyzed milk was similar to the one described by Warner and Sutton (21). The major modification consisted of the use of a specially designed photolyzing chamber fabricated from a 12 x 12 in. cylindrical pyrex jar and a 5 x 13.75 in. pyrex tube. This cylindrical chamber was supported by a base that permitted the mercury vapor lamp to be set upright within the center tube of the cylinder. The mercury vapor lamp, a 400 watt lamp emitting rays longer than 3000 Å, was of the same type as previously used in this laboratory (21). Constant agitation of milk during the photolytic process was effected by bubbling nitrogen gas through it at four equidistant outlets. With this equipment, maximum destruction of riboflavin (approximately 97 per cent) was obtained in batches of 33 to 35 lb. of milk within 4.5 hr.

Fresh milk was obtained from the University Holstein herd at the time of milking and immediately photolyzed. During photolysis, the milk was allowed to reach a temperature of 60 to 65° C. for at least 30 min. in order to control the growth of microorganisms. The temperature of the milk during treatment was controlled by means of an electric fan. The photolyzed milk was stored at 42° F. and fed within a period of 48 to 60 hr.

Riboflavin was determined in untreated and photolyzed milk and in colostrum by the fluorometric method of Hand (4) with a few minor modifications.

In addition to destroying riboflavin, photolysis may destroy other compounds of importance in the nutrition of the young calf. A survey of literature indicated that vitamin A, thiamine and pyridoxine may be sufficiently reduced by
photolysis that some compensation should be made in order that such milk might be used in evaluating the riboflavin requirements of the calf without the development of other nutritional deficiency complications. Previous experiments (21) had indicated the extent of vitamin A destruction. Consequently, the effects of light treatment on thiamine and pyridoxine content of milk were studied. Thiamine in untreated and photolyzed milk was determined by the method of Hodson (5). Pyridoxine was determined by microbiological methods (1, 19). The results are given in table 1. The destruction of thiamine averaged

\[
\text{TABLE 1}
\]

The effects of photolysis on the thiamine and pyridoxine content of milk

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thiamine</th>
<th>Pyridoxine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ/ml.</td>
<td>Loss (%)a</td>
</tr>
<tr>
<td>None</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Photolysis</td>
<td>0.28</td>
<td>12.5</td>
</tr>
<tr>
<td>None</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Photolysis</td>
<td>0.27</td>
<td>9.7</td>
</tr>
</tbody>
</table>

\(a\) In samples in which 97.7% of the riboflavin was destroyed.
\(b\) In samples in which 96.8% of the riboflavin was destroyed.

11.1 per cent in samples of milk in which 97.7 per cent of the riboflavin had been destroyed. Pyridoxine destruction was greater, averaging 64.8 per cent when riboflavin was destroyed to the extent of 96.8 per cent.

The destruction of thiamine was not considered sufficiently great to be a complicating factor in this experiment. In the case of pyridoxine, however, the experimental procedure was modified, as described later in this report, to determine whether the destruction was great enough to produce deficiency symptoms. Vitamin A in the form of an oil concentrate was fed daily at the rate of 20,000 I.U. per calf. The vitamin A oil was first emulsified with a small amount of milk in a Waring blender in order to facilitate mixing with the milk fed. The riboflavin supplement was added to the milk at each feeding in the form of a water solution. Fluorescence readings on the stock solution were taken intermittently and fresh riboflavin solutions were prepared when losses in potency were apparent. Riboflavin was determined in every batch of photolyzed milk and the amounts of riboflavin added at each intake level were adjusted accordingly. The milk was not supplemented with iron and copper in view of the work of Knoop et al. (9).

The experimental animals consisted of nine male dairy calves, all dropped in the University Dairy Herd. They were taken from their dams at birth and placed in individual pens having raised expanded metal floors. No bedding was used.

The calves were fed their dams’ colostrum for the first 48 hr., after which they were fed the experimental diets for a period of 8 wk. The riboflavin intake during the colostrum feeding period was recorded. Sulfathalidine was administered in every case of scours or diarrhea. Water was available at all times.
The nine calves used in the experiment were grouped to receive dietary treatments as indicated in table 2.

**TABLE 2**

**Grouping and dietary treatment of calves**

<table>
<thead>
<tr>
<th>Group</th>
<th>Calf</th>
<th>Treatment of Milk</th>
<th>Approximate daily riboflavin intake (γ/kg. body weight)</th>
<th>Supplemental pyridoxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A-0</td>
<td>Photolyzed</td>
<td>4 (remaining in milk)</td>
<td>None</td>
</tr>
<tr>
<td>II</td>
<td>J-I</td>
<td>Untreated</td>
<td>115 (in milk)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>J-II</td>
<td>Photolyzed</td>
<td>115 (supplemented)</td>
<td>None</td>
</tr>
<tr>
<td>III</td>
<td>J-25</td>
<td>Photolyzed</td>
<td>25 (supplemented)</td>
<td>Quantity equal to that in untreated milk.</td>
</tr>
<tr>
<td></td>
<td>S-25</td>
<td>Photolyzed</td>
<td>25 (supplemented)</td>
<td>None</td>
</tr>
<tr>
<td>IV</td>
<td>J-35</td>
<td>Photolyzed</td>
<td>35 (supplemented)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>H-35</td>
<td>Photolyzed</td>
<td>35 (supplemented)</td>
<td>Quantity equal to that in untreated milk.</td>
</tr>
<tr>
<td>V</td>
<td>J-45</td>
<td>Photolyzed</td>
<td>45 (supplemented)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>G-45</td>
<td>Photolyzed</td>
<td>45 (supplemented)</td>
<td>None</td>
</tr>
</tbody>
</table>

* Letter indicates breed.

The milk was fed warm (37 to 40° C.) at 12-hr. intervals at the rate of 10 per cent of the body weight per day. The calves were weighed weekly and the quantity of milk fed adjusted after each weighing.

Each week the calves were placed in a 4 × 1.5 ft. metabolism stall with an expanded metal floor, and a 24-hr. urine specimen was collected for riboflavin assay. Collection was made in an 8-qt. brown glass bottle which contained approximately 25 ml. of concentrated HCl. Contamination with feces was prevented by a water-proof feces bag attached to a blanket put over the calf during the collection period. The total volume of urine collected was measured and representative samples were stored, following the instructions of Slater and Morell (16). In general, riboflavin was determined in the urine within a week by the method of Slater and Morell (17). No major modifications were made except that the photolyzing apparatus sometimes was used instead of sunlight to destroy riboflavin as required in the procedure.

At the end of the experiment, the calves were slaughtered and all except A-0, J-I, J-II and G-45, whose carcasses were used in another experiment, were autopsied in the Veterinary Clinic. In every case, however, the rumen content was collected and incubated at 37° C. for 20 hr. The riboflavin content of the liquid rumen contents before and after incubation was determined. An increase during the incubation period was considered to be indicative of the rate of riboflavin synthesis in the rumen of the calves at the time of slaughtering. The method used for riboflavin determination in the rumen liquid was the same as that used for milk.

**RESULTS**

The growth rates of the calves are presented graphically in fig. 1. The Ragsdale standard (15) is included for purpose of comparison. The 24-hr. urinary
TABLE 3

The urinary excretion of riboflavin by calves receiving various levels of riboflavin (mg./100 lb. (45.3 kg.) body weight)

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>Riboflavin intake (mg./kilo./d.)</th>
<th>24 hr. urinary riboflavin excretion at age of:</th>
<th>Av.(\bar{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 d.</td>
<td>1 wk.</td>
</tr>
<tr>
<td>A-0</td>
<td>4</td>
<td>0.86</td>
<td>0.07</td>
</tr>
<tr>
<td>J-25</td>
<td>25</td>
<td>0.14(\bar{b})</td>
<td>0.18</td>
</tr>
<tr>
<td>S-25</td>
<td>25</td>
<td>0.44</td>
<td>0.14</td>
</tr>
<tr>
<td>J-35</td>
<td>35</td>
<td>1.01</td>
<td>0.08</td>
</tr>
<tr>
<td>H-35</td>
<td>35</td>
<td>1.73</td>
<td>0.09</td>
</tr>
<tr>
<td>J-45</td>
<td>45</td>
<td>0.19(\bar{b})</td>
<td>0.18</td>
</tr>
<tr>
<td>G-45</td>
<td>45</td>
<td>1.00</td>
<td>0.87</td>
</tr>
<tr>
<td>J-I</td>
<td>115(\bar{d})</td>
<td>2.26</td>
<td>3.06</td>
</tr>
<tr>
<td>J-II</td>
<td>115</td>
<td>0.14(\bar{b})</td>
<td>3.42</td>
</tr>
</tbody>
</table>

\(\bar{a}\) First collection excluded.
\(\bar{b}\) First collection made at the age of 4 to 5 d.
\(\bar{c}\) Riboflavin supplementation had been discontinued for 3 d. prior to this collection.
\(\bar{d}\) Average riboflavin intake in untreated milk.

The urinary excretion of riboflavin are given for each calf in table 3. The results of the first collection were not included when the average daily excretion for the entire experiment was calculated. These results were omitted in an attempt to eliminate the influence of the possible difference in riboflavin storage at birth, as well as the differences in intake during the colostrum feeding period.

Control calves. The growth of the J-I and J-II calves was practically identical and similar to the Ragsdale growth standard (15) (fig. 1). Both calves appeared normal, thrifty and active during the entire experiment. They showed no deficiency symptoms whatsoever. There was no physical evidence of pyridoxine deficiency in calf J-II, even though no pyridoxine supplement was fed. A normal healthy condition of the lungs and intestines was noted in these calves at slaughter. The rumen of J-I was small and contained a few hair balls.
but no fluid. It is possible, however, that some liquid, originally present in the rumen, was lost during slaughtering. A few hair balls were found in the rumens of all calves used in this experiment. The rumen of J-II contained approximately 500 ml. of liquid. During incubation, the riboflavin content of this liquid increased about 2.5 times. This is an indication that at the time of slaughtering some riboflavin-synthesizing microorganisms were present in the rumen of this calf.

The growth of the negative control (A-0) was poor and irregular (fig. 1). Excessive lacrimation and salivation appeared when the calf was 2 wk. old. Early during the third week diarrhea occurred which did not respond to sulfathalidine treatment and persisted until the termination of the experiment. When the milk allowances were reduced for a few days, however, the feces became more solid, but diarrhea invariably reappeared when the calf was brought back on full feed. At the end of the experiment, this calf was unthrifty, possessed a rough hair coat and showed definite signs of dry, scaly dermatitis, especially apparent in the area back of the ears. Excessive shedding persisted from the third week of the experiment on; however, severe alopecia as previously reported (21, 23) was not observed. When the animal was sacrificed at the age of 62 days, the rumen contained about 600 ml. of liquid. The riboflavin content of this liquid increased from 0.09 to 0.37 mg. per liter during the incubation period. It is possible, therefore, that some riboflavin was synthesized in the rumen of this calf. This phenomenon may possibly account for the periods of relatively good growth observed during the fourth and sixth week of the experiment. A condition of severe catarrhal enteritis and gangrenous pneumonia was observed when the calf was slaughtered.

25 γ of riboflavin per kilogram of body weight. J-25 grew well during the first 3 wk. of the experiment. During the fourth week, scours appeared, and the animal lost weight (fig. 1). From this time on, an unthrifty condition gradually developed. Excessive shedding accompanied the development of a rough hair coat. Sulfathalidine treatment was without beneficial effects in times of diarrhea. Excessive salivation was observed. The post-mortem examination showed pneumonia, severe catarrhal enteritis and “white spotted” kidneys as the gross pathology. Anemia, characterized by a low erythrocyte and leucocyte count and low hemoglobin content, also was observed.

S-25 grew normally during the first 6 wk., after which growth stopped and body weight remained practically unchanged until the termination of the experiment. At that time excessive loss of hair, a rough hair coat and excessive salivation were the most prominent external symptoms of deficiency. Diarrhea never appeared. The post-mortem examination showed a mild catarrhal enteritis and “white spotted” kidneys. No pneumonia was observed. A normocytic hypochromic type of anemia as observed in J-25 also was noted in S-25.

Some liquid was collected from the rumen of both J-25 and S-25 but incubation effected no change in the riboflavin content of this liquid. There was no indication that pyridoxine supplementation improved the photolyzed milk diet of S-25.
35 γ of riboflavin per kilogram of body weight. These two animals (J-35 and H-35) grew well until the end of the experiment (fig. 1). No external symptoms of deficiency were observed, except for somewhat excessive shedding, especially in calf H-35. Both calves apparently remained thrifty and in good health throughout. Diarrhea, however, appeared in J-35 toward the end of the experiment. Mild catarrhal enteritis and "white spotted" kidneys were revealed by the post-mortem examination. Normocytic hypochromic anemia, as previously noted in calves J-25 and S-25, also was observed in both J-35 and H-35. The rumens of these animals contained some liquid. The riboflavin content of this liquid was not changed during the incubation test.

There were no differences in the performance of these calves which could be attributed to pyridoxine supplementation in the diet of calf H-35.

45 γ of riboflavin per kilogram of body weight. The response of G-45 in growth, thriftiness and general health was excellent until termination of the experiment (fig. 1). No signs of deficiency whatsoever were observed. A normal condition of the viscera, especially the lungs and intestines, was noted at the time of slaughter.

J-45 had diarrhea during the first week of life and lost weight. After recovery, growth was resumed at a normal rate until the fourth week. During that week, for no apparent reason, the calf lost weight, but resumed normal growth during the fifth to seventh week. During the last week of the experiment, diarrhea which responded to the sulfathalidine treatment occurred and the calf again lost weight so that the over-all growth was fairly poor (fig. 1). It may be noted, however, that during the periods of good growth the gain in weight was normal according to the Ragsdale standard. In general, J-45 remained thrifty and very active. No external signs of riboflavin deficiency were observed during the entire course of the experiment. The post-mortem examination showed an entirely normal condition to prevail throughout. No anemia was observed.

The rumen of G-45 contained some liquid which, during the incubation test, showed no change in riboflavin content.

DISCUSSION

Photolyzed milk, as prepared in this experiment and supplemented with vitamin A, was found to be a satisfactory basal ration for the study of riboflavin nutrition in the young calf. When the photolyzed milk diet was fed without riboflavin supplementation (A-0) riboflavin deficiency symptoms developed, which were similar to those previously reported (21, 23). On the other hand, when this basal diet was supplemented adequately with riboflavin, the symptoms did not appear and the performance of the calf fed this ration (J-II) was identical to that of a similar calf (J-I) fed untreated milk. It appears, therefore, that riboflavin is the only limiting factor in photolyzed whole milk supplemented with vitamin A when fed to calves up to 8 weeks of age.

The destruction of thiamine and pyridoxine by the process of photolysis apparently did not complicate the results. In fact, the destruction of thiamine as
a result of the treatment was small and the destruction of pyridoxine, even though considerable, obviously was without significance for the young calf.

When the photolyzed milk ration was fed without supplementation or when riboflavin was supplemented at the 25 microgram level, gross deficiency symptoms developed. Post-mortem examination showed catarrhal enteritis and “white spotted” kidneys as the gross pathology. These symptoms of riboflavin deficiency were associated with anemia and, in some cases, pneumonia. The latter condition was reported to develop in lambs fed a riboflavin-deficient ration (3). The anemia observed was of the normocytic hypochromic type accompanied by marked decreases in erythrocyte (8.4 to 3.4 millions per ml.) and leucocyte (6,350 to 2,700 per ml.) counts. This type of anemia was found to be similar to the type of anemia reported to be associated with riboflavin deficiency in swine (24) and monkey (2, 20).

When riboflavin was supplemented at the 35 γ level, the calves grew normally and showed no external signs of riboflavin deficiency except, perhaps, for excessive shedding. The post-mortem examination, however, showed a mild catarrhal enteritis and “white spotted” kidneys but no pneumonia. Normocytic hypochromic anemia was observed.

The calves fed riboflavin at the 45 γ level showed no evidence of abnormalities that definitely could be attributed to a deficiency of riboflavin. They showed no external signs of deficiency whatever and post-mortem examination revealed a normal condition to prevail throughout. Anemia was not observed.

The fact that the riboflavin excretions on intakes of 25 and 35 γ per kilogram body weight were essentially the same indicates that the higher of these levels may still be below the minimum daily requirement. On the basis of these observations it appears that the minimum daily riboflavin requirement is between 35 and 45 γ per kilogram of body weight. This requirement is in good agreement with that reported for other mammalian species of comparable age (2, 6, 10, 12, 13, 14, 18).

The question of the significance of microbiological synthesis of riboflavin in the rumen of the milk-fed calf is still unanswered. The failure to observe an increase in riboflavin during the incubation of rumen fluid from a number of the calves in this experiment may be interpreted as indicating that riboflavin-synthesizing organisms were not present. In the case of the two calves where there was evidence of riboflavin synthesis during the incubation of the rumen fluid, evidence of such synthesis in vivo could not be detected by urine analysis.

SUMMARY

Nine male calves representing five dairy breeds were used to determine the minimum riboflavin requirement of the dairy calf up to 8 wk. of age.

The basal riboflavin-deficient diet consisted of whole milk in which approximately 97 per cent of the riboflavin had been destroyed by photolysis. The photolyzed product contained an average of 0.04 mg. of riboflavin per liter. This photolyzed milk was supplemented with vitamin A and the various levels of riboflavin studied.
It was found that calves receiving 35 \( \gamma \) or less riboflavin per kilogram of body weight daily developed symptoms of riboflavin deficiency. The deficiency symptoms were more severe at the lower levels. The calves receiving 45 \( \gamma \) or more of riboflavin per kilogram body weight showed no symptoms that could be attributed to a deficiency of the vitamin.

The data obtained indicate that the urinary excretion of riboflavin is a good index of the nutritional status of the calf as far as riboflavin is concerned.

The data obtained indicate that the minimum daily riboflavin requirement of the male dairy calf up to 8 wk. of age is between 35 and 45 \( \gamma \) per kilogram of body weight.

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REFERENCES