SYNTHESIS OF VITAMIN B IN THE RUMEN OF THE COW.*

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In our studies concerning the vitamin B requirement of calves, one of us produced conclusive evidence that a calf will grow normally to maturity and produce normal offspring on a ration that carries an insufficient amount of the vitamin B complex to support growth and well being in rats (1). In later investigations we determined that vitamin B in milk is not dependent on the presence of this vitamin in the ration of the cow (2). Three cows that were fed for over 2 years, throughout their growth period, on a ration that was decidedly deficient in vitamin B, were used in this study. The evidence obtained in these investigations appeared to indicate that cattle, and possibly all other ruminants, possess the ability to synthesize vitamin B. Damon (3), Kuroya and Hosoya (4), Heller et al. (5), Scheunert and Schieblich (6), Sunderlin and Werkman (7), and others have made contributions to our knowledge of the possible synthesis of vitamin B complex by bacteria. The present paper is intended to set forth the results of an investigation which was designed to determine whether the microorganisms present in the rumen of one of our experimental cows were responsible for the synthesis of vitamin B complex.

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A pure bred Holstein heifer, Penstate Homestead Jessie, No. 924062, was made the subject of this study. She was one of a group of seventeen experimental animals, and had grown to maturity on the experimental ration, which was made up from corn gluten meal, cane sugar, commercial casein, polished rice, corn-starch, pearled hominy, dried sugar beet pulp, cod liver oil, and mineral supplements. This ration was demonstrated, through rat feeding trials (1), to be practically devoid of the vitamin B complex.

A permanent fistula about 3½ inches in diameter was made in the rumen of the experimental heifer through the left side. This provision afforded an easy means of sampling the rumen contents. A special pack, after the method used by Schalk and Amidon (8), was provided for keeping the fistula tightly closed so that normal conditions within the rumen would be maintained. The heifer continued to thrive (on the ration deficient in vitamin B) and gave every evidence of functioning normally as she had done on the experimental ration, prior to the operation.

Our first step in the investigation consisted in testing out alcoholic extracts from the fermented rumen contents. 12 hours after feeding, portions of about 30 pounds of the wet fermented feed were removed from the rumen and placed in a 5 gallon milk can. The fermented material was incubated for 5 days at a temperature of 37°, with occasional stirring of the mixture in order that the bacterial flora might multiply and produce, if possible, the maximum quantity of vitamin B. This procedure was considered preliminary, and precautions were taken to provide an extract sufficiently concentrated to eliminate possible difficulties in feeding technique. A total of 50,530 gm. of the fermented rumen contents, representing four separate samplings, was used in preparing the extract. This represented 6252 gm. of dry matter, the average percentage of dry matter being 11.06 per cent.

The fermented rumen contents were stored in 5 gallon stone jars. 95 per cent ethyl alcohol was added to the jars in liberal amounts because of the large amount of moisture present. It was the aim to provide a solution of approximately 70 per cent alcohol. The jars containing the mixture were allowed to stand for 1 week at room temperature with occasional stirring each day.
The liquid was then filtered off and evaporated to dryness at a temperature of 50°, after the usual practice of preparing vitamin B extracts. 2226 gm. of extract carrying 1200 gm. of dextrin, were so prepared that 1 gm. of this extract represented 25.4 gm. of the original fresh fermented rumen material.

Rat Feeding Experiments with Rumen Extract.

The feeding technique used in this and later feeding trials is essentially that described by Dutcher, Francis, and Combs (9). The animals were placed on experiment when they were 21 days of age, and their average weight was about 40 gm. Males and females were used in equal numbers, and litter mates were distributed throughout the groups. Each rat was kept in an individual cage provided with a screen bottom (10). Growth records were obtained and food intake noted.

The animals refused to eat the ration containing rumen extract for the reason that it carried a decided butyric acid odor. 3 per cent of olive oil incorporated in the ration proved to make it palatable. The ration finally adopted (Ration 103) was made up as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>18</td>
</tr>
<tr>
<td>Salts 185*</td>
<td>3</td>
</tr>
<tr>
<td>Agar</td>
<td>2</td>
</tr>
<tr>
<td>Olive oil</td>
<td>3</td>
</tr>
<tr>
<td>Dextrin</td>
<td>24</td>
</tr>
<tr>
<td>Rumen extract</td>
<td>50</td>
</tr>
</tbody>
</table>


The controls also receive an equivalent of olive oil in their ration. Each rat was given 4 drops of Squibb’s cod liver oil daily to insure an adequate supply of vitamins A and D.

The average growth curve for the two groups is shown in Chart I. In the 8 week period the groups receiving the rumen extract made an average gain in weight of 5.2 gm. per week (Curve I).

The growth curves indicate clearly that the rumen extract must have supplied the vitamin B complex since all animals maintained a constant rate of growth throughout the 8 week period. The
controls (Curve II, Chart I) were significantly different although they continued to live (some of them for as long as 10 weeks), but their increases were very slight during the 8 week period.

The evidence obtained in this feeding experiment seemed to indicate quite clearly that vitamin B must have been synthesized by bacteria or other microorganisms. The next step was to make a study of the microflora in the rumen.

**Bacterial Study of Rumen Microflora.**

The work of Damon (3) on acid-fast bacteria which synthesized the vitamin B complex, suggested that we might be dealing with a similar situation in the rumen of our experimental cow. We had also expected to find wild yeasts. Direct microscopic examination of stained smears of the rumen contents revealed the presence of neither acid-fast bacteria nor yeasts. Samples plated with plain nutrient agar revealed approximately 2,225,000 bacteria per gm. of rumen contents but no yeast colonies were found. What we did find was an almost pure culture of a bacterium that produced a small colony about 1 to 2 mm. in diameter. The colonies developing upon the surface showed a pale lemon-yellow pigment. The subsurface colonies developed no pigment but when transfers were made to agar slants, they proved to be identical with the surface colonies. A search of the literature revealed that our organism had never been described. We have, therefore, designated it *Flavobacterium vitarumen*. Platings on plain nutrient agar of several samples showed the results indicated in Table I. The predominance of this type of colony in our first plates prompted us to make preparations for a test of its ability to synthesize the vitamin B complex.
Plain nutrient agar medium, made from the dehydrated Bacto-Nutrient Agar manufactured by the Digestive Ferments Company, was placed in 32 ounce bottles of the Blake type and sterilized for 20 minutes at 15 pounds pressure. The bottles were placed in a horizontal position in order that the agar would solidify on one side of the bottle and provide the maximum area for growth. The media in these bottles were then inoculated with a water suspension of a pure culture of the organism and incubated for 48 to 72 hours at 37°. The growth, which was not abundant, was then washed from the surface of the media with sterile water and the washings were evaporated to dryness at a temperature of approximately 50°. The amount of growth secured in this manner was less than 1 gm. of dried material per bottle. We were able, however, to prepare a sufficient quantity of dried bacterial cells in the form of a meal to make the necessary test for vitamin B in the rat feeding experiments.

*Flavobacterium vitarumen* is rod-shaped, being 0.5 to 1.5 microns $\times$ 0.5 – 3.0 microns in size. It has rounded ends, does not form endospores, generally occurs singly but occasionally in pairs, and is non-motive. It differs from the vast majority of the genus *Flavobacterium* in that it is Gram-positive.

Surface agar colonies are 1 to 4 mm. in diameter, while the imbedded colonies are somewhat smaller. Gelatin colonies are very similar to the agar colonies. Other cultural characteristics are as follows: Agar slant, filiform; chromogenesis, pale lemon-yellow; gelatin stab, no liquefaction; sugar broths, no gas produced; sucrose, dextrose, and maltose broth, acid produced;

**TABLE I.**

<table>
<thead>
<tr>
<th>Date</th>
<th>Total No. of bacteria per gm</th>
<th>Approximate percentage of <em>Flavobacterium vitarumen</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Feb. 21</td>
<td>2,225,000</td>
<td>98</td>
</tr>
<tr>
<td>&quot;  26</td>
<td>8,500,000</td>
<td>98</td>
</tr>
<tr>
<td>Mar. 4</td>
<td>1,840,000</td>
<td>97</td>
</tr>
<tr>
<td>&quot;  17</td>
<td>1,850,000</td>
<td>96</td>
</tr>
<tr>
<td>June 30</td>
<td>26,300,000</td>
<td>95</td>
</tr>
<tr>
<td>July 13</td>
<td>27,450,000</td>
<td>90</td>
</tr>
<tr>
<td>Aug. 1</td>
<td>34,500,000</td>
<td>50</td>
</tr>
</tbody>
</table>
lactose, xylose, dulcitol, mannitol, sorbitol, and inositol broth, no acid produced; starch agar, no diastase; cellulose, no fermentation; lead acetate agar, no growth; Endo's medium, no growth.

Litmus milk is fermented with the production of acid. There is a slight reduction of the litmus but no curd is formed.

It does not form a ring of pellicle growth on any of the broth media. The growth clouds the liquid and finally settles. It does not form indole or skatole and nitrates are reduced.

Rat Feeding Experiments with Dried Bacteria.

The feeding technique was the same as that employed in the previous test on the rumen extract. The dried bacterial meal was fed daily in 0.5 gm. doses to each rat as a supplement to the basal ration. In Curve I, Chart II, the result of this feeding test is presented. The curve represents the average growth of ten rats. The average weekly gain per rat was 6.8 gm.

At the same time another group of rats, some of which were litter mates of those represented in Curve I, were fed the basal ration supplemented with 0.5 gm. of Fleischmann's special yeast. In Curve II the average growth of this group is presented. The average weekly gain per rat in this group was 6.4 gm.

There was some question as to whether the media (dehydrated nutrient agar) which were used in growing the large quantities of bacterial cells, did not carry appreciable amounts of the vitamin B complex. To test out this point, a liter of medium made from Bacto-Nutrient Agar was shaken with 10 gm. of Lloyd's reagent.
The mixture was filtered and the filtrate evaporated to dryness. The residue was fed to rats in daily 0.5 gm. doses with the result as presented in Curve IV, Chart II. A similar test was made with autolyzed yeast, prepared as described by Seidell (11). Curve III represents the average growth of this group. A control group was also fed on the basal diet with the results portrayed in Curve V.

**SUMMARY AND CONCLUSION.**

Investigations were conducted on the fermented rumen contents of a Holstein cow, representative of a group of seventeen animals that were grown to maturity on a ration highly deficient in vitamin B complex. Alcoholic extracts of the fermented rumen contents were proved potent in the vitamin B complex through rat feeding trials.

One bacterium of the genus *Flavobacterium* was found about 90 per cent predominant in the rumen microflora. This organism was grown in large quantities on vitamin B-free media and fed to rats to the extent of about (12 per cent) of dried bacterial cells in a synthetic vitamin B-free ration. This test proved the bacterial cells to be highly potent in the vitamin B complex.

The results of this study warrant the conclusion that the vitamin B complex was produced in the rumen of our experimental cow by bacterial fermentation. This result offers a satisfactory explanation as to why cattle, unlike any other species of animal yet studied, have the ability to grow to maturity, to produce normal offspring, and to produce milk of normal dietary composition, on a ration that carries an insufficient amount of vitamin B complex to support growth and well being in rats.

Credit is due Dr. J. F. Shigley, of the Department of Animal Husbandry, for veterinary service in caring for the experimental heifer with the permanent fistula.

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Vitamin B Synthesis in Cow

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