The results of early studies (1, 2, 3) concerning the influence of diet upon the antiscorbutic potency of cow’s milk, appeared to indicate that the vitamin C content of the milk paralleled that of the ration. During the same period, however, findings were reported (4) which led to the opposite conclusion, namely, that the ration received by cows had no influence on the antiscorbutic potency of their milk.

Since the development of chemical methods for the quantitative determination of the antiscorbutic factor, which was shown to be ascorbic acid, further differences of opinion have arisen concerning the factors which influence the vitamin C content of cow’s milk. As a result of recent work (5, 6, 7) the influence of breed and stage of lactation have been emphasized. Several workers (5, 8, 9) have attributed variations in the ascorbic acid content of milk to the season of the year. Other investigators (10, 11, 12, 13, 14) have concluded that the vitamin C content of cow’s milk tends to be quite constant and is independent of the ration of the cow. Opposed to this view are those who still contend that the ascorbic acid content of the diet is a factor which cannot be ignored (6, 15).

A number of investigators have suggested that the cow is able to synthesize vitamin C (9, 13, 16, 17, 18, 19). How or where this synthesis occurs is not understood, although one investigator (30) has claimed that the vitamin C of cow’s milk is synthesized by the udder parenchyma and that the synthesis depends largely upon the condition of the udder.

The failure of numerous experiments involving standard dairy rations to establish clearly and conclusively the importance of the ascorbic acid content of the diet with regard to the amount of the vitamin in the milk, made it seem desirable to repeat previous work, but to alter the procedure by supplementing standard rations with known amounts of pure synthetic ascorbic acid. Moreover, it was desired to increase the significance of customary milk ascorbic acid analyses by determining, simultaneously, the amount of ascorbic acid in the blood and in 24-hour samples of urine.
If, in the above proposed studies, it could be shown conclusively that the vitamin C content of milk is independent of the ration of the cow, it was hoped that some explanation could be obtained for the anomalous results just mentioned. It was desired, for example, to find new evidence for the synthesis of ascorbic acid in the cow. Further, it was hoped that some clue might be found regarding both the metabolic fate of ascorbic acid and the factors which influence its elimination from the body in the milk and in the urine.

EXPERIMENTAL

Methods and Apparatus. One or both of two chemical methods of estimation were used in all ascorbic acid analyses. These were the Tillmans titration method (20) employing standard 2,6-dichlorobenzenoneindophenol, and the newer Roe furfural method (21), which estimates total ascorbic acid (both reduced and reversibly oxidized ascorbic acid).

Milk samples were taken in a special apparatus which has been shown to preserve all of the ascorbic acid of the milk in the reduced form (22). The use of this apparatus permitted the quantitative determination of the vitamin by the convenient indophenol titration method.

Blood samples were taken in the conventional oxalated tubes under paraffin oil. For each test, 20 to 30 ml. of blood were taken from the jugular vein of the cow. As soon as the sample was obtained, the collection apparatus was placed inside a dark glass receptacle containing ice and water and was taken immediately to the laboratory for analysis. Essentially the Farmer and Abt macro-method (23) for plasma ascorbic acid was used for routine blood analysis.

Successful collections of the total 24-hour urinary excretion, free from fecal contamination, were made by employing a special rubber urine tube developed by Forbes and coworkers (24). The 18-liter carboys, which were used as receivers in the collection apparatus, were painted black to exclude light and were charged with enough glacial acetic acid to give a final concentration of about 5 per cent by volume. Addition of stick metaphosphoric acid to the acetic acid appeared to give no better results than when acetic acid was used alone; consequently, the addition of metaphosphoric acid was discontinued. In urinary ascorbic acid analyses, both the indophenol titration and the Roe furfural method were employed, with the exception of the earliest work on one of the cows which was done before the furfural technique had been reported. This double analysis seemed desirable in view of the lack of unanimity among various workers concerning the specificity of present methods for the determination of ascorbic acid in urine. Moreover, it appeared probable that some of the vitamin would be unavoidably oxidized to dehydroascorbic acid during the collection of a 24-hour sample. Such has since been shown to be true with human urine (25). The furfural method
is particularly valuable in this case because it determines both dehydro- and reduced ascorbic acids.

It was recognized at the outset that it would be desirable to eliminate from the projected work the effect of breed differences by limiting the experiments to one breed of dairy cow. Holstein cows were eventually chosen, largely because they were available for experimental purposes at the time this work was started.

**Effect of a Standard Ration.** In order to have values which might later be used to compare with those obtained during administration of ascorbic acid, each cow was maintained on a standard ration for an interval of time during which analyses were made to determine the concentrations of ascorbic acid in the blood, the milk, and the urine. The averages of the values obtained during typical five-day test periods are given in table 1. Five-day

<table>
<thead>
<tr>
<th></th>
<th>S. R.</th>
<th>C. S.</th>
<th>G. C.</th>
<th>D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg. ascorbic acid per mL.</td>
<td>0.019</td>
<td>0.021</td>
<td>0.021</td>
<td>0.020</td>
</tr>
<tr>
<td>Mg. ascorbic acid per 100 mL. plasma</td>
<td>0.53</td>
<td>0.58</td>
<td>0.50</td>
<td>0.48</td>
</tr>
<tr>
<td>Mg. ascorbic acid per day in urine—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indophenol titration</td>
<td>45.7</td>
<td>52.7</td>
<td>54.1</td>
<td>26.8</td>
</tr>
<tr>
<td>Furfural method</td>
<td>910.5</td>
<td>833.1</td>
<td>643.5</td>
<td></td>
</tr>
</tbody>
</table>

S. R.—Standard ration.
C. S.—Standard ration supplemented with 50 to 100 grams of ascorbic acid mixed with corn silage.
G. C.—Standard ration supplemented with 50 grams of ascorbic acid in gelatin capsules.
D.—Standard ration supplemented with 50 grams of ascorbic acid administered by drenching.

* The values given in Table 1 represent the averages obtained in experiments employing from two to four cows, except in the case of the ascorbic acid administered by drenching, where the values are from an experiment with a single cow.

sampling periods were considered representative for any diet, in view of the fact that all cows received a particular ration for several days, or even weeks, prior to the actual collection of samples.

**Effect of Ascorbic Acid Added to Corn Silage.** In choosing methods for administration of massive amounts of ascorbic acid, it was decided to administer some of the vitamin mixed with a small amount (3–5 pounds) of corn silage. It was found that 50–200 grams (1,000,000–4,000,000 International Units) of ascorbic acid placed in such a mixture were consumed by a cow in a period of about 10 minutes. In contrast, ascorbic acid mixed with grain was eaten very slowly, if at all.

Experimental periods were designed to include a two-day interval during which the cow received the standard ration alone, followed by a three-day period during which she received the standard ration supplemented each day with a certain amount of crystalline ascorbic acid mixed with corn silage,
which was succeeded by a post-administrative period of two days during which the animal again received only the standard ration. The same procedure was used in subsequent experiments during which the ascorbic acid was administered in gelatin capsules and by drenching. The data from such experimental periods are summarized in table 1.

From a comparison of values in table 1, it is apparent that supplements of ascorbic acid administered by three different methods had no significant influence upon the concentrations of ascorbic acid in the blood, milk, or urine of the cows used in these experiments.

Effect of Ascorbic Acid Injected Intravenously. Rasmussen and coworkers (26) reported that intravenous injection of ascorbic acid resulted in a marked temporary rise in the vitamin C content of the milk of the ewe and cow. It was desired to repeat this work and to enlarge upon it by making analyses of blood and urine as well as of milk. Therefore, experiments were performed during which ascorbic acid was injected into the blood stream via the jugular vein. 24 grams of crystalline ascorbic acid, dissolved in sterile water, were given in this manner on each of two or three successive days. The effect of these ascorbic acid injections was studied with three cows with corresponding results. Typical values are given for one of the cows in table 2.

**TABLE 2**

<table>
<thead>
<tr>
<th>Day</th>
<th>Mg. ascorbic acid per 100 ml. plasma</th>
<th>Mg. ascorbic acid per ml. milk</th>
<th>Mg. urinary ascorbic acid per day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Indophenol titration</td>
<td>Furfural method</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.58</td>
<td>0.021</td>
<td>24.3</td>
</tr>
<tr>
<td>2</td>
<td>0.58</td>
<td>0.020</td>
<td>34.6</td>
</tr>
<tr>
<td>3*</td>
<td>2.74**</td>
<td>0.020</td>
<td>6922.9</td>
</tr>
<tr>
<td></td>
<td>0.019</td>
<td>0.022</td>
<td>13311.8</td>
</tr>
<tr>
<td>4*</td>
<td>4.42**</td>
<td>0.024</td>
<td>9870.0</td>
</tr>
<tr>
<td></td>
<td>0.028</td>
<td>0.028</td>
<td>13632.6</td>
</tr>
<tr>
<td>5*</td>
<td>4.86**</td>
<td>0.030</td>
<td>14715.2</td>
</tr>
<tr>
<td></td>
<td>0.029</td>
<td>0.027</td>
<td>18194.0</td>
</tr>
<tr>
<td>6</td>
<td>0.86</td>
<td>0.028</td>
<td>8813.6</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>0.022</td>
<td>20923.8</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.021</td>
<td>161.2</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.020</td>
<td>117.8</td>
</tr>
</tbody>
</table>

* 24 grams of ascorbic acid dissolved in 100 ml. of sterile water were injected into the jugular vein. The multiple values for milk ascorbic acid represent each of the three daily milking periods.

** Blood analyses were made on samples taken 1½ hours after injection of ascorbic acid.
The intravenous injection of ascorbic acid produced unmistakable increases in the concentration of that vitamin in the milk and urine as well as in the blood. A very large portion of the injected ascorbic acid which could be accounted for appeared in the urine. In the case of one cow, this amounted to over ninety per cent of the total ascorbic acid injected. These results correspond closely to those recently reported (31) for experiments in which ascorbic acid was injected into goats.

Effect of Ascorbic Acid Injected Subcutaneously. The experiments described thus far seemed to indicate that ascorbic acid administered by non-injection methods never reached the blood stream in significant amounts. Further, data from the intravenous injection experiments showed that the concentration of ascorbic acid in the milk was definitely increased if large amounts of the vitamin reached the blood stream. In order to further substantiate these findings, it was desired to administer some ascorbic acid by a method which would not place the vitamin directly in the blood stream but which would insure the ultimate arrival of large amounts in the circulation. Such a purpose was accomplished by setting up an experiment in which ascorbic acid was injected subcutaneously in regions around the cow's forelegs.

The effect of the subcutaneous injections of ascorbic acid closely resembled that of the intravenous injections. The influence of the subcutaneous injections upon blood, milk, and urinary ascorbic acid values was more gradual and somewhat less pronounced than in the case of the intravenous injections. During the course of three successive daily injections of 24 grams of ascorbic acid, the ascorbic acid titer of the milk was raised from 0.020 to 0.027 mg. per ml. and that of the urine from 600 to a peak of 15,000 mg. per day.

Studies with a Rumen Fistula. At this point in the experiments it appeared to be almost certain that the failure of massive amounts of ingested ascorbic acid to influence the concentration of the vitamin in the milk or to significantly alter its concentration in the blood and urine, could be attributed to a destruction of this substance in the rumen. To investigate this possibility, a rumen fistula was created in one of the cows. With this permanent opening leading directly into the largest compartment of the cow's stomach, it was possible to study the fate of ingested ascorbic acid by removal and analysis of partially digested food at intervals after feeding.

A rapid and pronounced destruction of ascorbic acid in the rumen was demonstrated by removal and analysis of samples of the rumen contents at regular intervals after feeding supplements of ascorbic acid and after insertion of the vitamin directly into the rumen. During such periods, the average concentrations of ascorbic acid in the blood plasma, in the milk, and in the urine were respectively 0.42 mg. per 100 ml., 0.019 mg. per ml., and 1260.5 mg. per day, values which do not differ significantly from those obtained during feeding of a standard ration alone.
The disappearance of ascorbic acid from the rumen contents during an experiment in which 150 grams of ascorbic acid were fed is shown graphically in figure 1. A preliminary report on this experiment has been given elsewhere (32).

In order to demonstrate that volume changes were not responsible for the pronounced decrease in the ascorbic acid concentration of the rumen contents as shown above, an experiment was performed in the laboratory with a controlled volume of rumen contents. 1000 ml. of rumen contents, from which the coarse particles of feed had been removed by straining through cheesecloth, were placed in a dark-glass, wide-mouth bottle. To this mixture, which had a pH of 6.50, was added 1000 mg. of crystalline ascorbic acid. After thoroughly mixing the contents, the bottle was loosely stoppered and placed in a water bath maintained at a temperature of 39° to 42° C. At intervals, samples were removed for analysis.

Figure 1 shows that the ascorbic acid disappeared just as it had in the in vivo experiments with the exception that the in vitro decrease proceeded at a more gradual rate. This slower rate of destruction of ascorbic acid in the in vitro experiment as compared to the in vivo experiments seems readily

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* By the term "rumen juice" is meant the liquid portion of rumen contents.
explained by the absence of the continual stirring and the circulation of gases so characteristic of the rumen.

**DISCUSSION OF RESULTS**

*State of Ascorbic Acid in Urine.* As soon as the furfural method was applied to the urine analyses it became apparent that the results obtained ranged from one and a half to thirty times as high as those obtained by the indophenol titration. During injections of ascorbic acid, however, this discrepancy narrowed to a point where, in some cases, the results obtained by the two methods almost coincided. These facts, together with the knowledge that the indophenol and furfural methods of analysis checked well when applied to 24-hour samples of rat urine collected under similar conditions (33), suggested that important amounts of the ascorbic acid excreted daily by a cow on a standard ration were excreted in the form of dehydroascorbic acid. In order to test this hypothesis, samples of urine were collected as they were excreted and analyzed at once. Table 3 gives the results obtained

<table>
<thead>
<tr>
<th>Cow No.</th>
<th>Reduced ascorbic acid Mg. per liter</th>
<th>Total ascorbic acid Mg. per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Indophenol titration</td>
<td>Furfural method</td>
</tr>
<tr>
<td>1</td>
<td>20.9</td>
<td>17.0</td>
</tr>
<tr>
<td>2</td>
<td>33.2</td>
<td>25.4</td>
</tr>
<tr>
<td>3</td>
<td>19.7</td>
<td>18.2</td>
</tr>
<tr>
<td>4</td>
<td>10.1</td>
<td>10.3</td>
</tr>
<tr>
<td>5</td>
<td>27.7</td>
<td>23.1</td>
</tr>
<tr>
<td>6</td>
<td>27.6</td>
<td>25.4</td>
</tr>
<tr>
<td>7</td>
<td>53.1</td>
<td>48.1</td>
</tr>
<tr>
<td>8</td>
<td>40.1</td>
<td>43.3</td>
</tr>
<tr>
<td>9</td>
<td>21.5</td>
<td>19.0</td>
</tr>
<tr>
<td>10</td>
<td>31.0</td>
<td>29.6</td>
</tr>
</tbody>
</table>

from Holstein, Jersey, Brown Swiss, and Ayrshire cows. These findings seem to indicate that all the ascorbic acid excreted in the urine of the dairy cow is in the reduced form. The greater values obtained in eight out of ten cases by the indophenol method are readily explained by the recognized tendency to go beyond the endpoint, which is obscured to an extent proportional to the concentration of the urine pigments. The greater values obtained by the furfural method for 24-hour samples of urine must indicate, therefore, that variable amounts of the excreted ascorbic acid are oxidized to dehydroascorbic acid during the interval between excretion and analysis, or that the urine contains appreciable amounts of non-ascorbic acid furfural precursors capable of forming a derivative with 2,4-dinitrophenylhydrazine. Tests for the latter interfering substances were made repeatedly and in no case showed the presence of concentrations sufficiently large to interfere with
the method. Consequently, it may be concluded that while all the ascorbic acid excreted in the urine is originally in the reduced form, some of it is oxidized to dehydroascorbic acid under the conditions involved in the collection of a 24-hour sample of urine.

**Destruction of Ascorbic Acid in the Rumen.** Several explanations might be given for the rapid and pronounced disappearance of ascorbic acid from the rumen after oral administration of massive amounts of the vitamin. It might be argued that the large amounts observed in the first two or three samples represented stages in the incomplete mixing of the ascorbic acid with rumen contents. Changes in the concentration of the rumen ascorbic acid could also be attributed to an intake of water by the animal, or by a passage of a portion of the rumen contents to other chambers of the stomach.

Another interpretation of the findings is that the very soluble vitamin was rapidly absorbed. This has been suggested by Riddell and Whitnah (27). These workers studied the fate of vitamin C in the rumen contents of a cow with a rumen fistula and in a steer at slaughter. In each case the rumen contents were found to contain less than one-tenth the vitamin C of green rye ingested twelve hours earlier. These workers explained the rapid disappearance of ascorbic acid from the rumen by suggesting that ascorbic acid was rapidly absorbed. The basis for this suggestion was the observation of a temporary doubling of the vitamin C content of the blood within 12 hours and a fivefold increase in the ascorbic acid content of the urine within 60 hours after green feed was first supplied.

The most plausible explanation, however, and the one demanded by our experimental data, is that a rapid and pronounced destruction of ingested ascorbic acid occurs in the rumen. Thus, while the other factors which have been mentioned undoubtedly have some influence on the results observed, it is hardly likely that they account for changes of the magnitude indicated in figure 1.

The incomplete mixing theory is especially inadequate. When a cow is eating, the rumen contracts about three times per minute and each contraction causes a flow of liquid throughout the rumen and its solid contents. While figure 1 indicates that the first sample was removed at zero time, it was actually taken about five minutes after the animal had eaten the last of the ascorbic acid treated silage or 15 minutes after she had started to eat. This means that the ascorbic acid had been subjected to 15 to 45 contractions of the rumen. Further, when the cow was slaughtered, it was found that the rumen contained 38 liters of liquid or semi-liquid material. If 150 grams of ascorbic acid were thoroughly distributed throughout this liquid, there should be a concentration equivalent to about 400 mg. per 100 ml. of juice. Figure 1 indicates that the concentration of ascorbic acid in the juice, as shown by analysis of the first sample removed, was about 185 mg. per 100 ml. of juice. From these facts, it would appear that fairly complete mixing had occurred, accompanied by extensive destruction of the vitamin.
Observations of this and other dairy cows show that they drink water infrequently. It is difficult to say how much and how often liquid material passes permanently from the rumen into other compartments of the stomach. Ewing and Wright (28), working with steers, found that the average rate of passage of food residues through the rumen and reticulum was 61 hours. In any event, credence in the volume-change explanation for the disappearance of ascorbic acid from the rumen is seriously discounted by the results of in vitro constant-volume experiments such as shown in figure 1.

Indirect evidence that ingested ascorbic acid is largely destroyed in the rumen rather than being rapidly absorbed is given in the blood, milk, and urine ascorbic acid values, which, with the possible exception of the urine, show no response to the feeding of 50 grams or more of ascorbic acid. Why there is always a small amount of ascorbic acid in the rumen, as there appears to be, is difficult to explain. Possibly the release of this vitamin from solid feed fragments proceeds gradually and at a rate slightly higher than the rate of destruction. We hope to continue the study of the factors responsible for the destructive effects described.

In the light of the demonstrated destruction of ascorbic acid in the rumen, it becomes clear why various methods of oral administration of the vitamin failed to produce a response in the milk or other body fluids. If the ascorbic acid administered in gelatin capsules had been able to survive rumen conditions and reach other compartments of the stomach, it seems likely that other results might have been obtained, for the ascorbic acid content of the milk of non-ruminating animals, e.g., guinea pigs (26) and humans (8, 29), has been shown to be influenced by diet. In work with the rumen fistula, however, it was found that gelatin capsules were dissolved and the ascorbic acid was released after 10–15 minutes in the rumen.

**SUMMARY**

1. Special equipment was employed which permitted the complete collection from dairy cows of 24-hour samples of urine free from any fecal contamination. It was found impractical, if not impossible, to preserve all the ascorbic acid in such urine samples in the reduced form.

2. By the simultaneous application of the indophenol titration and the furfural method of analysis to freshly excreted samples of urine, it was possible to show that ascorbic acid is excreted in cow’s urine in the reduced form.

3. Analysis of over 50 samples of blood obtained from four Holstein cows showed that the ascorbic acid content of the plasma ranged from 0.43 to 0.62 mg. per 100 ml. when the cows received standard dairy rations.

4. Ascorbic acid was administered to Holstein cows (a) mixed with a small amount of corn silage, (b) in gelatin capsules, (c) in aqueous solution, (d) intravenous injection, and (e) by subcutaneous injection. Administration of as high as 100 grams (2,000,000 International Units) of ascorbic acid per day for three days by a non-injection method failed to increase the ascor-
bic acid concentration of the milk or blood and had only a slight effect on the concentration of the vitamin in the urine. It was only by the injection methods that a significant increase in the ascorbic acid concentrations of the blood, milk, and urine could be demonstrated. The greatest increase in milk ascorbic acid concentration during experiments in which 24 grams of ascorbic acid were injected intravenously on each of three successive days, was from 20 mg. per liter to 30 mg. per liter.

5. A rumen fistula was made in a Holstein cow. Experiments were performed in which this cow was fed as much as 150 grams (3,000,000 International Units) of synthetic ascorbic acid at one time; similar amounts were also placed directly in the rumen through the fistula opening. A rapid and pronounced destruction of ascorbic acid in the rumen was demonstrated by removal and analysis of samples of the rumen contents at regular intervals. Ascorbic acid added to rumen contents in vitro and stored in a dark-glass, stoppered receptacle at 39°–42° C. disappeared at much the same rate as that of the in vivo experiments. In making analyses, both the indophenol titration and the Roe furfural method were employed.

ACKNOWLEDGMENTS

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