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FLAVOR AND VITAMIN STABILITY IN FLUID MILK

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The factors involved in the flavor and vitamin stability of fluid milk have not been established with any marked degree of agreement despite the numerous studies published to correlate these variables. The purpose of this report is to present some of the particularly controversial issues as well as a possible reconciliation developed from studies made in this laboratory.

Vitamin Deterioration

A partial explanation of the relationship of riboflavin, ascorbic acid, light, and oxygen in milk was presented by Hand and coworkers (11) in 1938. They demonstrated that when all ascorbic acid and riboflavin were removed from milk and then ascorbic acid replaced, very little ascorbic acid was lost until riboflavin was added. Similarly, when the riboflavin was removed from milk by adsorption or destruction, the ascorbic acid became more stable. When the oxygen was removed from milk, the ascorbic acid loss was reduced, but not to the same extent as when the riboflavin was eliminated. Thus, the authors concluded, "Lactoflavin is the sole agent in milk responsible for the sensitivity of ascorbic acid to light." These findings were further verified by later studies (12).

The photo-oxidation of riboflavin in milk was also described by Williams and Cheldelin (24), who reported that 64 percent was lost when milk was boiled in a lighted room for 45 minutes, but only 5 percent was destroyed when identical samples were boiled in the dark. The fact that the loss of riboflavin may be as high as 85 percent on exposure of milk to sunlight in a period of 2 hours has also been demonstrated, together with the practically total loss of ascorbic acid within the first 30 minutes of the experiment (15). The same workers (15) showed that two identical samples of milk would lose riboflavin in direct proportion to the intensity of the light falling on each sample. In this connection, Stamberg and Theophilus (22) observed that the destruction of riboflavin by light was greater in raw milk than in pasteurized. The least destruction was incurred by homogenized-pasteurized milk. Although not expressed by the authors, here is excellent evidence of the possible role played by enzymes in milk deterioration, although phosphatase itself in raw milk merely accelerates the breakdown which ultimately occurs in processed milk.

Flavor Deterioration

The oxidized flavor which becomes evident in milk after exposure to light, air and prolonged storage conditions has been variously reported (3, 4, 7, 8, 10). However, the exact origin and nature of this objectionable quality has not been described, nor has the relationship between the development of the oxidized flavor and the concentration of both ribo-

flavin and ascorbic acid in milk been clearly explained. Krukovsky and Guthrie (18, 19) have claimed that ascorbic acid definitely accelerated the development of oxidized flavor in milk and that the maximum acceleration occurred when the ascorbic acid was a mixture of the reduced and dehydro forms. Rapid oxidation of ascorbic acid beyond the dehydro stage presumably eliminated its prooxidant effect.

The 1938 work of Hand, Guthrie, and Sharp (11) referred to studies carried out by Sharp in 1936 in which it was found that addition of 0.005—0.01 percent ascorbic acid to milk delayed the development of oxidized flavor, thus establishing an apparent contradiction to the 1945 and 1946 work of Krukovsky and Guthrie (18, 19). Chilson and coworkers (2) have found that the addition of 1.5 grams ascorbic acid (0.003 percent) to 100 pounds of milk prevented the formation of oxidized flavor even after the seventh day of dark refrigerated storage, whereas the control samples developed an oxidized flavor after 2 days of such storage. Milk fortified with ascorbic acid, however, rapidly lost it on exposure to sunlight or on treatment with hydrogen peroxide. Nevertheless, the authors found that no oxidized flavor occurred in the milk even after a 5-day storage period. This does not appear to detract from the work of Krukovsky and Guthrie (18, 19), who have emphasized the development and control of oxidized flavor under conditions similar to those described in the Chilson paper. However, neither group attempted to correlate the riboflavin content itself with oxidized flavor.

Recently, Holmes (14) made a study of mare's milk in order to learn why ascorbic acid disappeared from cow's milk ten times faster than from mare's milk when both were stored under the same conditions. While Holmes did not mention the oxidized-flavor relationship, his paper has significance in this regard, as will be indicated later. The rapid destruction of ascorbic acid was first assumed to be due to the fact that cow's milk contained much more riboflavin than mare's milk; Holmes decided to study the effect of adding riboflavin to samples of mare's milk in order to observe the reaction upon the reduced ascorbic acid content. The sample enriched with 2 mg. of riboflavin per liter lost 3.8 percent reduced ascorbic acid daily, and the sample treated with 4 mg. per liter lost 4.1 percent ascorbic acid; but at the end of a 5-day storage period in the dark at 10°C., both samples retained about 85 percent of their original reduced ascorbic acid. These data were interpreted as indicating that riboflavin was not the principal factor in causing the rapid disappearance of ascorbic acid from milk stored in the dark at 10°C. The interesting graph presented by Holmes illustrating these data will be discussed later. In the interim, the conclusion that riboflavin was not the principal cause of the ascorbic acid loss in mare's milk adds to the complexity of the problem. This study does not appear to be a reaction activated by prolonged light exposures, although the description of the experimental conditions does allow for considerable light radiation and aeration during the early stages of the investigation.

Mechanism for Vitamin and Flavor Deterioration

In 1945-46 (6) during an investigation of the conditions (light, temperature, storage period) affecting the vitamin content and general composition of raw and processed cow's milk, the writer observed that the concentrations of components determined to a large extent the storage stability of the milk. For example, the various batches of pasteurized milk contained the following ranges, per 100 ml. of sample, of what were considered to be the most critical components: 0.11–0.19 mg. of riboflavin, 1.0–1.9 mg. of ascorbic acid, 0.24–0.28 mg. of iron, and 0.020–0.024 mg. of copper. Thus, there were sufficient amounts of iron to catalyze the riboflavin oxidation and of copper to catalyze the destruction of ascorbic acid. When milk contained the lower riboflavin value, no oxidized flavor could be detected until the fourth day of dark refrigerated storage. The milk with the higher riboflavin content developed an oxidized flavor on the second day of storage. There was an approximately inverse proportion between the riboflavin and the ascorbic acid content in each sample, within the range previously stated. Also, during taste-panel studies of the oxidized flavor, a distinct odor developed together with the flavor deterioration and, in some samples, acceptability ratings could be defined as accurately by odor as by taste.

A continuation of these studies within recent months led to a possible explanation of the mechanisms which cause the deterioration of ascorbic acid, riboflavin, and flavor in milk on exposure to light, air, and storage. When the two isolated vitamins (ascorbic acid and riboflavin) and the two isolated minerals (copper and iron) were dissolved separately in pasteurized milk solutions, employing the procedure of Hand (11), the ascorbic acid and riboflavin proved to be fairly stable constituents in dark refrigerated storage over a 10-day period. The ascorbic acid solution did not exhibit any marked deterioration on exposure to air and light until the copper solution was added. The addition of the riboflavin solution merely accelerated the destruction of the ascorbic acid, and was unnecessary for the initiation or continuation of the reaction.

An attempt was made to prepare a riboflavin-free milk (11) which could be subjected to oxidized-flavor studies, but milk treated to remove the vitamin tasted so strange that "oxidized flavor" as such was considered but a minor defect. It was not possible to detect the progressive development of the oxidized flavor under this condition.

An ascorbic acid-free milk was readily prepared by exposing frozen pasteurized milk in flat enameled pans, 1 inch deep, to bright sunlight for 25 minutes. Ascorbic acid analyses were made by the direct titration method using the sodium 2,6-dichlorobenzenoneindophenol dye. Such milk did not develop an oxidized flavor until after the fourth day of dark refrigerated storage. When, however, such sunlight-exposed milk was only chilled, omitting light-protective measures entirely, the oxidized flavor could be detected at the end of the third day. It would thus appear that riboflavin must be

mainly the cause of the development of the oxidized flavor and not ascorbic acid.

A further investigation of the riboflavin activity was therefore advanced. Riboflavin in milk represents an intermediate complex, as in plant and animal tissues this vitamin is almost entirely combined with protein. In urine, the vitamin is all in the free state, whereas in milk it is partly free and partly combined. By using the fluorometric method of analysis (1) with and without hydrolysis, it was observed that two-thirds of the riboflavin present in fresh pasteurized milk is in the free form. The one-third which is combined represents an enzyme complex consisting of riboflavin, 2 molecules of phosphoric acid, one molecule of adenine, and a specific protein molecule.

In milk, there are present mainly 2 such yellow enzymes (23): diaphorase and xanthine oxidase, each one possessing a slightly different protein structure but otherwise retaining a structural similarity to each other. Both enzymes are oxidative. As the name, xanthine oxidase, indicates, it is capable of oxidizing xanthine or purine derivatives to uric acid. It also oxidizes a variety of aliphatic and aromatic aldehydes to the acid stage. The diaphorase (actually 2 exist) oxidizes the reduced forms of coenzymes 1 and 2 which act to decompose carbohydrates and release niacinamide from molecular combination. In the degradation of carbohydrates, many of the aldehydes are formed which are then oxidized by the xanthine oxidase. When xanthine oxidase acts upon its substrates in the presence of air, *hydrogen peroxide* is formed (23). This explains in part the accelerating action that the riboflavin complex has upon the destruction of ascorbic acid. However, the hydrogen peroxide *inhibits* the breakdown of the riboflavin enzyme unless catalase is present to destroy it, and milk contains only traces of catalase. Despite the fact that catalase is one of the most effective enzymes known, the peroxide present under these conditions is capable of destroying this enzyme completely. The yellow enzymes, however, ultimately yield their protein and riboflavin in free form on exposure to light. The writer observed that the most destructive radiations were in the range of 445-460 millimicrons.

Based on the information obtained that riboflavin was mainly responsible for the development of the oxidized flavor in milk, it was recalled that certain visual disturbances (dimness of vision) could be cured by riboflavin within a short time (21). The evidence indicated, therefore, that riboflavin might sensitize the retinal cells through conversion by light of substances present into a compound which is capable of stimulating the optic nerve. Thus, riboflavin can be considered a receptor pigment which can elicit a phototropic response (photo-oxidation) in compounds other than itself. Galston and Baker (9) demonstrated such a reaction during a study of the mechanism of light action on plant growth. They found that riboflavin could sensitize the photo-oxidation of various indole-containing compounds including the amino acid, tryptophane. The authors showed that the oxidation took place even when the amino acid was included within a protein molecule. In fact,

Galston found that all the enzymes tested were inactivated when suspended in riboflavin solutions subjected to moderate intensities of light. It is, therefore, reasonable to visualize the manner in which protein chains from milk and from the riboflavin enzymes can be readily oxidized and inactivated by way of the tryptophane link. Furthermore, oxidation of tryptophane gives rise to indole and indole derivatives, the taste and odor of which are well known.

Once the protein molecule is broken, the other amino acids are released to greater or smaller degree, thereby producing the sulfhydryl groups of cystine and methionine (5). The production of hydrogen sulfide under such circumstances is a natural sequence and this gas, even in the minute quantities formed, can be detected with moistened lead acetate paper. The degree of hydrolysis in milk when tryptophane is oxidized may be approximated by noting that the milk contains about 3.5 percent protein. The tryptophane content is 2.0 percent of the milk protein; methionine is 2.8 percent of this protein and cystine is 1.2 percent (5). While the usual proteolytic action in milk can produce protein fragments of peptide length, the tryptophane-sensitized oxidation may shorten these chains even more.

Another light-sensitive enzyme in cow's milk was described by Kay (16) and Krukovsky (17). The lipase (tributyrinase) Kay studied proved to undergo the same type of photo-destruction in the presence of riboflavin as that mentioned by Galston (9). In this instance, however, the reaction can give rise to the formation of butyric acid. The fact that this lipase is destroyed within 3 hours after exposure explains why no greater quantity of butyric tastes and odors are produced. Kay indicated that this lipase, as well as ascorbic acid, underwent continued decomposition in the presence of riboflavin even when the milk was returned to dark refrigerated storage after a short initial light exposure.

Because of the marked complexity of the activity of riboflavin (free and combined) in milk, there is a similar complexity of origins for the production of the oxidized flavor, some of which are:

1. Diaphorase oxidation of reduced coenzymes, the products of which are carbohydrate derivatives including aldehydes and pyridines of the niacinamide type.
2. Xanthine oxidase oxidation of aliphatic and aromatic aldehydes to the acid stage, production of uric acid and a peroxide.
3. Lipase action responsible for the production of butyric derivatives.
4. Photo-oxidation of tryptophane, producing indole and indole derivatives.
5. Proteolytic action giving rise to the sulfhydryl groups and hydrogen sulfide.
6. Bacterial action.

Thus, the initial concentrations of the enzymes and vitamins in the milk, together with light exposure, storage conditions, aeration, and sanitation, will largely determine the stability of that milk.

Discussion

Through the use of this information, it may be possible to evaluate the controversial statements cited

previously with some degree of correlation:

In general, the work of Hand and coworkers (11) was verified except for the declaration that riboflavin *alone* was responsible for the sensitivity of ascorbic acid to light. The presence of copper, dissolved oxygen, and sunlight catalyzed the decomposition of ascorbic acid beyond the dehydro-stage, forming peroxide (13). Because the deterioration of the ascorbic acid did not cease when the sample was placed in dark refrigerated storage, it was concluded that the peroxide present was sufficient to activate a chain reaction which slowly continued the oxidative destruction. There can be no doubt that the photo-sensitization of ascorbic acid in the presence of riboflavin is the principal reaction, but this reaction is not the only accelerator of ascorbic acid inactivation.

Concerning the observation by Stamberg and Theophilus (22) that the photo-oxidation of riboflavin was greater in raw milk than in pasteurized milk, the writer found that phosphatase could hasten such destruction through ability to oxidize the two types of yellow enzymes at the phosphoric acid linkages, thereby releasing free riboflavin from an otherwise fairly stable complex. As is well-known, the phosphatase is completely destroyed by pasteurization, leaving the combined riboflavin complex unbroken through any action by this enzyme.

The reports by Krukovsky and Guthrie (18, 19) that ascorbic acid in milk accelerated the development of oxidized flavor must be carefully considered in conjunction with the work of Chilson (2). It is not sufficient to conclude that partial oxidation of the ascorbic acid might cause the acceleration rather than the inhibition of the oxidation of milk fat, for example, and that further rapid oxidation of the ascorbic acid might be expected to eliminate its pro-oxidant effect. This would lead to the assumption that the completeness and speed of the ascorbic acid oxidation would eliminate oxidized flavor formation. Since oxidized flavor ultimately occurs, even after the rapid oxidation of ascorbic acid, it is difficult to explain this flavor defect in terms of ascorbic acid action alone. Also, the mixture of the reduced and dehydroascorbic acids which Krukovsky (18, 19) proposed as the accelerator of oxidized flavor appears to rely mainly upon the o-quinoid-type structure of dehydroscorbic acid for the oxidative action. At the normal pH of milk, 6.6, the dehydroascorbic acid is rapidly converted to an irreversible, physiologically inactive product. The speed of this reaction may be considered only a minor point in favor of the prevention of oxidized flavor.

However, the action of peroxides upon fats to cause "reversion" is well known. Thus, the oxidized flavor in milk is acknowledged as being partly due to the peroxide action upon the milk fat. The peroxide quantities released from the ascorbic acid oxidation and the xanthine oxidase activity are exceedingly minute (0.002%), however, when compared with the 0.1% or more of hydrogen peroxide used to preserve milk experimentally. Duplication by the writer of the work of Chilson (2),

with higher peroxide preservative concentrations, revealed that it is possible to oxidize the entire ascorbic acid present in milk within minutes, and under these same conditions, sterilize the fluid and oxidize much of the free riboflavin to lumichrome. The lumichrome is capable of photosensitizing the oxidation of tryptophane and other components, but performs this activity at a much slower rate than riboflavin itself (9b). Thus, the development of the oxidized flavor is delayed but not eliminated through the use of hydrogen peroxide.

The fortification of milk with ascorbic acid such as was done by Chilson (2) and Hand (11) serves to remove, temporarily, the available oxygen required for the photo-sensitizing reaction of riboflavin. Chilson stated that while such fortified milk did lose all of its ascorbic acid rapidly when exposed to sunlight or to a solution containing peroxide, it did not develop oxidized flavor until the fifth day of storage. Thus, a temporarily protective quality seems to have been conferred upon the milk by either the excess ascorbic acid or the peroxide treatment. It appears that the keto-gulonic acid breakdown products from ascorbic acid are of a peroxide type and act in a manner analogous to hydrogen peroxide. On the other hand, it is difficult to conceive of the keto-gulonic oxidation products functioning as antioxidants. Fortification of milk with ascorbic acid therefore appears to be most advantageous when that milk is retained in dark refrigerated storage.

With reference to the work of Holmes (14) on the riboflavin enrichment of mare's milk to hasten the ascorbic acid destruction, it is necessary to note the comparative values of both mare's and cow's milk as listed in table 1.

Analyses for water, fat, protein, sugar, ash compared favorably with Sherman (20) for both types of milk. The ascorbic acid and riboflavin values are averages for cow's milk and were determined by the writer (6), whereas the ascorbic acid average for mare's milk was obtained from Holmes (14). The riboflavin content of mare's milk was difficult to ascertain (6) because of the wide variation observed, but very few samples contained more than 0.02 mg. per 100 ml. An average of 54 percent of the riboflavin in mare's milk was in the combined form.

A survey of the comparative data on mare's and cow's milk shows the fat, protein, and vitamin values to be significant factors. The writer could detect no oxidized flavor in pasteurized mare's milk which had been stored in a dark refrigerator

TABLE 1.—ANALYSIS OF MARE'S AND COW'S MILK

	Water (percent)	*Fat (percent)*	*Protein (percent)*	*Sugar (percent)*	*Ash (percent)*	*Ascorbic acid (mg/100 ml.)*	*Riboflavin (mg/100 ml.)*
Cow's milk..	87.00	4.00	3.35	4.90	0.75	1.5	0.15
Mare's milk.	90.75	1.20	2.00	5.70	0.35	13.5	0.02

for 8 days, whereas cow's milk had an oxidized flavor after 2 days of identical storage. Apparently, the high ascorbic acid content presented conditions typical of an unusually well-fortified milk. The low fat content of the mare's milk was also a factor in maintaining its superior storage qualities. The extremely low riboflavin value in mare's milk coupled with the low protein content, provided a poor basis for the photo-sensitized reaction between riboflavin, ascorbic acid, and tryptophane. Thus, one would expect that dark refrigerated storage of mare's milk would bring about very little deterioration of riboflavin and flavor, and such was the case. However, based on the previously discussed peroxide activated chain reaction, it is not surprising to note that Holmes found ascorbic acid continuing to deteriorate even in the dark. His conclusion, therefore, that riboflavin was not the principal factor in causing the rapid disappearance of ascorbic acid from milk stored in the dark at 10° C. (14) seems only partially tenable as both ascorbic acid and xanthine oxidase deterioration give rise to peroxide. The riboflavin is still a potent accelerator of this reaction, as there were short intervals of light exposure and no precautions were taken to remove available oxygen from the samples initially.

Holmes (14) illustrated the course of ascorbic acid deterioration in the presence of riboflavin-enriched mare's by three curves: one for normal milk and two for milk to which riboflavin had been added, as previously described. The first curve appears to be sharply divergent from the other two. Nevertheless, all three curves are very nearly parallel after the first day. Thus, as long as free riboflavin is present, particularly after the unavoidable light exposures in preparing the samples for storage and for analysis, the ascorbic acid loss is comparatively sharp and precipitous for the first day. Then, on the second day, the riboflavin complex, being fairly stable, does not induce a very great ascorbic acid loss until hydrolysis of this complex occurs. Once this has taken place, free riboflavin is again available for furnishing the means for further destruction of the ascorbic acid.

Summary and Conclusions

1. Factors which have been found to influence the stability of milk include: ascorbic acid (reduced and dehydro), riboflavin, lipase, xanthine oxidase, diaphorase, copper, iron, dissolved oxygen, light, and temperature.

2. Riboflavin accelerates the destruction of ascorbic acid in milk, but is unnecessary for the initiation or continuation of the reaction.

3. Riboflavin is mainly responsible for the development of the oxidized flavor in milk, whereas ascorbic acid is only secondarily so.

4. In fresh pasteurized milk, two-thirds of the riboflavin is in the free form. The other third is combined as an enzyme-complex consisting of riboflavin, 2 molecules of phosphoric acid, one molecule of adenine, and a specific protein molecule, the protein differing for the particular enzyme concerned (xanthine oxidase and diaphorase).

5. Riboflavin is responsible for the photo-oxidation of tryptophane in the milk protein, producing indole derivatives.

6. Sulfhydryl groups and hydrogen sulfide are detectable in milk undergoing light and storage deterioration.

7. The destruction of ascorbic acid appears to be a chain process which continues to completion once initiated in the milk, whereas the riboflavin loss can be markedly delayed by storing the milk, de-aerated, in a dark refrigerator.

8. A peroxide action upon the milk fat has been traced to the action of xanthine oxidase and ascorbic acid oxidation.

9. Fortification of milk with ascorbic acid results in the stabilization of the milk by the ascorbic acid functioning as a antioxidant which removes dissolved oxygen from the solution.

10. The use of peroxide in the preservation of milk is based upon sterilizing action, degradation of fatty oxides, oxidation of riboflavin to lumichrome, and the oxidation of ascorbic acid irreversibly to physiologically inactive products which do not bring about further objectionable changes in the milk.

11. The slower deterioration of mare's milk as compared with cow's milk may be due to the fact that mare's milk contains approximately ten times the amount of ascorbic acid and about one-eighth the riboflavin of cow's milk. The riboflavin in mare's milk is combined to a greater extent (54%) than the same vitamin in cow's milk (33%).

12. Milk contains a lipase which is responsible for the production of butyric acid.

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