Inhibition of L-Ascorbic Acid Oxidation by Type II Gas Hydrates

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Oxidation of L-ascorbic acid (vitamin C) was inhibited substantially by the gas hydrates (aqueous clathrates, clathrate hydrates) CCl_3F, CCl_2F_2, and C_3H_8 at temperatures in the range of 6° to −17° C. These Type II hydrates contain cavities of two sizes, only the larger of which can accommodate guest molecules of CCl_3F, CCl_2F_2, or C_3H_8. The smaller cavities, which constitute two-thirds of the total number, apparently absorb atmospheric oxygen with sufficient tenacity to prevent it from participating in oxidative reactions.

G as hydrates (clathrate hydrates, aqueous clathrates) are ice-like inclusion compounds wherein water, the "host" substance, forms hydrogen-bonded, cage-like structures that physically entrap molecules of a second molecular species, known as the "guest" or "hydrate former." Crystals of classical gas hydrates (composed of simple cages able to accommodate one guest molecule per cage) can exist in either a Type I or a Type II structure, both of which have cubic symmetry (Claussen, 1951; Stackelberg, 1949; Stackelberg and Müller, 1954). Size of the guest molecule dictates the structural type (Claussen, 1951), i.e., guest molecules too large for cavities of the Type I structure will promote formation of Type II hydrates.

Water molecules in the Type I structure are hydrogen-bonded in a way so that a unit cell contains two cavities, each with a diameter of 5.2 Å, and six larger cavities, each with a diameter of 5.9 Å. When all cavities are occupied by guest molecules, the molecular ratio (water/guest) is 5.75, whereas occupation of only the larger cavities results in a ratio of 7.67. Ethyl fluoride, methyl chloride, methyl bromide, chlorofluoromethane, and chlorodifluoromethane are examples of guest molecules which can fill only the larger cavities, and argon, methane, ethane, hydrogen sulfide, methyl fluoride, nitrous oxide, and carbon dioxide are guest molecules which can fill cavities of both sizes (Barduhn et al., 1960; Claussen, 1951; Müller and Stackelberg, 1952; Stackelberg and Müller, 1954). Water molecules in the Type II structure are hydrogen-bonded in a way so that a unit cell contains 16 cavities, each with a diameter of 4.8 Å, and eight larger cavities, each with a diameter of 6.9 Å. Complete occupancy of all cavities results in a molecular ratio (water/guest) of 5.65, whereas occupancy of only the larger cavities results in a ratio of 17. Dichlorodifluoromethane, trichlorofluoromethane, difluoroethane, chlorodifluoroethane, propane, and isobutane are molecules which are too large to form Type I gas hydrates, but are suitable for the Type II structure. These guest molecules can occupy only the larger cavities of the Type II structure (Claussen, 1951; Stackelberg and Müller, 1951, 1954).

Gas hydrates have been studied extensively and many of their characteristics are now well defined (Hagan, 1962; Huang et al., 1965; Jeffrey and McMullan, 1967; Lund et al., 1969; Mandelcorn, 1959, 1964; Palmer, 1950; Swern, 1957; van der Waals and Platteeuw, 1959; Van Hulle et al., 1966). One of the most interesting properties exhibited by many gas hydrates is an ability to exist above 0° (provided the pressure is sufficient). Several uses have been suggested for gas hydrates, but none has yet attained commercial importance (Barduhn, 1963; Barduhn et al., 1960, 1962; Glew, 1962;

Hagan, 1962; Huang et al., 1966; Pauling, 1961). An apparently unexplored area of potential importance is the effect of gas hydrates on vitamin stability. Reported here are effects of several kinds of gas hydrates on stability of L-ascorbic acid (vitamin C). This compound was chosen for study since it is an important vitamin that deteriorates at a significant rate in many foods during storage at refrigerated and subfreezing temperatures.

MATERIALS AND METHODS

Preparation of Gas Hydrates. All studies involving CCl_3F (f-11) hydrates were performed in cylindrical 65-ml glass bottles. Unless specified otherwise, each bottle was provided with two glass rods (8 mm o.d., 70 mm long), 8 mg L-ascorbic acid, 20 ml acetate buffer (0.02 M, pH 4.6) and 23 vol% f-11 (redistilled, DuPont). This amount of f-11 was theoretically sufficient to bind all the water in the form of hydrate crystals, assuming 95% occupancy of the Type II hydrate structure (Lund et al., 1969). The hydrate former (f-11) was added through a valve in the bottle closure to avoid loss of air from the head space.

Filled bottles were rotated for 1 hr at 100 rpm in an ice-water bath to form the hydrate (Lund, 1968; Lund et al., 1968). Control samples were prepared and treated in the same manner, except that f-11 was omitted. Handling of the samples beyond this point differed, depending on the desired temperature of reaction. Samples for study above 0° were allowed to equilibrate at the desired reaction temperature for 45 min, and the end of this period was regarded as "zero time." Samples for study at sub-zero temperatures were rotated in a Dry Ice-acetone bath, for 1 min at 100 rpm, then placed at the desired sub-zero temperature for 45 min, and the end of this period was regarded as "zero time." Overcooling and rewarming was done to help assure solid-liquid equilibrium at the sub-zero reaction temperature.

Samples containing C_3H_8 (propane; 99.1% pure; Matheson), or CCl_2F_2 (f-12; DuPont) were prepared in a manner similar to that described for the CCl_3F samples, except aerosol cans (143 ml, 202 × 214, double-epoxy lining with stainless steel caps) were substituted for glass bottles to accommodate the high vapor pressure of these hydrate formers.

Analyses. Just before removal of samples for analysis, 30 ml of 3% HPO_3 were added to each container to stop further oxidation of L-ascorbic acid (AA) and to facilitate melting of those samples which were stored below 0°. Duplicate samples were removed at 0 time and at several subsequent times, and each sample was analyzed for its AA content. Knowledge of AA content at various times permitted calculation of reaction rate constants.

Dehydroascorbic acid (DHA) and diketogulonic acid (DKG) contents were determined in the first (0 time) and last samples in some series.

L-Ascorbic acid and DHA were determined by visual titra-

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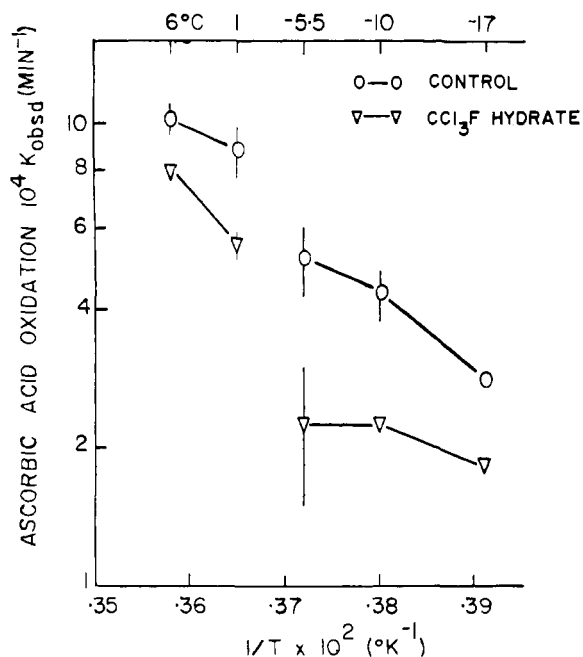
Figure 1. Short-term effect of CCl_3F hydrate on L-ascorbic acid stability at various temperatures. Range at each point represents two determinations. Differences between hydrate samples and controls, averaged over all temperatures, were highly significant
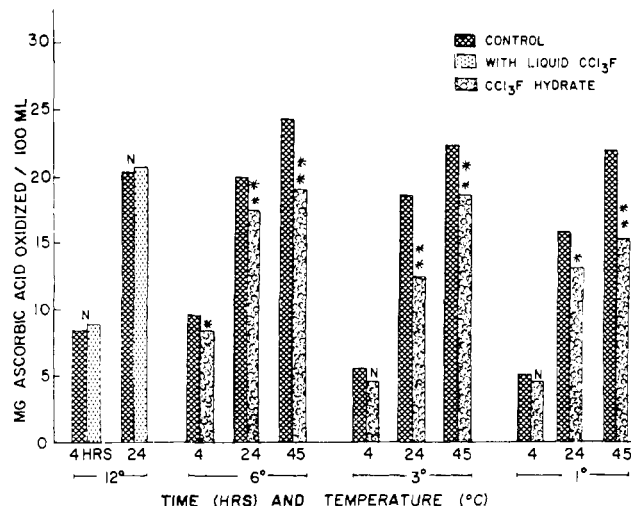

Figure 2. Long-term effect of CCl_3F hydrate on L-ascorbic acid stability at temperatures above 0°. **: Highly significant (1% level); *: Significant (5% level); N: Not significant
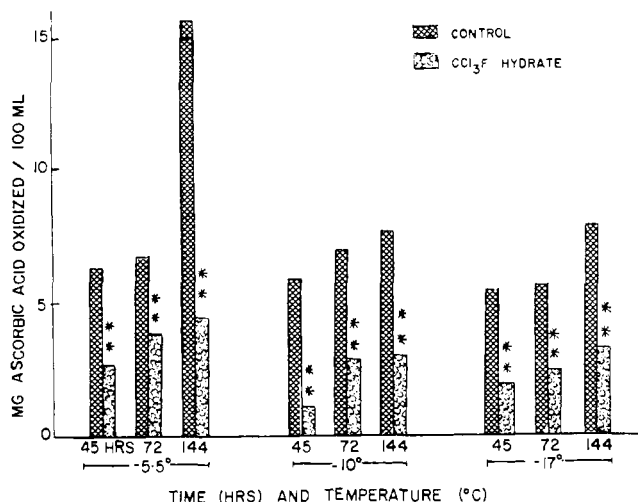

Figure 3. Long-term effect of CCl_3F hydrate on L-ascorbic acid stability at temperatures below 0°. **: Highly significant (1% level)

tion with a solution of 2,6-dichlorophenolindophenol in general accordance with the procedure described by the Association of Vitamin Chemists (1966). Since the original amount of AA was known, and the final amounts of AA and DHA were determined experimentally, it was possible to calculate the amount of DKG (actually DKG and its degradation products) in the following manner.

$$DKG = (original\ AA) - (final\ AA + DHA)$$

RESULTS AND DISCUSSION

Studies with CCl_3F (f-11). SHORT-TERM REACTIONS. These samples were reacted at temperatures of 6°, 1°, −5.5°, −10° and −17° C for periods up to 8 hr. For each temperature, the logarithm of the AA concentration was plotted against reaction time and a pseudo first order rate constant was calculated. These rate constants were plotted against temperature, as shown in Figure 1. When averaged over all temperatures, oxidation of AA was significantly less (1% level) in the presence of CCl_3F hydrate than it was in the controls. Differences at each of the temperatures were obviously significant, since there was no overlap of the replicated data.

Studies with CCl_3F (f-11). LONG-TERM REACTIONS. These samples were reacted at temperatures of 12°, 6°, 3°, 1°, −5.5°, −10°, and −17° C for periods up to 144 hr, and the results are shown in Figures 2 and 3. The ability of CCl_3F hydrate to inhibit oxidation of AA persisted throughout the entire storage period at each temperature, and almost all of the differences were statistically significant at either the 1 or 5% level. It also is evident that the protective effect of CCl_3F hydrate at any given temperature tended to increase with storage time, and was greater at temperatures below 0° than above.

Results obtained at 12° C (Fig. 2) are especially important, since CCl_3F hydrate crystals cannot form or exist at this temperature. The CCl_3F samples therefore contained CCl_3F in an uncombined liquid state, enabling determination of the direct effect of CCl_3F (no hydrate) on AA stability. It is

evident from tests at 12° C that liquid CCl_3F was, if anything, slightly detrimental to AA stability. The protective effect of CCl_3F hydrate on AA, as demonstrated in Figures 1, 2, and 3, must therefore be attributed mainly to some property of the hydrate crystals rather than to a direct consequence of CCl_3F.

The influence of CCl_3F hydrate on the kind and amount of oxidation products from AA was studied both above and below 0°, and the results are shown in Figure 4. After 45 hr at temperatures above 0°, the DKG contents of the hydrate samples and their respective controls did not differ significantly, whereas the DHA contents of the hydrate samples were about one-half (significant at 1% level) that of the controls. Thus at temperatures above 0°, differences in the amount of AA oxidized (total bar height) in the hydrate and control samples can be accounted for nearly entirely by the amount of DHA formed. Although biological activity of DHA is about 75-80% that of AA, its formation is considered undesirable, since it can be easily oxidized to vitamin-C-inactive DKG.

Oxidation products obtained at temperatures below 0° (−5.5°, −10° and −17° C) are indicated in Figure 4 and the results are qualitatively different from those obtained above 0°. After storage for 144 hr at sub-zero temperatures, nearly
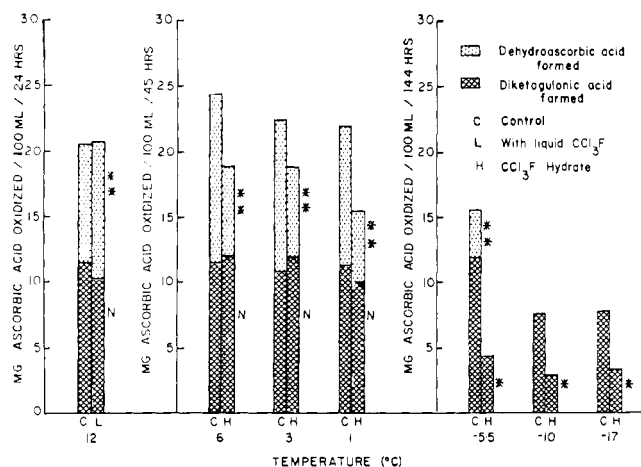
Figure 4. Oxidation products of L-ascorbic acid as influenced by CCl₃F hydrate. **∗∗**: Highly significant (1% level); ∗: Significant (5% level); N: Not significant
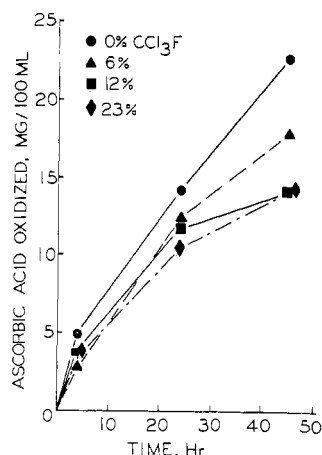

Figure 5. Oxidation of L-ascorbic acid as influenced by the amount of CCl₃F hydrate. Average range for each point is ±0.7 mg

all samples incurred a net loss of DHA, as compared to 0 time values, and in all instances the DKG contents of the hydrate samples were significantly (5% level) less than the DKG contents of corresponding control samples. Since DKG has no vitamin C activity, the protective action of CCl₃F hydrate appears especially important at sub-zero temperatures.

Some of the differences between samples stored above and below 0 may have occurred because the sub-zero samples were stored for longer times and were more completely solidified (controls contained ice) than the above-zero samples.

As is evident from tests at 12° C (Fig. 4), liquid CCl₃F did not significantly affect the total amount of AA oxidized or the amount of DKG formed, but it did result in significantly greater (1% level) amounts of DHA than in the controls. Differences in the kind and amount of oxidation products contained in the hydrate samples and controls therefore must be attributed almost entirely to the hydrate of CCl₃F.

Oxidation of L-Ascorbic Acid as Influenced by the Amount of CCl₃F Hydrate. Hydrate crystals were formed in AA samples containing 6, 12, and 23 vol% CCl₃F and rates of AA oxidation in these samples at 1° C were compared to rates in suitable control samples. From results in Figure 5 (especially the 45 hr data) it can be concluded that increased protection against oxidation of AA resulted as the level of CCl₃F was raised from 0 to 6 to 12%, but no additional protection was obtained when the level was raised from 12 to 23%.

Table I. Stability of L-Ascorbic Acid at 6° C as Influenced by Oxygen-Free Crystals of CCl₃F Hydrate[a]

Reaction time (hr)	mg L-ascorbic acid oxidized per 100 ml of solution[b]		Significance of difference (5% level)[c]
	Controls	Samples containing CCl₃F hydrate	
4	3.0	3.8	n.s.
25	12.4	11.4	n.s.
45	18.3	17.4	n.s.

[a] Samples initially contained 40 mg ascorbic acid per 100 ml of 0.02 M acetate buffer (pH 4.6). Treated samples contained 23 vol % CCl₃F. [b] Means of four replicates. [c] n.s. = not significant.

Buffer-Type and the Ability of CCl₃F Hydrate to Inhibit Oxidation of L-Ascorbic Acid. The ability of CCl₃F hydrate to inhibit oxidation of AA was determined in the presence of buffers of disodium phosphate-citric acid, potassium phosphate-citric acid and sodium acetate-acetic acid, each at 0.02M and pH 4.6. The antioxidant effect was essentially independent of buffer type.

Mechanism of Protection. Since air contains 21% oxygen, oxidation of AA proceeds readily in its presence. Oxygen also is a "help gas" (a chemical which is incorporated into the hydrate structure as a supplement to the primary hydrate-former, and thereby aids stability of the hydrate) for partially-filled Type II hydrates, such as those formed from CCl₃F, C₃H₈, or CF₂Cl₂ (Barduhn et al., 1960; Stackelberg, 1949; Stackelberg and Meinhold, 1954; Villard, 1897). If oxygen functioned as a help gas in the gas hydrates used in these experiments, then part of the oxygen present within the sample containers would have been physically entrapped within the hydrate structure and unavailable for reaction with AA. This appears to be a possible mechanism by which Type II hydrates inhibit oxidation of AA. To test this hypothesis, samples containing AA, buffer, and CCl₃F (23 vol%) were exposed to hydrate-forming conditions in an atmosphere of pure nitrogen. This presumably caused nitrogen (also a help gas) to occupy most of the cavities which were unfilled by CCl₃F. Samples were then stored at 6° C in the presence of air. Control samples (no CCl₃F) were treated in the same manner. Failure of these CCl₃F hydrate crystals to exert their normal protective effect on AA would be strong evidence in favor of the oxygen-entrapment hypothesis.

Results in Table I indicate that oxidation of AA during 45 hr at 6° C was not inhibited significantly by the presence of crystals of CCl₃F hydrate which previously had been formed in an atmosphere devoid of oxygen. The extent to which crystals of CCl₃F hydrate lose their ability to inhibit oxidation of AA when formed in the presence of pure nitrogen is more clearly illustrated by comparing results in Table I with data from tests at 6° C in Figure 2. The CCl₃F hydrate samples in Figure 2 were protective to the extent of 2.6 mg AA at 24 hr and 5.3 mg at 45 hr, whereas the 24 and 45 hr values for the oxygen-free CCl₃F hydrate samples in Table I were, respectively, 1 mg and 0.9 mg. Oxygen entrapment therefore appears to be the mechanism by which CCl₃F hydrate exerts its protective effect on AA.

Careful consideration of conditions employed to obtain results reported in Table I provides additional evidence that CCl₃F, as such, contributes little, if anything, to the ability of CCl₃F hydrate to inhibit oxidation of AA. All samples in Table I were initially rotated (agitated) for 1 hr at 0°. Hydrate samples were fluid for a substantial portion of this
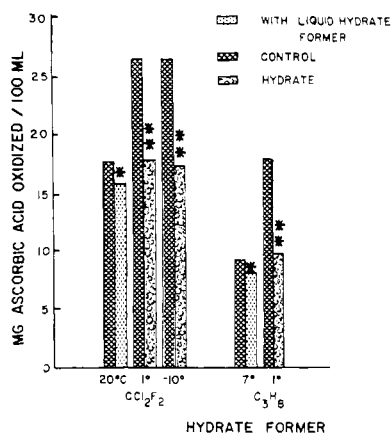
Figure 6. Stability of L-ascorbic acid as influenced by various hydrates and hydrate formers. **: Highly significant (1% level); *: Significant (5% level)

time, so close contact was maintained between AA and CCl_3F. Some uncombined CCl_3F surely remained after hydrate formation and this afforded continued contact between CCl_3F and AA. Hydrate crystals were formed as a shell on the inner surface of each bottle, thus assuring good contact between oxygen and AA. Since the combined presence of liquid CCl_3F and CCl_3F hydrate (crystals were devoid of oxygen) had no significant effect on the stability of AA, there is little doubt that CCl_3F alone, at temperatures in the range of 0–6° C, had no significant effect on the stability of AA.

Studies with Other Hydrates. CCl_2F_2 (f-12) and C_3H_8 (propane) both form Type II gas hydrates, and they were studied to determine if Type II gas hydrates, other than CCl_3F hydrate, are able to inhibit oxidation of AA. Shown in Figure 6 is the amount of AA oxidized as influenced by the direct effect of liquid CCl_2F_2 and C_3H_8 (reaction temperatures were chosen to be above temperatures at which these hydrates can form or exist), and by the effects of their hydrates. Only the 0-time data is presented, since differences between controls and corresponding treated samples did not change significantly during the remainder of the test period. Both CCl_2F_2 and C_3H_8 (no hydrate) slightly retarded oxidation of AA as compared to respective control samples. Protective effects of the hydrates of CCl_2F_2 and C_3H_8 were, however, substantially greater than direct effects of the unhydrated liquid chemicals, thus suggesting that these hydrates behave much like CCl_3F hydrate in their ability to inhibit oxidation of AA.

Based on results of these experiments, it would be expected that all gas hydrates of the Type II and partially-filled Type I structures will inhibit oxidation of AA when used under conditions prevailing in this study. Furthermore, the oxygen-entrapping ability of Type II and partially-filled Type I hydrates could be of value in any system wherein an oxygen scavenger is desirable, assuming of course that conditions are suitable for stability of the hydrate.

Implications. Gas hydrates ordinarily are not encountered in nature because most of them are unstable at normal pressure. However, when the temperature is lowered, the pressure required decreases until eventually normal atmospheric pressure is sufficient for stabilization. The temperature at which this occurs varies greatly, depending on the hydrate. For example, at one atmosphere pressure, decomposition temperatures for various hydrates are as follows (hydrate, °C): carbon dioxide, −56° (Van Hulle, 1969); methane, −29° (Stackelberg and Müller, 1954); xenon, −3.4° (Stackelberg and Müller, 1954); and methyl chloride, 7.5° (Stackel-

berg and Müller, 1954). It is thus possible that hydrates could form either intentionally or unintentionally in products which are stored or processed at a low temperature. If, for example, aqueous samples are stored in the presence of Dry Ice at −78°, carbon dioxide hydrate could form and likely does so. Other examples involve the use of gas hydrate formation to concentrate fluid food materials in a fashion similar to freeze concentration (Glew, 1962; Huang et al., 1966) and use of dichlorodifluoromethane (fluorocarbon-12) for freezing foods (Alaburda et al., 1968; Lawler and Trauberman, 1969). In the first instance, gas hydrates are intentionally formed in direct contact with fluid foods, and if Type II hydrates are employed, the oxygen content of the air would be reduced substantially. In the second instance, liquid fluorocarbon-12 is allowed to boil in direct contact with food materials, thereby providing conditions suitable for formation of Type II fluorocarbon-12 hydrate on the surface of foods and on the condenser used to recover vapors of fluorocarbon-12. This process is in use commercially. Again, oxygen in the air would be depleted by this procedure, and oxidative reactions, including oxidation of AA, would be inhibited.

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