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Evaluation of heat-conduction microcalorimetry in pharmaceutical stability studies VII. Oxidation of ascorbic acid in aqueous solution

M. Angberg [a], C. Nyström [a] and S. Castensson [b]

[a] *Department of Pharmaceutics, Uppsala University and* [b] *Kabi Pharmacia AB, Uppsala (Sweden)*

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Summary

Heat-conduction microcalorimetry at 25.0°C has been evaluated as a method of predicting the stability of a drug undergoing complex degradation in aqueous solution. The oxidation of ascorbic acid was used as a model reaction. The influence on the microcalorimetric response of various amounts of ascorbic acid, oxygen and hydrogen ion in the solution, and of the addition of a metal complex binder or an antioxidant was investigated. In general, the appearance of the heat flow curves and the calculation of the heat evolved gave a clear indication of how the stability was influenced. The accuracy of the microcalorimetric response was investigated by comparing it with an HPLC assay technique. A linear relationship between the cumulative heat and the amount oxidized was obtained. The slope, described as the apparent enthalpy change, was 224 kJ/mol. It is concluded that microcalorimetry can be used as a simple stability indication technique, at normal storage temperatures, for a complex degradation reaction. However, for reactions that include an antioxidant (sodium metabisulfite), which in itself undergoes a continuous major chemical reaction, the appearance of the heat flow curves cannot be related to the stability of the ascorbic acid.

Introduction

Oxidation is one of the most common decomposition mechanisms for pharmaceutical compounds. Oxidation/reduction reactions are characterized by the transfer of one or more oxygen and hydrogen atoms or the transfer of electrons. The fact that many compounds are oxidized is a consequence of the omnipresence of oxygen in the environment and it is also relevant that many drugs exist in a reduced form. Kinetically there is a large activation energy to overcome for many oxidation reactions, which means that they will not proceed spontaneously at any measurable rate, even if molecular oxygen is present. However, the oxidation reaction can be catalysed by radiation from the sun and artificial light, and by trace quantities of metal ions. Oxidative decomposition occurring in pharmaceutical preparations often involves autooxidation reactions, which

Correspondence to: M. Angberg, Department of Pharmaceutics, Biomedical Centre, Box 580, S-751 23 Uppsala, Sweden.

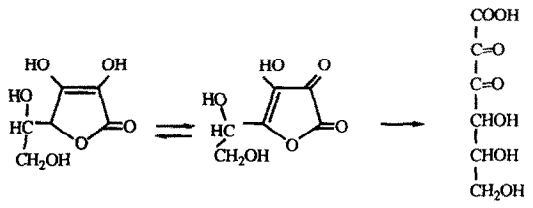
involve a free radical chain process and are induced by the above catalysts (Connors et al., 1986; Lachman et al., 1986).

Even if the rate equation many times is simple, the oxidation reaction involves complex reaction pathways with multiple intermediates and the understanding of the mechanisms behind these reactions is often poor. Furthermore, the reproduction of kinetic studies of oxidation sensitive substances is often difficult, because several environmental factors are difficult to control, e.g., the oxygen content, the radiation intensity and the concentration of metal ions. Additionally, the performance of stability studies at high temperatures has a contradictory effect, as the reaction rate will increase, but the solubility of oxygen in the solution decreases. The temperature dependence of many factors that influence the rate is largely unknown and the activation energy is different for the different reactions. This makes it difficult to extrapolate rate constants at accelerated conditions to normal temperatures (Connors et al., 1986; Lachman et al., 1986).

One well-known substance that undergoes oxidation under aerobic conditions in aqueous solution is ascorbic acid (vitamin C). Ascorbic acid is oxidized reversibly to dehydroascorbic acid by removing a pair of hydrogen atoms. This is followed by irreversible hydrolysis to give diketogulonic acid, which then undergoes oxidation to threonic and oxalic acids (Akers, 1982; Hajratwala, 1985; Anik, 1986). Ascorbic acid (the *l*-form) and dehydroascorbic acid exist together in equilibrium in biological systems and have the same biological activity (Remington's Pharmaceutical Sciences, 1985). The first part of the degradation of ascorbic acid is taken from Anik (1986) and shown in Scheme 1.

Investigations into the oxidation of ascorbic acid and efforts that have been made to improve its storage stability, e.g., exclusion of air/oxygen, adjustment of pH, reduced metal ion contamination and avoidance of light, have been reviewed by Hajratwala (1985). Additives such as antioxidants, chelating agents and surfactants have been used. Solvents other than water may also be utilized.

Many studies have shown that the oxidation



Scheme 1. The degradation of ascorbic acid.

rate for ascorbic acid is slower at low pH values (Nord, 1955; Taqui Khan and Martell, 1967; Fyhr and Brodin, 1987). The pH of the solution will affect the ratio of the ascorbate ion and the neutral ascorbic acid. A proposed mechanism is that the ascorbate ion has higher reactivity than the neutral molecules in forming the metal complex that is involved in catalysing the oxidation reaction (Taqui Khan and Martell, 1967). The redox potential also depends on pH. At lower pH the ascorbic acid is less readily oxidized (Lachman et al., 1986).

Heat-conduction microcalorimetry can be used as a stability indicating technique. It monitors the heat flow from the processes in a sample as a function of time. This method has been used to investigate the hydrolysis of acetylsalicylic acid (Angberg et al., 1988, 1990) and of ampicillin (Oliyai and Lindenbaum, 1991), both in aqueous solution, and the degradation of cephalosporins in the solid and the dissolved states (Pikal and Dellerman, 1989). Published studies on oxidation processes include an investigation of the autooxidation of lipids (Raemy et al., 1987) and lovastatin in the solid state (Hansen et al., 1989). Pikal et al. (1989) have reviewed some of these and other stability studies. Buckton and Beezer (1991) have reviewed the application of microcalorimetry in the field of physical pharmacy and Buckton et al. (1991) in more biologically based studies. Angberg et al. (1991a,b, 1992a,b) have with the microcalorimetric technique investigated the interaction of water vapour with various powders. The number of published studies are few and the applicability of the microcalorimetric technique in pharmaceutical stability studies has by no means been fully represented.

The object of this study was to evaluate the microcalorimetric technique at 25.0°C on a complex degradation reaction, such as the oxidation of ascorbic acid, in aqueous solution. The microcalorimetric response was investigated by testing a range of factors known to influence the stability. The results were compared to those from an HPLC assay technique measuring the rate and extent of the oxidation reaction.

Material and Methods

Materials

Ascorbic acid and sodium metabisulfite were obtained from Apoteksbolaget (Sweden); EDTA disodium salt dihydrate (EDTA; Titriplex® III), acetic acid 0.1 M (Titrisol®) and sodium hydroxide 1 M (Titrisol®) were all purchased from Merck (Germany). The acetic acid and sodium hydroxide solutions were prepared with ultrapure water from a Milli-Q® UF plus system ($QPAK_2$), Millipore Corp. (MA, U.S.A).

Methods

The microcalorimetric technique The microcalorimeter system used was the 2277 Thermal Activity Monitor, TAM (Thermometric AB, Sweden), equipped with four heat-conduction microcalorimeters, model 2277-201. This system has been described by Suurkuusk and Wadsö (1982). The reactions proceed at essentially isothermal conditions. The heat from physical and/or chemical processes in the sample vessel results in a heat flow signal, dQ/dt in μW (μJ/s), monitored as a function of time, which ideally is proportional to the rate of the process. If several processes proceed at the same time, the heat flow signal will comprise the sum of all these processes. The cumulative heat, Q in mJ, is obtained by integrating the heat flow curve. Exothermic heat flow signals are given positive signs in this paper. The experimental temperature was 25.0°C.

The sample solutions were filled until the desired weight was obtained, using a glass pipette, generally in disposable glass vials (approx. 3.3 ml) and more seldom in stainless-steel vessels (approx. 4.5 ml). The glass vials were sealed with a teflon coated rubber disc and an aluminium cap and the steel vessels were closed with a steel lid lined with a teflon disc. The vessels were placed within the microcalorimetric system for a 30 min temperature equilibration, and then inserted into the measurement position. At this time the collection of data by the computer started, referred to as $t = 0$. The reference vessels were filled with water. The technique has been described previously (Angberg et al., 1988, 1990).

Buffer preparation Acetic acid 0.1 M and NaOH 1.0 M were prepared beforehand and equilibrated with the ambient atmosphere. The pH of the acetic acid solution was measured with a pH-meter (Hanna instruments, model HI 8418, Italy) and was changed by adding NaOH until the desired pH was obtained. The pH of the buffers was in the range 3.0–6.5. Buffers of pH 4.0 and 5.0, which are in the range where acetic acid has a good buffer capacity, have mainly been used ($pK_a = 4.8$, Martin et al., 1983). For the buffers that contained sodium metabisulfite or EDTA, the powders were dissolved in the buffer solution, before the solution was added to the ascorbic acid powder.

Ascorbic acid solution preparation Ascorbic acid solutions of 0.01 and 0.10% w/v were prepared by dissolving the ascorbic acid (0.010 and 0.100 g, $M_w = 176.1$ g/mol) in the appropriate buffer solution (100.0 ml) and stirring for 5–7 min. The powder is taken to be 100% pure ascorbic acid in the calculations.

The oxygen content was reduced in some of the ascorbic acid solutions by purging about 15 ml with nitrogen gas (AGA, Sweden) through a sintered glass filter for 10 min. The gas was passed through a 0.2 μm membrane filter unit, Millex®-FG_{50} (Millipore Corp., MA, U.S.A.) in order to remove particulate contamination, i.e., metal particles from the gas tube.

The investigated variables are known to influence the oxidation rate of ascorbic acid. The measurements have, when possible, been performed as comparative stability measurements, since four microcalorimeters could be used simultaneously, i.e., the response for one solution has been compared to the response for the same solution that has been treated differently in just

one variable. Differences that are difficult to control for solutions made on different occasions, such as trace quantities of metal ions, are hereby avoided.

The oxygen content The oxygen content in the sample vessel was varied by filling the vessels either almost totally or partly with solution, the latter combined with a large head space volume containing air. The oxygen content in the totally filled vessels was reduced in some solutions by nitrogen purging. The concentrations of oxygen in air and dissolved in water at 25°C are shown in Table 1.

The ascorbic acid content Most measurements were performed with a 0.10% w/v ascorbic acid solution, but a 0.01% w/v solution has also been tested. The amount of ascorbic acid in the samples was dependent on the filling weight. However, to measure the concentration dependency, the glass vials were filled with decreasing weights of the 0.10% w/v ascorbic acid solution: 2.0, 1.5, 1.0 and 0.5 g. To obtain approximately the same oxygen content for all samples, the buffer solution was added up to 2.0 g.

The metal ion content It is difficult to avoid trace quantities of metal ions in the solution (Fyhr and Brodin, 1987), and metal ions were present as contaminants in this study. To reduce the concentration of 'active' metal ions the complexing agent, EDTA, was added to some solutions. The microcalorimetric response for an EDTA solution alone was also monitored. The influence of the sample vessel material on the oxidation rate was investigated by measuring ascorbic acid solutions, 0.10% w/v, pH 4.9, with or without 0.10% w/v EDTA, in glass vials and stainless-steel vessels. The stainless-steel vessels cannot be used at pH values below 4, since at these levels a disturbing heat flow occurs, probably due to corrosion (Suurkuusk and Wadsö, 1982).

The hydrogen ion content The pH was measured after the addition of ascorbic acid to the buffers and the values finally investigated were pH 3.0, 3.9, 4.9, 5.3 and 5.7. By changing the pH, the concentration of the neutral, mono-ionic and di-ionic forms of ascorbic acid will differ (pK_{a1} = 4.3 and pK_{a2} = 11.8 at 25°C; Martin et al., 1983).

The antioxidant content Antioxidants are added to protect oxidation-sensitive substances in pharmaceutical formulations. Sodium metabisulfite ($Na_2S_2O_5$), a sulfurous acid salt, was used in the concentrations 0.05 and 0.005% w/v. The solutions, pH 4.9, containing antioxidantia were investigated alone or in combination with ascorbic acid, 0.10% w/v.

The HPLC analysis A rapid quantitative reversed-phase HPLC determination method of *l*-ascorbic acid was developed to evaluate the accuracy of the microcalorimetric technique. The oxidation product, dehydroascorbic acid, shows poor UV absorptivity at low wavelengths (Ziegler et al., 1987).

The solutions prepared for the microcalorimetric measurements were analysed with HPLC to obtain a 100% value for the ascorbic acid. Directly after the microcalorimetric measurement was terminated, an HPLC analysis was performed on the solution. This means that the microcalorimetric response could be correlated to the disappearance of ascorbic acid in that specific sample. For some measurements a value at the microcalorimetric $t = 0$ was analysed, to measure the decrease during the 30 min equilibration time.

The HPLC system consisted of a continuous flow solvent delivery pump (LDC ConstaMetric® III, FA, U.S.A.) equipped with an injector (Reodyne 7125, CA, U.S.A.) with a 20 μl loop and a variable wavelength absorbance detector (LDC UV SpectroMonitor® III, FA, U.S.A.), connected to a recorder (Hitachi, Japan). Peak areas were measured with an integrator (model 3390A, Hewlett-Packard, PA, U.S.A.). A wavelength of 245 nm was used. A 125.0 × 4.0 mm stainless-steel column packed with LiChrosorb RP-8 (5 μm) (Merck, Germany) was attached to a 4 × 4 mm stainless steel precolumn (guard column) packed with LiChrospher RP-8 (5 μm) (Merck, Germany). The retention time was 1.90 min. The mobile phase was 0.25% phosphoric acid/water, 1 ml/min. Stock standard solutions contained between 1 and 10 μg/ml of ascorbic acid. A standard curve of the peak area against concentration showed a linear relationship, $r^2 = 0.999$.

The HPLC analysis was not influenced by the addition of either EDTA or sodium metabisulfite.

Results and Discussion

The oxygen content

The heat flow curves for the decomposition of a 0.10% w/v ascorbic acid solution at pH 4.9 and 3.9 over 71 h are shown in Fig. 1. The curves from glass vials totally filled with solution, (a) and (b), fall almost to zero within 25 h. The curves from glass vials filled with 2.0 g solution, (c) and (d), change direction after about 2 h (pH 4.9) and after 4 h (pH 3.9) and decrease less rapidly. These curves do not fall to zero within the experimental time, and become almost parallel, with pH 3.9 on the lower level. The initial parts of the curves differ with pH. At pH 4.9, the curves start with high heat flow signals which decrease rapidly. At pH 3.9, the curves increase during the first hour to a maximum value, much lower than at pH 4.9, and decrease thereafter. For both pH values, the maximum at the beginning is higher for the solutions with the largest amount of ascorbic acid, i.e., the totally filled vials.

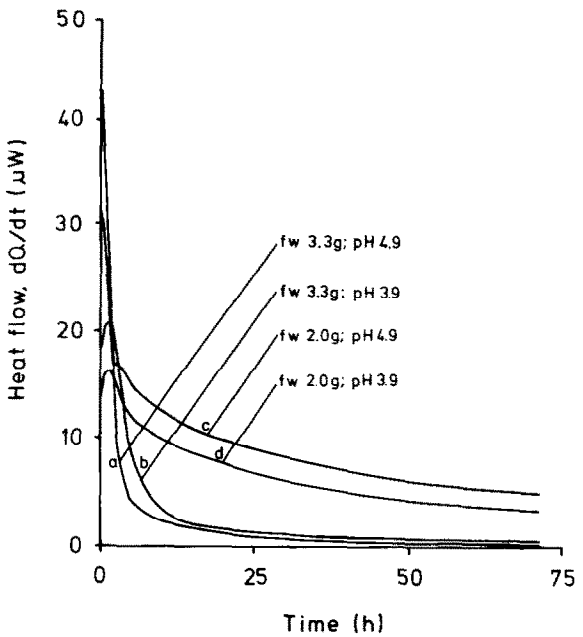


Fig. 1. Heat flow curves for 0.10% w/v ascorbic acid solutions in glass vials. The measurement time was 71 h. Curve (a) fw (filling weight) 3.3 g, pH 4.9; curve (b) fw 3.3 g, pH 3.9; curve (c) fw 2.0 g, pH 4.9; curve (d) fw 2.0 g, pH 3.9.

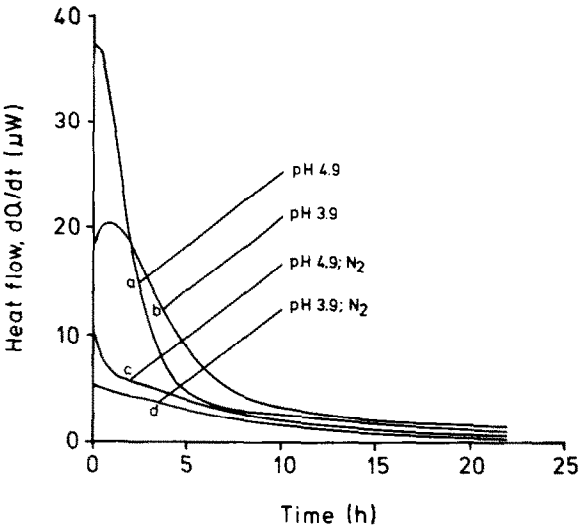


Fig. 2. Heat flow curves for 0.10% w/v ascorbic acid solutions in glass vials. The filling weight was 3.3 g and the measurement time was 22 h. Curve (a) pH 4.9; curve (b) pH 3.9; curve (c) pH 4.9, purged with nitrogen; curve (d) pH 3.9, purged with nitrogen.

If the microcalorimetric response in Fig. 1 had been obtained for a substance with unknown stability characteristics, the obvious conclusion is that the availability of oxygen is important and that an oxidation reaction may be involved in the degradation of the substance. Furthermore, it is evident that the pH of the solution influences the reaction, i.e. the intensity of the reaction at pH 3.9 seems to be lower than at pH 4.9. That the oxygen content is important can be further investigated by microcalorimetry, by simply purging the solution with nitrogen gas before enclosing it in the sample vessel and comparing it with the response for an untreated solution. In Fig. 2, the changed appearance of the heat flow curves on purging with nitrogen can be seen for a 0.10% w/v ascorbic acid solution in totally filled glass vials over 22 h at pH 4.9 and 3.9. The solutions purged with nitrogen show a very decreased heat flow, due to the fact that there is very little oxygen to react with.

It has been suggested that oxygen is consumed in the oxidation reaction through complex formation and free radical reactions (Taqui Khan and Martell, 1967; Fyhr and Brodin, 1987). In those

studies, there was an excess of oxygen during the measurements, since it had been actively added to the solution by purging. This is not the case in this study, in which there was a stagnant solution with a head space containing air, enclosed in the sample vessel.

In Table 1, the percentages of oxidized ascorbic acid, obtained by HPLC for the measurements presented in Figs 1 and 2, are shown. As the initial concentrations of ascorbic acid and the filling weights are known, the amount of ascorbic acid in μmol that has oxidized can be calculated (Table 1). The amount oxidized is much less for the totally filled vials than for the partly filled vials. The amount decomposed is around 10% for the totally filled vials, almost independent of either pH or measurement time, and this is reduced to about 4% for the solutions purged with nitrogen. For the partly filled vials, the amount oxidized is larger at pH 4.9 than at pH 3.9.

The amount of oxygen (in the form of $\frac{1}{2}O_2$) in the sample vials was calculated, so as to be able to relate it to the amount of oxidized ascorbic acid (Table 1). The samples that are described as performed in 'totally filled' vials, have in reality a head space volume of around 0.05 ml, which cannot be neglected. This volume was needed to avoid any leakage and thus vaporization of water, which would cause a high endothermic heat flow. For the totally filled vials (Table 1), the amount that has oxidized is approximately the same as the calculated amount of oxygen available, for the stoichiometric relationship $\frac{1}{2}O_2$ molecule to 1 ascorbic acid molecule (Nord, 1955). This shows that oxygen is continuously needed in the oxidation process and that it is probably not a propagating free radical mechanism involved, concluded also by Fyhr and Brodin (1987). Furthermore, the calculations show that there is a surplus of oxygen for the measurements performed with a 2.0 g solution, which contains approx. 11.4 μmol ascorbic acid. The oxygen content in the head space, though small, in the filled vials, explains the heat flow that occurred with the solution purged with nitrogen (Fig. 2). This is theoretically enough to oxidize 0.9 μmol of ascorbic acid and a result similar to this was actually obtained (Table 1).

As shown in Fig. 1, there is a rapid decrease in the heat flow curves at the beginning, especially at pH 4.9, independent of whether the vials are totally or partly filled. The proposed explanation is that the oxygen dissolved in the solution is rapidly consumed. For the totally filled vials the

TABLE 1

The oxidation of ascorbic acid in aqueous solution, 0.10% w / v in glass vials

Fig./ curve	Measurement time (h)	pH (−)	Purged with N_2 ±	Approx. filling weight (g)	Approx. amount oxidized ascorbic acid		Approx. amount $1/2O_2$		
					(%) [c]	(μmol) [d]	Solution [e]	Head space [f] (μmol)	Total
1/a	71	4.9	−	3.3 [a]	8.9	1.6	1.5	0.9	2.4
1/b	71	3.9	−	3.3 [a]	12.4	2.3	1.5	0.9	2.4
1/c	71	4.9	−	2.0 [b]	75.6	8.6	0.9	22.3	23.2
1/d	71	3.9	−	2.0 [b]	60.1	6.8	0.9	22.3	23.2
2/a	22	4.9	−	3.3 [a]	10.0	1.8	1.5	0.9	2.4
2/b	22	3.9	−	3.3 [a]	11.0	2.0	1.5	0.9	2.4
2/c	22	4.9	+	3.3 [a]	4.2	0.8	~0	0.9	0.9
2/d	22	3.9	+	3.3 [a]	4.6	0.9	~0	0.9	0.9

[a] Head space volume approx. 0.05 ml.
[b] Head space volume approx. 1.3 ml.
[c] Obtained from the HPLC analysis.
[d] Calculated value (the ascorbic acid was set as 100% pure).
[e] The amount O_2 dissolved per ml H_2O saturated with air is 0.23 μmol at 25°C (Connors et al., 1986).
[f] The O_2 content per ml air is 8.59 μmol at 25°C (Connors et al., 1979).

reaction rate decreases to near zero, because the oxygen cannot be replaced (except for the small amount in the head space). For the partly filled vials, the reaction continues with oxygen from the head space which dissolves in the solution. When this process begins, the heat flow curves change direction (Fig. 1). It is therefore only at the beginning that the oxygen seems to be in great excess, directly available in the solution. This was further supported by the curves for the solutions purged with nitrogen (Fig. 2). They did not have the high signals at the beginning, because there was no oxygen dissolved in the solution. However, the small oxygen content in the head space filled with air dissolved, which resulted in a low heat flow signal until that oxygen had been consumed.

For the most part, oxidative degradations of pharmaceutical compounds follow first-order or second-order kinetics (Lachman et al., 1986). First-order dependence for the oxidation of ascorbic acid in an excess of oxygen has been demonstrated (Taqui Khan and Martell, 1967; Blaug and Hajratwala, 1972; Fyhr and Brodin, 1987). However, the availability of oxygen, which is dependent on the dissolution rate of oxygen from the head space into the solution, will influence the kinetics during the course of the measurements in this study.

The percentage decrease in ascorbic acid after different lengths of time were analysed by HPLC to investigate the kinetics of the study. The same combinations of filling weights and pH as in Fig. 1 were followed for 1, 2, 4 and 22 h in the microcalorimeter and the HPLC analysis was performed directly after that. A $t = 0$ value, i.e. the time when the microcalorimetric measurement started, about 30 min after the 100% value of the total amount of ascorbic acid was analysed, was also measured.

In Fig. 3a, the logarithm of the percentage of ascorbic acid left, obtained by the HPLC technique, was plotted against time starting at $t = 0$. For the vials that were totally filled, the decrease is rapid initially, but the slope levels out after about 4 h, because of the shortage of oxygen. For the measurements performed in the partly filled vials in a surplus of oxygen, the decrease of the amount of ascorbic acid formed approximately straight lines, partly due to the fact that there was a long interval between measurements ending after 4 and 22 h. The slope at pH 4.9 is steeper (2.1×10^{-2} h^{-1}) than at pH 3.9 (1.6×10^{-2} h^{-1}).

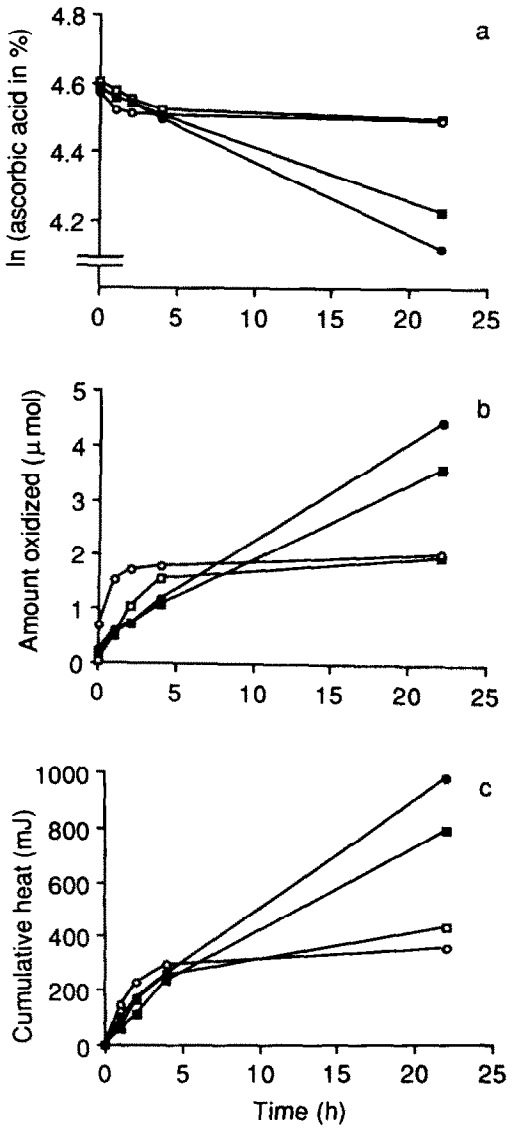


Fig. 3. Microcalorimetric measurements for 0.10% w/v ascorbic acid solutions in glass vials, combined with HPLC analysis of the ascorbic acid content in per cent before and after the microcalorimetric measurement. (○) fw (filling weight) 3.3 g, pH 4.9; (□) fw 3.3 g, pH 3.9; (●) fw 2.0 g, pH 4.9; (■) fw 2.0 g, pH 3.9. (a) The ln(ascorbic acid in %) plotted as a function of time. (b) The amount of oxidized ascorbic acid plotted as a function of time. (c) The cumulative heat plotted as a function of time.

However, if the HPLC results are investigated more thoroughly, it can be shown that there is a slightly higher rate at the beginning of the reaction. This corresponds well with the microcalorimetric result. This shows that the oxidation reaction does not follow first-order kinetics, which is expected with the type of measurement technique utilized in this study.

In Fig. 3b, the amount of oxidized ascorbic acid in μmol has been plotted as a function of time. This takes into consideration that, initially, the amount of ascorbic acid in each vial is different, due to the filling weight. At $t = 0$, some ascorbic acid has already been oxidized, especially for the totally filled vial at pH 4.9. In Fig. 3c, the cumulative heat values obtained from the integrated heat flow curves have been plotted. These curves resemble those in Fig. 3b closely, which demonstrates the good correlation between the amount oxidized and the microcalorimetric response. However, at the beginning, the amount oxidized is much larger than the cumulative heat can account for, because the cumulative heat cannot be obtained for the 30 min temperature equilibration period. This was especially noticeable for the measurements in the totally filled vials.

It can be concluded that the oxidation rate is determined to a large extent by the supply of oxygen. At the beginning, the oxygen in the solution will be available for the oxidation reaction. However, for most of the measurement time the reaction is influenced by the dissolution of oxygen from the head space. This is more clearly demonstrated by the primary heat flow curves in Fig. 1 than from the HPLC result in Fig. 3a. However, the HPLC technique has been used below to obtain a measure of the rate for partly filled vials, so as to be able to separate different measurements.

The ascorbic acid content

In a sample for which the oxygen content is limited, the amount of oxidized ascorbic acid will be determined mainly by the oxygen content, if the measurement continues for long enough. However, the concentration of ascorbic acid will influence the oxidation rate.

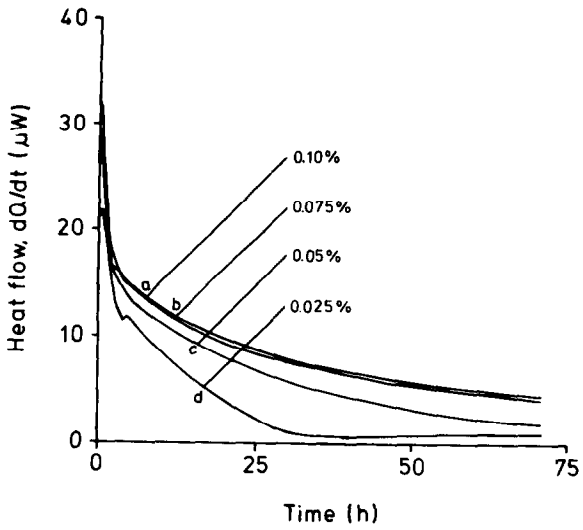


Fig. 4. Heat flow curves for different concentrations of ascorbic acid solutions in glass vials at pH 4.9. The filling weight was 2.0 g and the measurement time was 71 h. Curve (a) 0.10% w/v; curve (b) 0.075% w/v; curve (c) 0.05% w/v; curve (d) 0.025% w/v.

In measurements performed in a surplus of oxygen, the oxidation rate will also differ according to the concentration of ascorbic acid. In Fig. 4, the heat flow curves are shown for different concentrations of ascorbic acid, 0.10 to 0.025% w/v, but with the same weight, 2.0 g, in the sample vials. The amount of ascorbic acid was 11.3 for curve (a); 8.6 for curve (b); 5.8 for curve (c); and 2.8 μmol for curve (d). The measurements lasted for 71 h, the pH was 4.9 and glass vials were used. Curves (a) and (b) are very similar, which indicates that the amount oxidized is almost the same. The amount oxidized was 8.4 μmol for curve (a) and 7.8 μmol for curve (b). This means that there is 2.9 μmol of ascorbic acid left for curve (a) and 0.8 μmol left for curve (b). Curve (c) starts similarly to those of the higher concentrations, but approaches zero much more rapidly. The amount oxidized is about 5.4 μmol, which means that there is only 0.4 μmol ascorbic acid left after 71 h. For the lowest concentration, curve (d), the signal fell to near zero after about 30 h and the HPLC analysis after 71 h showed as expected that the ascorbic acid was depleted. However, there is a low signal between

30 and 71 h, but this heat flow may come from the degradation of dehydroascorbic and diketogulonic acids, as there is no lack of oxygen. For the totally filled vials, shown earlier, in which the oxygen is limited, further degradation will probably proceed at a very slow rate, especially the oxidation of diketogulonic acid.

If the HPLC results for the samples used for curves (a)–(c) in Fig. 4 are treated as first-order kinetics as in Fig. 3a, but only including the initial and final concentrations of ascorbic acid, the rate constants increase as the initial concentration decreases. This is expected, because the relative amount of oxygen will be higher as the initial concentration of ascorbic acid decreases. This is again dependent on the availability of oxygen.

With the microcalorimetric technique, it is possible to test further whether it is the oxygen or the substance that is the depleted reactant. This is done when the heat flow curve approaches a near zero value, by opening the sample vessel and exchanging the head space with fresh air, or for a totally filled vessel, by reducing the solution volume and exchanging that with air. Thereafter, the sample vessel is closed and another microcalorimetric measurement is started. If the heat flow signal stays near zero, it is the substance that is depleted. However, if the signal is significantly above zero, this shows that it was the oxygen that was the depleted reactant.

Metal ion content

Metal ions like copper and iron will increase the oxidation rate, as shown in several studies (Dekker and Dickinson, 1940; Nord, 1955; Taqui Khan and Martell, 1967; Fyhr and Brodin, 1987). It is difficult to control the content of metal ions, since only trace amounts are needed to catalyse the oxidation rate. The metal ions may come from many different sources, for example water, beakers and other laboratory equipment utilized in the sample preparation. The sample vessels themselves may also be contaminated with metal ions. The washing procedure is therefore very important if the metal ions are to be avoided totally (Fyhr and Brodin, 1987). In this study, there has been no special effort made to minimize the metal ion contamination. The glass equipment was washed with an ordinary laboratory washing machine, which concludes with two rinses of distilled water. The glass vials were used as received and the stainless-steel vessels were rinsed with water (Milli-Q® UF plus) several times.

To reduce the active metal ion content it is common to add complex binders, like EDTA. In Fig. 5a, the heat flow curves are shown for a 0.10% w/v ascorbic acid solution at pH 4.9 with different filling weights in glass vials. The measurements lasted for 22 h. Curves (a) and (b) are from solutions that have no EDTA added and curves (c) and (d) are from solutions containing 0.10% w/v EDTA. The addition of EDTA changes the appearance of the curves substantially, but the amount degraded (in μmol) corresponds well with the heat evolved. For the totally filled vials, the amount oxidized differed only slightly between the solutions 11.4% (2.1 μmol) for curve (a) and 8.1% (1.5 μmol) for curve (c), because the oxygen content will determine the amount degraded if the measurements are long enough. However, the appearance of the curves, which represents the rate, is very different between the two and it seems clear that the metal ions catalyse the oxidation process. For the glass vials filled with 1.1 g, 60.4% (3.7 μmol) was oxidized, curve (b), compared to only 16.8% (1.0 μmol) for curve (d). This shows that at a surplus of oxygen, the addition of EDTA was very important for both the extent and rate of the oxidation over the measurement time of 22 h. A 0.10% w/v EDTA solution alone did not show any heat flow response.

Glass vials were mainly used in this study, because this is the most common pharmaceutical packaging material for solutions. However, to investigate if the oxidation reaction was influenced by the sample vessel material, stainless-steel vessels were also used. The result is shown in Fig. 5b, for the same type of solutions as in Fig. 5a. The volumes of these vessels are larger than the glass vials, but the filling weights have been chosen to give approximately the same relationship between the solution and head space volumes as in the glass vials. Furthermore, the heat flow curves in Fig. 5b have been recalculated to corre-

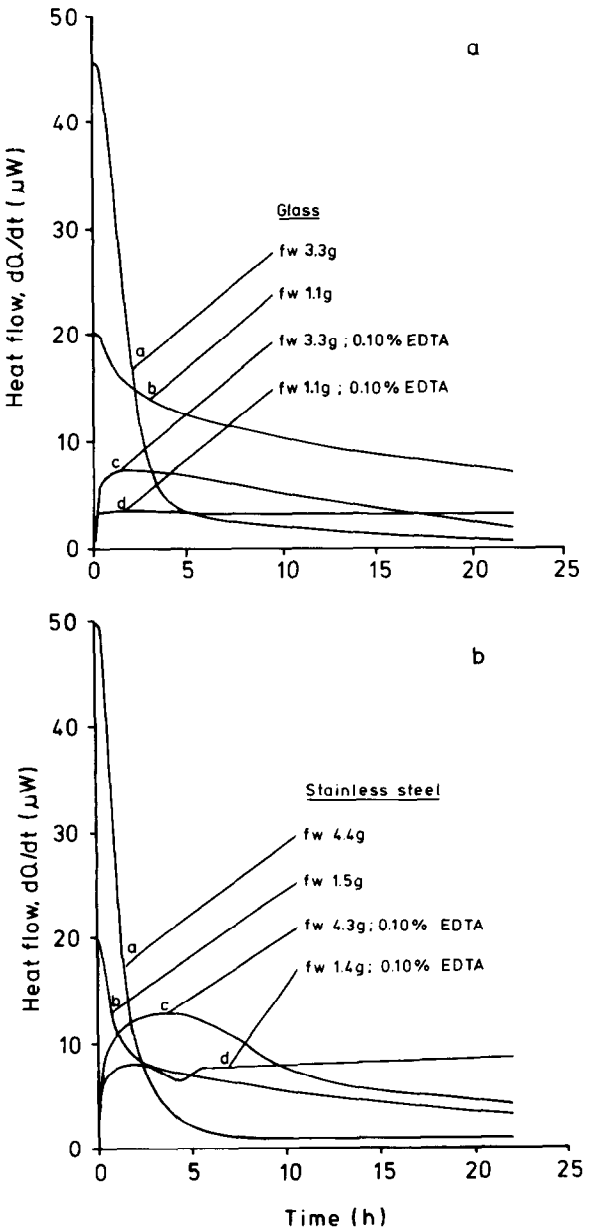


Fig. 5. Heat flow curves for 0.10% w/v ascorbic acid solutions, pH 4.9. The measurement time was 22 h and the filling weights differed as shown in the figure. (a) Measurements in glass vials. (b) Measurements in stainless-steel vessels. Curves (c) and (d) included 0.10% w/v EDTA, which the curves (a) and (b) did not. The heat flow curves for the stainless-steel vessels have been diminished to correspond to the amount of ascorbic acid in the glass vials.

spond to the amount of ascorbic acid in the glass vials and they are therefore reduced in comparison to the actual response.

A comparison between measurements performed in glass vials and those in the stainless-steel vessels shows that for the totally filled vessels, the heat flow curves (a) were quite similar, with a decrease of 11.4% (2.1 μmol) for the glass vial and 9.3% (2.3 μmol) for the steel vessel. Again, it seems to be the amount of oxygen available that determines the amount decomposed. For the partly filled vessels, the (b) curves, the percentage decrease was lower for the steel vessel, 34.8% (3.0 μmol) compared with 60.4% (3.7 μmol) for the glass vial. This shows that the stainless-steel vessels are less detrimental to the ascorbic acid than the glass vials. The curves corresponding to the measurements in the steel vessels that included EDTA are very different from the other curves. The addition of EDTA even increased the amount oxidized. For curve (c) 13.5% and for curve (d) 39.8% were oxidized. The reason for this has not been further investigated.

The appearances of the heat flow curves differed more when the steel vessels were used than when utilizing the glass vials. However, the cumulative heat evolved corresponded well with the amount of ascorbic acid that had oxidized as measured by HPLC. Therefore, the heat flow curves probably gave a good representation of the oxidation reaction.

The hydrogen ion content

In Fig. 6, the heat flow curves obtained in the pH range 3.0–5.7 are shown. The ascorbic acid solutions were 0.10% w/v, the filling weight 2.0 g and glass vials were used. The measurement time was 22 h. The acetic acid buffer capacity was far from excellent at the outer limits of the pH range investigated, but this was a compromise so as to keep the same buffer system in all measurements. The heat flow response increased as the pH of the solution increased, corresponding to the amount oxidized as obtained by HPLC: pH 3.0, 13.5%; pH 3.9, 30.6%; pH 4.9, 38.9%; pH 5.3, 44.0% and pH 5.7, 46.4%.

The rate constants were calculated as for

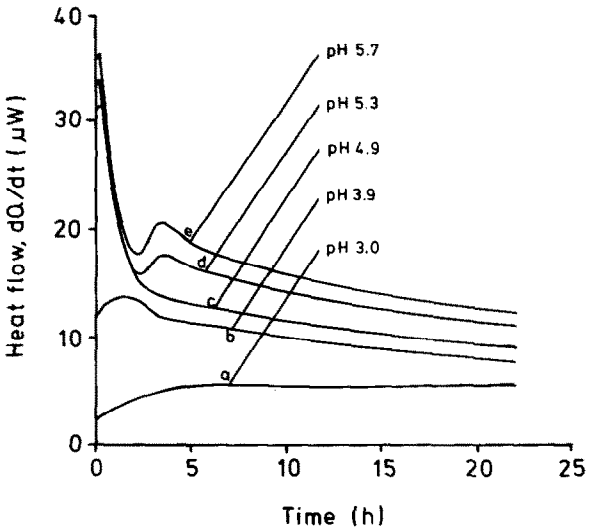


Fig. 6. Heat flow curves for 0.10% w/v ascorbic acid solutions in glass vials. The filling weight was 2.0 g and the measurement time was 22 h. Curve (a) pH 3.0; curve (b) pH 3.9; curve (c) pH 4.9; curve (d) pH 5.3; curve (e) pH 5.7.

first-order kinetics from the HPLC data at the different pH values and the results were plotted as a pH-rate profile in Fig. 7. A similar profile is obtained for the logarithm of the cumulative heat obtained by integrating the heat flow curves for the same measurements, also shown in Fig. 7. This shows how the microcalorimetric data can be utilized to indicate the stability of a process. A

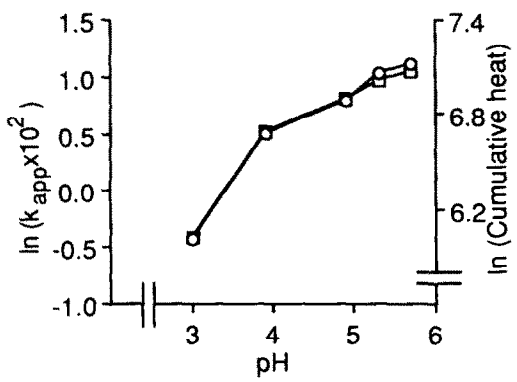


Fig. 7. The pH-rate profile for the measurements shown in Fig. 6. The logarithms of the apparent first-order rate constants (□) and the cumulative heat values over 22 h (○) have been plotted against pH.

similar approach was earlier used for a hydrolysis reaction (Angberg et al., 1990).

The redox potential varies with pH for weak acids. At lower pH values, the ascorbic acid is, therefore, less prone to undergo oxidation (Lachman et al., 1986). Furthermore, the pH determines the concentrations of the neutral, mono- and di-ionic forms of ascorbic acid. In this study, the acid range has been investigated and it is therefore only the first dissociation constant, $pK_{a1} = 4.3$, that is of importance. Blaug and Hajratwala (1972), showed that the oxidation rate was largest around pK_{a1}, but that study was performed at 67°C. However, most studies have shown that the reaction rate increases as pH increases in the acid range. The oxidation occurs by parallel reactions with the neutral ascorbic acid molecule and the ascorbate ion (Taqui Kahn and Martell, 1967; Fyhr and Brodin, 1987).

Taqui Khan and Martell proposed that in excess oxygen and when metal ions are present, a complex is formed between the ascorbate ion (HA^-) and the metal ion (Me^{n+}). This is followed by an attack of oxygen (O_2) that forms a new complex, ($MeHA^-(O_2)$). After three pre-equilibrium steps, the rate-determining reaction follows, in which an electron is transferred through the metal ion to the oxygen. A similar complex can be formed with the neutral ascorbic acid molecule (H_2A). However, the metal ion has higher reactivity towards the ascorbate ion than to the neutral molecule. As the concentration of ascorbate anions increases with the pH, the oxidation rate increases.

As can be seen in Fig. 6, the heat flow curves show very different appearances if the pH is above, around or below the pK_{a1}, especially during the first hours. The heat flow curves may therefore be explained by the following. At a pH above pK_{a1}, the concentration of ascorbate ions is high. The equilibrium complex ($MeHA^-(O_2)$) can easily be formed with the oxygen dissolved in the solution and the oxidation reaction proceeds rapidly. At the highest pH values, pH 5.3 and 5.7, the curves show an increase in heat flow about 4 h after the first rapid decrease followed by a less steep slope. The reason for this increase is not known, but may again be connected with the

speed at which the oxygen is consumed and the rate at which it can be replenished in the solution. At a pH below pK_{a1}, the concentration of ascorbate ions and the formation rate of the metal complex are lower. At pH 3.9, for example, the heat flow curve reaches a maximum after about 1 h. At pH 3.0, the heat flow signal is very low, due to the low concentration of ascorbate ion. Even if the metal complex model is not correct, the difference in reactivity for the neutral ascorbic acid molecules and the ascorbate ions still holds. However, Fig. 6 shows that for most of the measurement in the sample vessel, the oxidation reaction is influenced by the dissolution rate of oxygen from the head space. When the concentration of ascorbate ions in the solution increases, due to the pH, the faster the dissolved oxygen is consumed and the faster the dissolution rate.

The antioxidant content

The antioxidants used in pharmaceutical products show large structural variety, which will influence their mechanistic behaviour (Akers, 1982). An antioxidant may protect an oxidation sensitive substance by being more readily oxidized, or as a chain inhibitor of radical-induced decomposition. In this study, sodium metabisulfite (abbreviated to bisulfite) has been used. Bisulfite functions mainly by being preferentially oxidized and it reacts with oxygen with the stoichiometric relationship 1 $Na_2S_2O_5$: 1 O_2 (Akers, 1982). It can also function as a free radical inhibitor (Lachman et al., 1986).

In Fig. 8a, the heat flow curves for bisulfite solutions alone at pH 4.9 are shown. Glass vials were used and the measurement time was 22 h. Curves (a) and (b) correspond to 0.05% w/v bisulfite and a filling weight of 3.3 g (a) and 2.0 g (b). Curve (c) corresponds to a 0.005% w/v bisulfite solution and a filling weight of 2.0 g. Curves (a) and (c) show approximately the same heat flow response, but for curve (a), which contains 8.5 μmol bisulfite, it is the oxygen that is depleted, and for curve (c) it is the 0.5 μmol bisulfite. For curve (b), a very high heat flow response with some fluctuations was obtained and the reaction did not end within the experimental time.

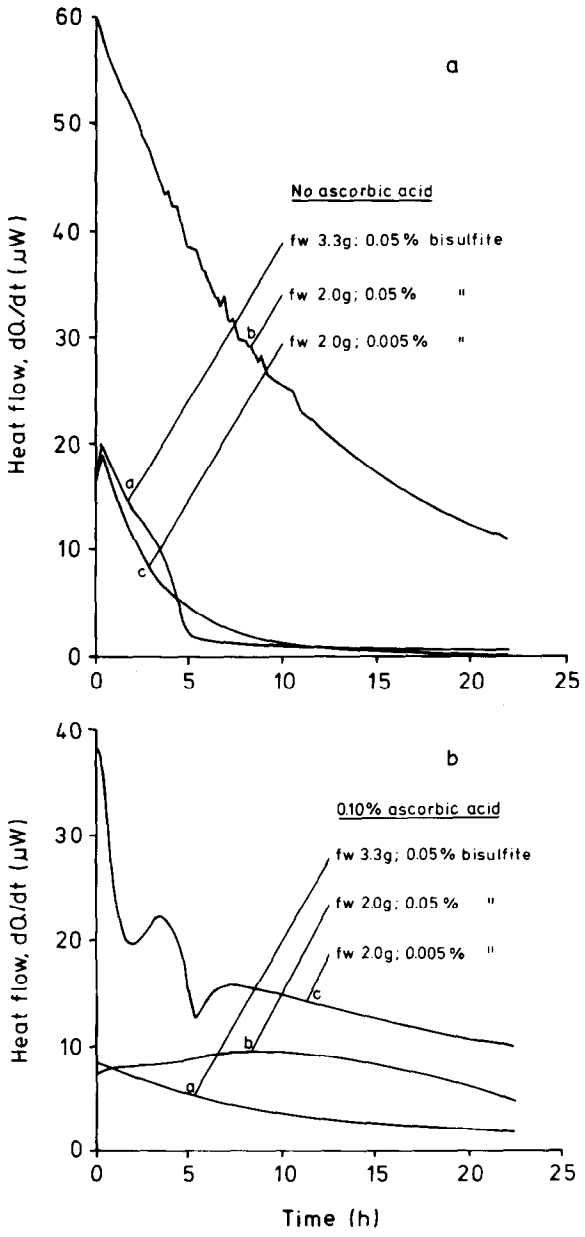


Fig. 8. Heat flow curves for sodium metabisulfite (bisulfite) solutions alone or together with 0.10% w/v ascorbic acid in glass vials and pH 4.9. The measurement time was 22 h. (a) Measurements with only bisulfite. Curve (a) fw (filling weight) 3.3 g, 0.05%; curve (b) fw 2.0 g, 0.05%; curve (c) fw 2.0 g, 0.005%. (b) The same combinations of filling weights and bisulfite concentrations, but including also ascorbic acid.

The heat flow curves presumably correspond to the reaction between bisulfite and oxygen. These results demonstrate the importance of measuring all additives separately by microcalorimetry, to avoid misinterpretation of the heat flow curves for the mixture.

In Fig. 8b, the same types of solutions as in Fig. 8a are shown, but including 0.10% w/v ascorbic acid. For curve (a), the amount of oxidized ascorbic acid was very low during the measurement, only 0.4 μmol (2.1%). This means that the ascorbic acid was partly protected compared to when it was alone, probably caused by the limited oxygen content being shared between the two reactions. For curve (b), the amount of oxidized ascorbic acid was 1.1 μmol (9.5%), which is also much less than for ascorbic acid alone. Curve (c) has the lowest concentration of bisulfite, 0.005% w/v. The amount of ascorbic acid oxidized is approximately the same as without the antioxidant, 4.2 μmol (37.1%), demonstrating the lack of protective effect. The appearance of this curve gives the impression that there are different processes that dominate at different time intervals. The concentration of bisulfite was also much lower than recommended, as it normally ranges between 0.025 and 0.10% (Akers, 1982).

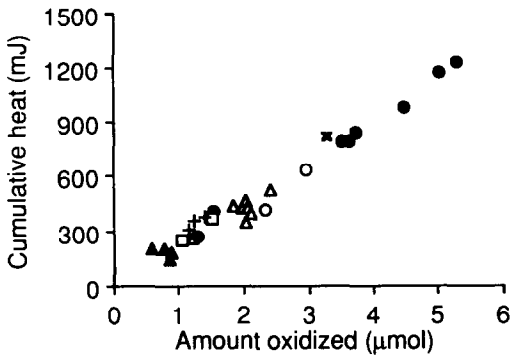


Fig. 9. Cumulative heat plotted as a function of the amount of ascorbic acid in μmol that has oxidized during the microcalorimetric measurement. The slope that can be drawn is the apparent enthalpy change of the process, 224 kJ/mol. The measurements have been performed under different conditions mainly with a 0.10% w/v ascorbic acid solution. (△) Totally filled glass vials, (●) partly filled glass vials, (▲) N_2-purged solutions in totally filled glass vials, (□) including EDTA in totally and partly filled glass vials, (○) totally and partly filled stainless-steel vessels, (×) including EDTA in totally and partly filled stainless-steel vessels and finally (+) 0.01% w/v ascorbic acid in totally and partly filled glass vials.

The measurements were performed with the intention of showing the complexity of the microcalorimetric response when two major competing reactions proceed in the solution with different reaction rates. The above results for the mixed samples, especially curve (b), are not easily interpreted. The heat evolved during that measurement is much lower for the combination than for either component alone. One explanation might be that the bisulfite functions as terminator of radical reactions (Lachman et al., 1986). Fyhr and Brodin (1987) conclude that the oxidation takes place by catalysis in which radicals participate, rather than through a propagating radical mechanism. However, there are several other reactions that may influence the effectiveness of bisulfites as antioxidants (Lachman et al., 1986), which means that many different reactions may influence the heat flow response.

The apparent enthalpy change

In Fig. 9, the cumulative heat has been plotted against the amount oxidized in μmol for all measurements lasting 22 h, except those including antioxidants. As can be seen, there is a linear relationship. The slope is referred to as the apparent enthalpy change, which means the enthalpy change corresponding to the heat from all the various reactions in the oxidation process obtained by microcalorimetry, divided by the amount oxidized in μmol, as obtained by HPLC. The slope gives a value of 224 kJ/mol ($n = 30$). The linear relationship shows that the microcalorimetric technique can be used as a stability indicating technique for the oxidation of ascorbic acid.

If the 71 h measurements are also included ($n = 38$), the slope of the line increases somewhat, to 262 kJ/mol, because of high cumulative heat values for some of the measurements that showed a large decomposition. The main reason is probably that the further degradation of the dehydroascorbic acid proceeds in many steps. These later decomposition steps become more dominant for an extended measurement time in a

surplus of oxygen, which will increase the cumulative heat and thus the apparent enthalpy change.

Conclusions

The microcalorimetric technique is shown to be a reliable method of indicating stability at 25.0°C for a complicated degradation reaction, such as the oxidation of ascorbic acid. The cumulative heat evolved and the amount of ascorbic acid oxidized showed a linear relationship. However, when several major competing reactions proceed, such as when an antioxidant is added, the microcalorimetric response becomes very difficult to interpret.

The sensitivity of the primary heat flow curves was used to describe subtle changes in the reaction rate. The oxidation rate is strongly pH-dependent. The conditions under which the measurements were made, i.e., the amount and the availability of oxygen in the sample vials, substantially influenced the rate and extent of the oxidation reaction.

For complicated reaction mechanisms dependent on environmental changes, the microcalorimetric measurements should preferably be performed as a comparative stability study, in which the response for one sample is compared to that for another sample with only one variable changed at a time. With the TAM this is easily achieved, because of the multichannel configuration.

The heat flow curves associated with the solutions in the stainless steel vessels were different from those associated with the glass vials, but the amount oxidized could still be related to the heat evolved.

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