ACTA CHEMICA SCANDINAVICA 2 (1948) 97—108

Effect of Oxidation-Reduction Potential on the Stability of Ascorbic Acid in Milk and in Fermented Milk

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It is a well-known fact that ascorbic acid is easily destroyed through oxidative processes. Destruction is brought about through autoxidation as well as through catalytic oxidation caused by heavy metals or different enzyme systems. In investigations on the oxidation of ascorbic acid in various biological materials, different substances or mechanisms have been found which have an inhibitory effect on this reaction. Such mechanisms are known to occur above all in animal tissues and fluids [1—4] but vegetable materials, particularly those containing appreciable quantities of ascorbic acid, also, have protective properties [5—9]. The nature of the protective mechanisms has until now remained obscure, although several substances — glutathione, cysteine and some other amino acids, proteins, etc. — as well as enzymatic systems have been assumed to be responsible for the protection of the oxidation. Surprisingly little attention has been paid to the effect of the oxidation-reduction potential, although the equilibrium ascorbic acid — dehydroascorbic acid is theoretically wholly dependent on the electrode potential of the medium. In a medium of high potential, ascorbic acid is oxidized to dehydroascorbic acid, which, being unstable, gradually disappears, thus causing losses in the vitamin C potency of the system. The effect of the oxidation-reduction potential is important not only from the theoretical point of view but also from the practical, because the potentials of various foodstuffs are greatly different.

The behaviour of ascorbic acid in milk and particularly its role in the development of the »oxidized» flavors has been an object of much research

* The author wishes to express his sincere gratitude to Mr. Ahti Harjanne for his valuable assistance throughout the investigation. Thanks are also due to Dipl.eng. Raakel Porvari, who assisted in some experiments.

work. It should be here stated only that ascorbic acid in milk has been found to be comparatively unstable. In addition to its photo-chemical oxidation by daylight [10–12] it is slowly oxidized also in the dark and at low temperatures [13]. In the commercial handling of milk, when milk is exposured to light, contaminated with metals, etc., the losses of ascorbic acid are very high. This fact has a great practical significance because milk is, in those countries where it is amply consumed, an important source of ascorbic acid.

There seem to exist no investigations of the stability of ascorbic acid in different kinds of fermented milks, although these milk products have a large use in many countries. For example, in Finland buttermilks, either natural churned buttermilk or different kinds of cultured buttermilks made from whole or skim milk, are in common use. Bacterial cultures are nearly always strongly reducing systems, often lowering the oxidation-reduction potential of the medium to a very low level, and this holds true also for fermented milk. It was therefore interesting to know whether ascorbic acid is protected against oxidation better in fermented milk than in ordinary milk and, if so, whether the effect is actually due to the low oxidation-reduction potential or only to the acidity of the fermented milk. In order to get information on this problem the experiments reported herein were performed.

EXPERIMENTAL

Experiments with *Lactobacillus acidophilus* grown in an artificial medium

Esselen and Fuller [14] and Esselen [15] have found that certain bacteria, particularly members of the coliform group, are able to inhibit the oxidation of ascorbic acid in culture media. Some strains of *Escherichia coli* were even found to be able to reduce dehydroascorbic acid to ascorbic acid. Concerning the behaviour of the lactic acid bacteria there are in literature conflicting reports. In the experiments of Esselen [15] *Streptococcus lactis* was found to exert a weak, and *Lactobacillus acidophilus* a strong inhibitory action on the oxidation of ascorbic acid. Tkachenko [16] stated that *L. bulgaricus*, *L. acidophilus*, and *L. leichmannii* were able to reduce dehydroascorbic acid into ascorbic acid, while Harowitz-Wlasowa and Buchman [17], on the other hand, found the same bacteria to exert a destructive action on ascorbic acid.

An experiment made to determine the stability of ascorbic acid in cultures of *L. acidophilus* is given in the following.

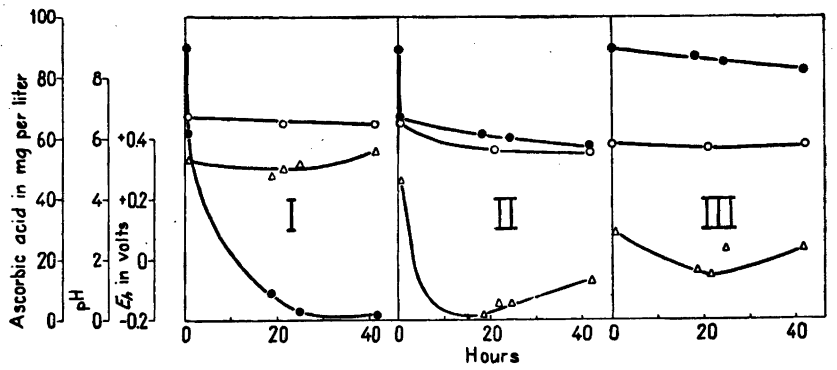

Fig. 1. Stability of added ascorbic acid in cultures of Lactobacillus acidophilus. I. Sterile medium. II. Culture inoculated simultaneously with the addition of ascorbic acid. III. Culture inoculated the day before the addition of ascorbic acid.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

The nutrient medium had the following composition:

NaCl	2 g
K_2HPO_4	2 »
$MgSO_4 \cdot 7\ H_2O$	1 »
Glucose	10 »
Lactose	10 »
Peptone	20 »
Tap water	1 l

200 ml of this medium together with 2 g of $CaCO_3$ were put in 250 ml Erlenmeyer flasks and sterilized in autoclave. One of the flasks was inoculated with *L. acidophilus*, allowed to stand for 20 hours at 37° C, whereupon 20 mg ascorbic acid (Merck), dissolved in water and sterilized with »Jena G 5 auf 3» bacterial filter, were added. In the second flask ascorbic acid was added immediately after the inoculation. The third flask was kept sterile and only ascorbic acid was added to it. In addition the experiment included two control flasks without ascorbic acid, one of which was inoculated and the other kept sterile. The flasks were incubated at 37° C and samples were taken at the beginning of the experiment and then afterward at certain intervals.

The pH and E_h measurements were made by potentiometric methods. All the E_h values were calculated for pH = 6. Ascorbic acid was determined by 2,6-dichlorophenol indophenol titration after the solution had been made acid with trichloroacetic acid, and centrifuged. The results are given in Fig. 1. In addition it should be mentioned that no substances able to reduce dichlorophenol indophenol were formed in the inoculated control without ascorbic acid.

It can be seen from Fig. 1 that cultures of *L. acidophilus* have a distinct protective effect upon the oxidation of ascorbic acid. While in the sterile medium (I) ascorbic acid was completely destroyed in 25 hours, the destruction range was only 33 % in culture II, which had been inoculated simultaneously with the addition of ascorbic acid. In culture III, where ascorbic acid was added into a medium inoculated the day before, the destruction was insignificant. The E_h curves show the dependence of the destruction upon the oxidation-reduction potential. In the sterile medium with high potential, the oxidation has occured very rapidly, while in culture III the low potential has almost completely inhibited the oxidation. In culture II oxidation has taken place during the beginning of the experiment while the oxidation-reduction potential was still high; the rapid fall of the potential, caused by bacterial growth, has, however, inhibited further oxidation. The slight changes of pH during the experiment have no effect on the oxidation; as a separate experiment showed, the rate of oxidation of ascorbic acid in the above conditions was practically independent of the pH in the interval between 4 and 7.

Experiments with milk

The *milk* used in the experiments was milked into glass vessels and kept in dark flasks until the experiment was started (usually 6 to 8 hours later).

Determination of ascorbic acid was performed according to Lunde [18]. 10 ml of milk were precipitated with 6 ml of 20 % trichloroacetic acid. After centrifuging or filtering, an aliquot part was titrated with 0.001 *N* solution of 2,6-dichlorophenol indophenol.

Total vitamin C was determined according to Gunsalus and Hand [19]. 10 ml of milk was mixed with 1 ml of resting suspension of *Escherichia coli* and allowed to stand at 40° C in order to reduce dehydroascorbic acid to ascorbic acid. After 15 minutes 6 ml of 20 % trichloroacetic acid were added, filtered, and titrated with 2,6-dichlorophenol indophenol. With fermented milks the pH was adjusted to 6.2—6.6 before the incubation.

In order to discover the *effect of pH on the stability of ascorbic acid in milk* some experiments were performed in which the pH of milk was adjusted to desired values by addition of acids.

Because lactic acid seemed to accelerate the oxidation of ascorbic acid, an experiment was made in which in addition to lactic acid (P. G. VI, Schering-Kahlbaum) the effect of metaphosphoric acid (glacial, sticks, Schering) and sulphuric acid (for forensic purposes, Merck) also were tested. It was stated that lactic and metaphosphoric acids distinctly accelerated the destruction of ascorbic acid as compared with a milk sample to which no acids were added. Sulphuric acid was without effect. The destructive action of lactic and metaphosphoric acids was probably due to their content of heavy metals; the copper content of the lactic acid used was according to determination 1.0 μg per ml.

To eliminate the effect of heavy metals, lactic acid was distilled twice *in vacuo* whereby its copper content fell to a value below 0.02 μg per ml. With this acid the pH of three 40 ml milk samples in large test tubes was adjusted to 4.2, 5.2, and 6.0, respectively. A

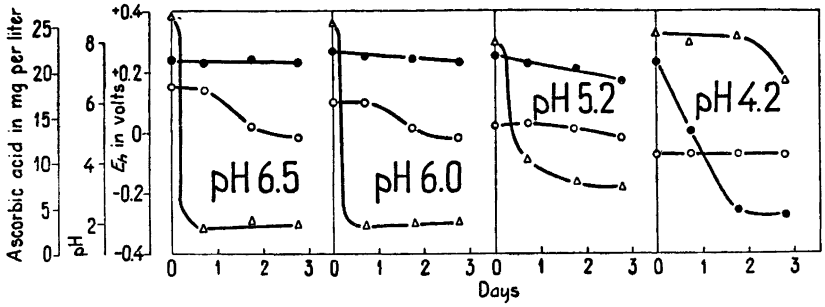

Fig. 2. Effects of pH and E_h on the stability of ascorbic acid in milk.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

fourth, a control tube, contained ordinary milk (pH = 6.5). The tubes were equipped with electrodes for the E_h measurements and allowed to stand in the dark at room temperature (about 20° C). At certain intervals samples for pH and ascorbic acid determinations were taken. The results are given in Fig. 2.

It can be seen from Fig. 2 that the stability of ascorbic acid has been practically independent of the pH of milk. In samples with starting pH of 6.5 or 6.0 hardly any destruction of ascorbic acid has taken place and even in the 5.2-sample destruction has been very slight. At the lowest pH, 4.2, ascorbic acid has been rapidly destroyed, but this is evidently not owing to the acidity but to the high oxidation-reduction potential of that sample. The E_h curves show distinctly the dependence of the stability upon the oxidation-reduction potential. In 6.5- and 6.0-samples there is an abrupt fall in the curves owing to the bacterial growth activities, and it is seen that ascorbic acid has been almost completely protected from oxidation. Even in the 5.2-sample a rapid fall in the E_h curve and a corresponding great stability of ascorbic acid can be noticed. In the 4.2-sample the bacteria have no longer been able to grow. The potential has therefore remained at the original high level, with the result that ascorbic acid has been largely destroyed. These results show that the stability of ascorbic acid in milk is not dependent on the acidity, but on the oxidation-reduction potential of milk.

Some of the typical experiments concerning the *stability of ascorbic acid in milk and in fermented milk* will be given in the following.

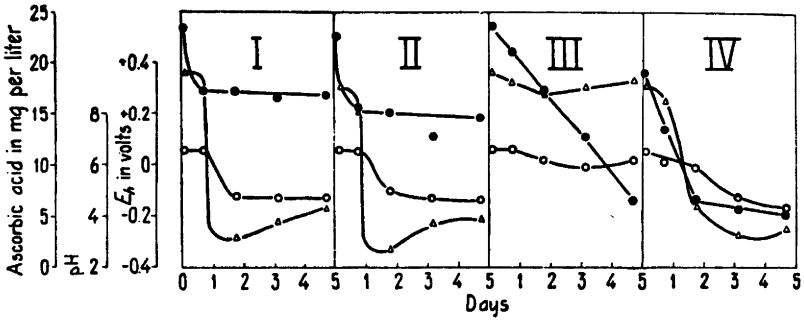

Fig. 3. Stability of ascorbic acid in milk. I. Milk stored at 20° C. II. Skim milk stored at 20° C. III. Milk stored at 1—3° C. IV. Pasteurized milk stored at 20° C.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

The following samples were included in the experiment:

I. Milk.
II. Skim milk.
III. Milk, kept in an ice-box (1—3° C).
IV. Milk, pasteurized at 72° C for 30 seconds.
V. Milk, fermented with a starter (the ordinary butter culture).
VI. Milk, fermented with *Lactobacillus acidophilus.*
VII. Milk, fermented with a ropiness-causing culture of *Streptococcus lactis.*

140 ml of milk were placed in cylinder-shaped 250 ml flasks, wrapped in thick brown paper and equipped with electrodes for the E_h measurements. All the flasks except III were kept at room temperature. Samples for pH and ascorbic acid determinations were taken at certain intervals, the first 1 ½ hours after the inoculation of flasks V—VII. The results are given in Fig. 3 and Fig. 4.

It can be seen from Fig. 3 that a remarkable fall in the ascorbic acid content of all the samples during the beginning of the experiment has taken place. In cases I and II, milk and skim milk stored at room temperature, the bacterial growth has quickly and abruptly lowered the oxidation-reduction potential, and subsequently only a slight decrease in ascorbic acid can be noticed. In pasteurized milk the fall of E_h has been weaker and much more ascorbic acid has been destroyed. In case III where milk was stored in an ice-box, the E_h has remained high during the whole period of the experiment, and a continuous, rapid fall in ascorbic acid has occurred.

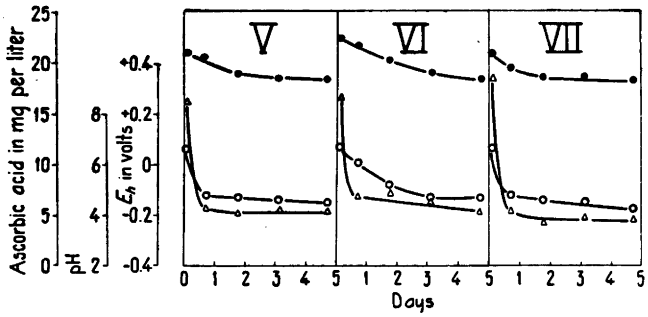

Fig. 4. Stability of ascorbic acid in fermented milks. V. Fermented with starter. VI. Fermented with Lactobacillus acidophilus. VII. Fermented with Streptococcus lactis. All stored at 20° C.

●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

Fig. 4 shows that in all fermented milks the stability of ascorbic acid has been fairly good. Owing to the inoculation with bacteria, the potential of these samples has begun to fall at the very beginning of the experiment and only slight losses of ascorbic acid have occurred.

Garret, Arnold, and Hartman [20] have stated that factors associated with the individual cow have a marked influence on the stability of ascorbic acid in milk. This fact could be noticed also in the present investigation. The milk used in different experiments was usually taken from one cow only, and marked variations in the stability of ascorbic acid was observed. As the E_h measurements showed (compare, *e. g.*, the E_h curves of milk in Fig. 2 and Fig. 3, respectively) the abrupt fall of the potential occurred sometimes earlier, sometimes later, probably depending on the extent of the bacterial contamination of milk. This observation gives one possible explanation for the variations in the stability of ascorbic acid in different milk samples and in the milk of different cows. Variations in the air content of milk offer another explanation. It is probable that relatively more air is mixed in the milk of a cow with low milk production than in that of a cow with high production.

In the experiments described above attention was paid to the changes in the amounts of reduced ascorbic acid only, the amounts of dehydroascorbic acid not being determined. In some experiments, however, also the *total vitamin C* was determined, and its changes during the storage of milk and fermented milks were investigated. The results of such an experiment, made with pasteurized milk (62—63° C, 30 minutes), are given in Fig. 5.

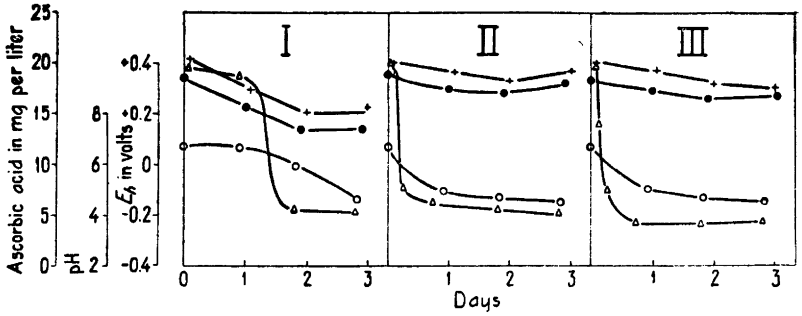

Fig. 5. Stability of total vitamin C and of ascorbic acid in milk and in fermented milks. I. Pasteurized milk. II. Pasteurized milk fermented with starter. III. Pasteurized milk fermented with Streptococcus lactis. All stored at 20° C.

+——+ *Total vitamin C*
●——● *Ascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

Although the differences are not very great it can be distinctly observed also in this experiment that the reduced ascorbic acid has disappeared more rapidly in ordinary milk than in fermented milks. In addition it is observable that the course of the curve representing total vitamin C and of that representing reduced ascorbic acid, are quite parallel. Accordingly, the dehydroascorbic acid formed by the oxidation of ascorbic acid is rapidly destroyed (*cf.* Hand[13]) and a decrease observed in the amount of reduced ascorbic acid means thus also that a decrease in the total vitamin C potency of milk has taken place.

In order to know whether the fermented milks are able only to inhibit the oxidation of ascorbic acid, or whether they can also reduce dehydroascorbic acid to ascorbic acid, experiments were arranged where dehydroascorbic acid (prepared by iodine oxidation according to Stewart and Sharp[21]) was added to fermented milks. Fig. 6 shows the course of an experiment with milk fermented by a ropiness-causing culture of *Streptococcus lactis*. It is seen that the amount of dehydroascorbic acid has fallen rapidly, while that of ascorbic acid has continuously increased. Exactly the same picture was obtained from milk fermented with ordinary starter. In uninoculated milk, the added dehydroascorbic acid disappeared more rapidly still, but no reduction to ascorbic acid could be noticed.

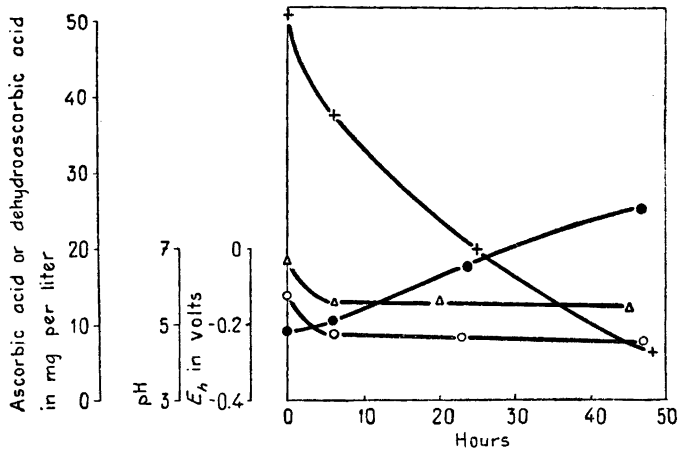

Fig. 6. Reduction of added dehydroascorbic acid in fermented milk. (Stored at 20° C).

●——● *Ascorbic acid*
\+ —— + *Dehydroascorbic acid*
○——○ *pH*
△——△ E_h *(pH = 6.0)*

DISCUSSION

The oxidation of ascorbic acid is a problem of much interest both from the theoretical and the practical points of view. Numerous investigations have been made concerning the mechanism of oxidation as well as concerning the stability of ascorbic acid in different foodstuffs and under different conditions. It has been shown that foods contain heavy metals and enzymes which are able to oxidize ascorbic acid and, on the other hand, substances or systems which tend to inhibit this oxidation. Since ascorbic acid is very stable in strongly acid solutions, it has been commonly assumed that also the acidity of foodstuffs has a marked influence on the stability, ascorbic acid being more stable in acid than in neutral materials. Very little attention has been paid to the direct importance of the oxidation-reduction potential, although great variations occur in the potentials of different foodstuffs.

In the experiments described above the influence of the acidity, as well as that of the oxidation-reduction potential on the stability of ascorbic acid in milk and fermented milks was investigated. Some experiments were made also with cultures of *Lactobacillus acidophilus* in an artificial medium. It was shown that the stability of ascorbic acid was neither in milk nor in the culture medium used dependent on the acidity in the pH range tested, *i. e.*, between

pH 4 and 7. In general, it seems probable that the differences of acidity occurring in foodstuffs have no influence or only a slight one on the stability of ascorbic acid.

The oxidation-reduction potential, on the other hand, was shown to have a distinct effect on the stability of ascorbic acid. While ascorbic acid was rapidly destroyed in a sterile culture medium, only very slight destruction could be noticed when it was added into a culture of *L. acidophilus*, in which the bacterial growth had brought down the oxidation-reduction potential. In fresh milk with its high initial potential a rapid fall in the ascorbic acid content, and in the total vitamin C content as well, was shown to take place, until bacterial growth had brought the oxidation-reduction potential to a low level. In fermented milks, on the other hand, the decrease in the ascorbic acid content was only very slight. Dehydroascorbic acid when added to fermented milks was to a large extent reduced to ascorbic acid.

It is probable that the effect of the oxidation-reduction potential on the stability of ascorbic acid similar to that observed in milk and in fermented milk has importance also in other foodstuffs. Furthermore it is possible that the level of the oxidation-reduction potential of foods is significant also for the stability of other readily oxidizable food constituents, such as vitamin A, carotene, vitamin E, »essential fatty acids,» etc. — There are great differences in the oxidation-reduction potentials of different foodstuffs. The E_h values of certain fruits and vegetables, for example, are known to be widely differing (*cf.* Jørgensen [22]). On the other hand, it is well known that great variations occur in the ascorbic acid contents of different vegetable foodstuffs. It will be an interesting problem to investigate the stability of ascorbic acid and other easily oxidizable and reducible constituents in such foodstuffs in the preparation of which fermentation processes take place, as bread, butter, cheese, salted meat, sauerkraut, beer, etc.

From the practical point of view it would be important to know, first, the fate of »reduced» foodstuffs in the alimentary canal, and, second, how to choose and prepare such foodstuffs, the constituents of which are in a »reduced» state. The oxidation-reduction potentials of the different parts of the alimentary canal are to date very little known. In the mouth the conditions evidently are suitable for oxidations, due to the presence of air, but in the stomach the potential is probably already lower. In the intestines with vigorous bacterial growth strongly reducing conditions evidently prevail. Noticeable oxidation of food constituents could be expected to take place above all in the mouth and perhaps still in the stomach. As far as ascorbic acid is concerned it has been shown by Jenkins [23], however, that only small irreversible losses may occur during mastication. According to Wacholder [24], saliva,

gastric juice, and the digestive juice of the duodenum contain some protective agents which inhibit the oxidation of ascorbic acid and dehydroascorbic acid, until absorption takes place in the duodenum. As for ascorbic acid, at least, there thus seems to exist no great danger of destruction once the food has come into the mouth.

Treatment of foodstuffs in such a way as to avoid the oxidative processes as much as possible, is a problem on which an enormous number of investigations has been performed. Efforts have been made to find out methods by the use of which the losses of valuable food constituents as well as the development of objectionable flavors could be avoided. Important progress in this direction could be effected by an increased use of such food products as have natural reducing properties. Such products are, *e. g.*, fermented milks produced in appropriate ways. Ordinary milk stored in the cold often takes on, as result of oxidative processes, objectionable flavors which make the milk distasteful to the human palate. In a reducing environment the off flavors do not form readily (*cf.* Greenbank [25]); the lowering of the E_h can even remove slight flavors already developed. Because bacterial growth rapidly lowers the oxidation-reduction potential of milk, the flavors do not develop when milk is fermented. On the contrary, fermented milks often take on flavors which make them very pleasing to many people. Fermented milks are commonly used in Finland and the other Scandinavian countries and in many other countries as well, and they are known to be valuable therapeutic foodstuffs. In the light of newer knowledge an increased use of fermented milks can well be recommended. The explanation of the different aspects which have significance for the use of fermented milks in nutrition, requires, however, much research work, including nutrition experiments both with animals and human beings.

SUMMARY

The main part of the experiments described deal with the effect of the oxidation-reduction potential on the stability of ascorbic acid in milk and in fermented milks. Some experiments were made also with cultures of *Lactobacillus acidophilus* in an artificial medium.

Ascorbic acid was found to be very stable when it was added into a culture of *L. acidophilus*, where the bacterial growth had brought down the oxidation-reduction potential. In the sterile medium with its high potential, on the other hand, ascorbic acid was rapidly destroyed. The stability was not dependent upon the pH within the range from 4 to 7.

Acidifying of milk with lactic acid down to pH 6.0, 5.2, or 4.2, respectively, did not increase the stability of ascorbic acid. When fresh milk was stored at

room temperature its ascorbic acid content, as well as the total vitamin C content decreased at first rapidly. The fall ceased, however, after bacterial growth had abruptly lowered the oxidation-reduction potential. When milk was stored in cold (1—3° C) the potential remained high during several days and the ascorbic acid content fell continuously.

The oxidation-reduction potentials of milks inoculated with a starter, *L. acidophilus*, or *Str. lactis*, respectively, fell rapidly. The losses of ascorbic acid in these fermented milks during storage at room temperature were very small.

Dehydroascorbic acid, added to fermented milks was to a large extent reduced to ascorbic acid.

The effect of the oxidation-reduction potential of foods on the stability of ascorbic acid and other easily oxidizable constituents is discussed. Attention is paid also to the fate of »reduced» foodstuffs in the alimentary canal as well as to an increased use of such foods, *e. g.*, fermented milks, in nutrition.

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Received March 31, 1947.