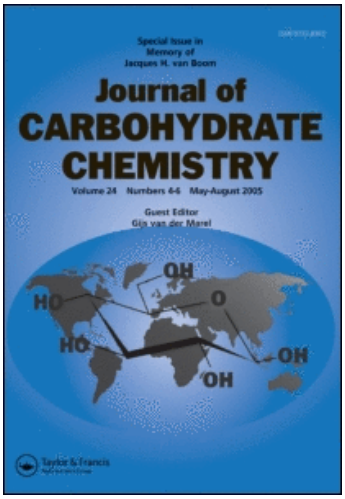

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A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY STUDY OF D-CELLOBIOSE DEGRADATION UNDER FENTON CONDITIONS

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ABSTRACT

Cellobiose was used to model chemical processes taking place during the weathering of cotton fiber cellulose. High-performance liquid chromatography (HPLC) showed that the products of cellobiose degradation were D-glucose and organic acids under Fenton-type conditions (ferrous ion plus H_2O_2). Hydrogen peroxide was added directly or photochemically generated *in situ* by the action of UV light upon aqueous ferrous ammonium sulfate. Effects on D-cellobiose degradation caused by added peroxide or ferrous ion were monitored at varying concentrations and under UV light and dark conditions. Increasing concentrations of peroxide or ferrous ion resulted in greater degradation. Samples exposed to UV light (350 nm) experienced greater degradation than those not exposed.

INTRODUCTION

The quality of cotton fiber diminishes from the time of boll opening to harvest because of a process termed weathering.[1-3] Although light has long been known to chemically affect cotton textiles,[4] little is known of how other parameters, in particular mineral content, may relate to the photochemical action of sunlight on cotton degradation. The principal component of cotton fiber is cellulose. To study the effects of light and other factors on cotton, it is useful to examine reactions of model systems. We have

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + {}^{\bullet}OH + -OH$$
$$Fe^{+2} + {}^{\bullet}OH \rightarrow Fe^{+3} + -OH$$
$$H_2O_2 + {}^{\bullet}OH \rightarrow H_2O + HO_2{}^{\bullet}$$
$$Fe^{+2} + HO_2{}^{\bullet} \rightarrow Fe^{+3} + HO_2-$$
$$Fe^{+3} + HO_2{}^{\bullet} \rightarrow Fe^{+2} + O_2 + H+$$

Scheme 1. Fenton reaction sequence.[15]

chosen cellobiose as such a model since it is the fundamental disaccharide unit of cellulose, consisting of two D-glucopyranose units of β-1→4 linkage.

In earlier work, the degradation of cellobiose was studied under ultraviolet (UV) light,[5,6] Fenton reaction,[7] and alkaline hydrogen peroxide (H_2O_2) conditions.[8-10] Stillings and Van Nostrand irradiated cellobiose for 108 h under a Uviarc lamp with precautions to exclude oxygen. They noted a slight evolution of carbon monoxide and carbon dioxide but concluded that this did not result from the photolysis of hemiacetal groups or glycosidic bonds.[5] Beelik and Hamilton irradiated cellobiose with light of 220 to 400 nm in the presence of air and, using paper chromatography, identified D-glucopyranosyl-β-(1→3)-D-arabinose, D-glucose and D-arabinose as reaction products.[6]

In the Fenton reaction,[11] iron in the +2 oxidation state (ferrous) reacts with H_2O_2, producing highly reactive hydroxyl radicals. This reaction has been extensively studied and there are several reviews on the subject.[12-14] The sequence of reactions given by Baxendale *et al.* is generally accepted (Scheme 1),[15] and would be applicable under neutral or acidic conditions.

In cotton fiber, H_2O_2 can be generated from the action of peroxidase enzymes present within plant cell walls.[16-22] Iron varies significantly (20-95 ppm) in the cotton fiber depending on growing conditions.[23]

Uchida and Kawakishi, using Cu(I) → Cu(II) as a modified Fenton reagent, studied the reactions of several disaccharides, including cellobiose with hydroxyl radical.[7] By analyzing the trimethylsilyl derivatives of the reduced oxidation products using GLC-MS, D-erythrose, D-arabinose, D-glucose, D-glucopyranosyl-β-(1→2)-D-erythrose and D-glucopyranosyl-β-(1→3)-D-arabinose were

found. It was not possible by their method, however, to distinguish between aldoses and their corresponding aldonic acids, since both were reduced to the alditol form and thus formed the same trimethylsilyl derivative.

Isbell and coworkers treated cellobiose and other disaccharides with alkaline hydrogen peroxide in both the presence and absence of iron salts and, for cellobiose, determined cellobionic acid, D-glucopyranosyl-β-(1→3)-D-arabinonic acid, D-glucopyranosyl-β-(1→2)-D-erythronic acid and formic acid to be products.[8-10] They determined that the rate of degradation of D-glucose is greatly increased when traces of either ferrous or ferric salts are included with the alkaline hydrogen peroxide.[9]

Still unknown are the combined effects of UV light from incident sunlight, ferrous ion and H_2O_2 on disaccharides such as cellobiose. An investigation into the effects of UV light on cellobiose in the presence of trace amounts of ferrous ion at concentrations typically found in weathered cotton fibers could clarify the potential for degradation of cellulose under weathered conditions. In this regard, high-performance liquid chromatography (HPLC) is a useful tool for the monitoring of cellobiose degradation products.

RESULTS AND DISCUSSION

Degradation of cellobiose under the influence of hydroxyl radicals generated by the Fenton reaction was first studied using relatively high concentrations of ferrous ammonium sulfate (FAS) and H_2O_2. Concentrations of FAS (5.1×10^{-3}M) with [H_2O_2] (2.2×10^{-2}M) caused significant degradation of cellobiose within 30 min in the dark. Evaluation of the HPLC chromatograms for cellobiose reacted with Fenton's reagent in the dark indicated major peaks for cellobiose (and co-eluates), D-glucose (and co-eluates) and formic acid. Extending the reaction time to 72 h did not result in any further degradation in the dark. In contrast, when cellobiose reacted with Fenton's reagent under UV light, additional degradation was observed with increasing reaction time. As seen in **Fig. 1**, increases in peak area were observed for degradation products, specifically in peaks obtained from the refractive index (RI) detector for D-glucose (and co-eluates) at *ca.* 9.5 min retention time (RT), and for formic acid at *ca.* 15.2 min, while

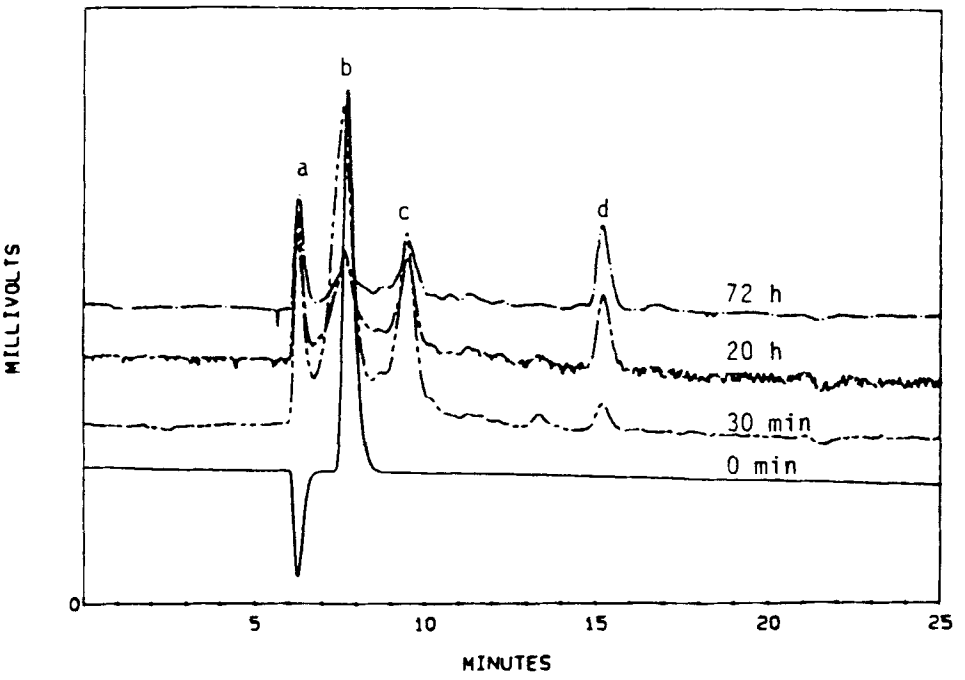


Figure 1. HPLC chromatograms (RI) of cellobiose (7.8 mM) reacted with FAS (5.1 mM) and H_2O_2 (22 mM) under UV light exposure for 0 min (_____), 30 min (___ _ _ ___), 20 h (___ _ ___) and 72 h (___ ___). Peak **a** is ammonium sulfate, **b** is cellobiose (and co-eluates), **c** is D-glucose (and co-eluates), **d** is formic acid.

decreases in peak area occurred for cellobiose at *ca.* 7.7 min. These results show that the Fenton reaction, which is complete within minutes in the dark,[24,25] is not the only mechanism operating when cellobiose is degraded in the presence of FAS, H_2O_2 and UV light. This more extensive degradation under UV exposure is likely a significant mechanistic component of cotton fiber weathering.

Additional verification that at high concentrations of FAS and H_2O_2, cellobiose degradation can occur in the dark was accomplished by varying the concentrations of either FAS or H_2O_2 while holding the other constant. The effect of varying [H_2O_2] while maintaining [FAS] (0.0051 M) on cellobiose degradation in the dark for 30 min is seen in **Fig. 2.** Upon increasing the H_2O_2 concentration from 0 to 33 mM, progressive degradation resulted in the formation of a major product with RT *ca.* 9.5 min and minor products at *ca.* 10.2 and 15.2

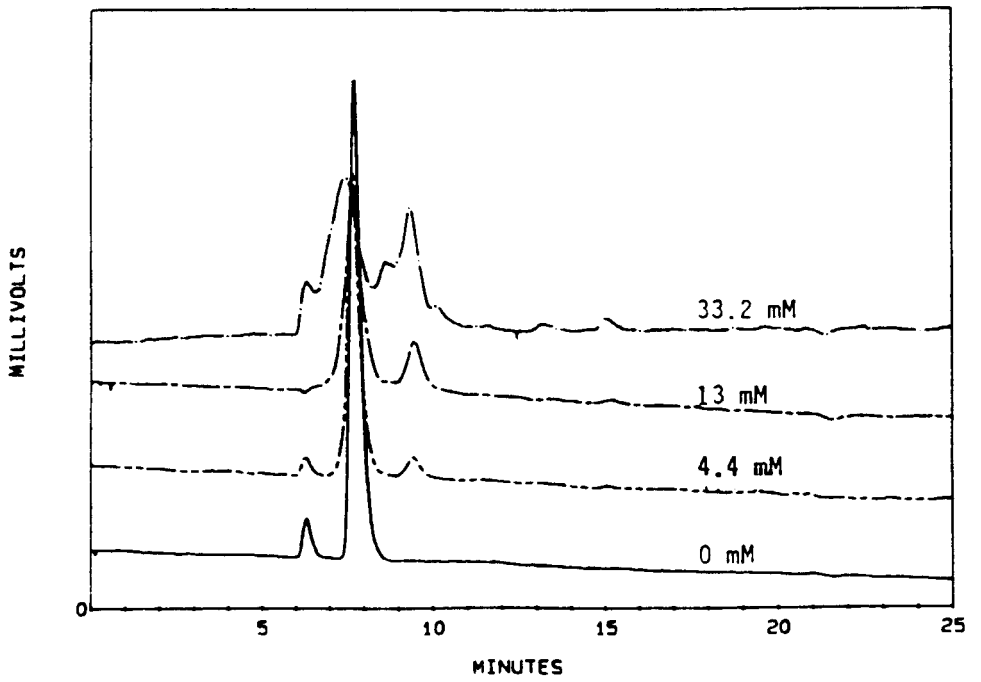


Figure 2. HPLC chromatograms (RI) of cellobiose (7.8 mM) reacted with FAS (5.1 mM) and varying [H_2O_2] for 30 min in the dark. [H_2O_2] = 0 mM (_____), 4.4 mM (___ _ _ ___), 13 mM (___ _ ___), 33.2 mM (___ . ___). Peak **a** is ammonium sulfate, **b** is cellobiose (and co-eluates), **c** is D-glucose (and co-eluates), **d** is formic acid.

min. The major product co-elutes with both D-glucose and D-*glucono*-δ-lactone standards. The minor products at 10.2 and 15.2 min co-elute with D-arabinose and formic acid standards, respectively. From the chromatogram of the reaction mixture with [H_2O_2] = 0, it is apparent that Fe^{+2} alone does not cause degradation of cellobiose in the dark. Similarly, H_2O_2 alone is not sufficient under dark conditions, at least in this time period. In another experiment, [H_2O_2] was held constant at 22 mM while [FAS] was varied from 0 to 1.02 mM. Slight degradation was seen in the 0.26 and 0.51 mM samples, but significant degradation was not seen until [FAS] was increased to 1.02 mM.

In contrast, cellobiose was degraded when exposed to UV light at much lower concentrations of FAS and H_2O_2 than those required to cause degradation in the dark. In addition, the presence of either

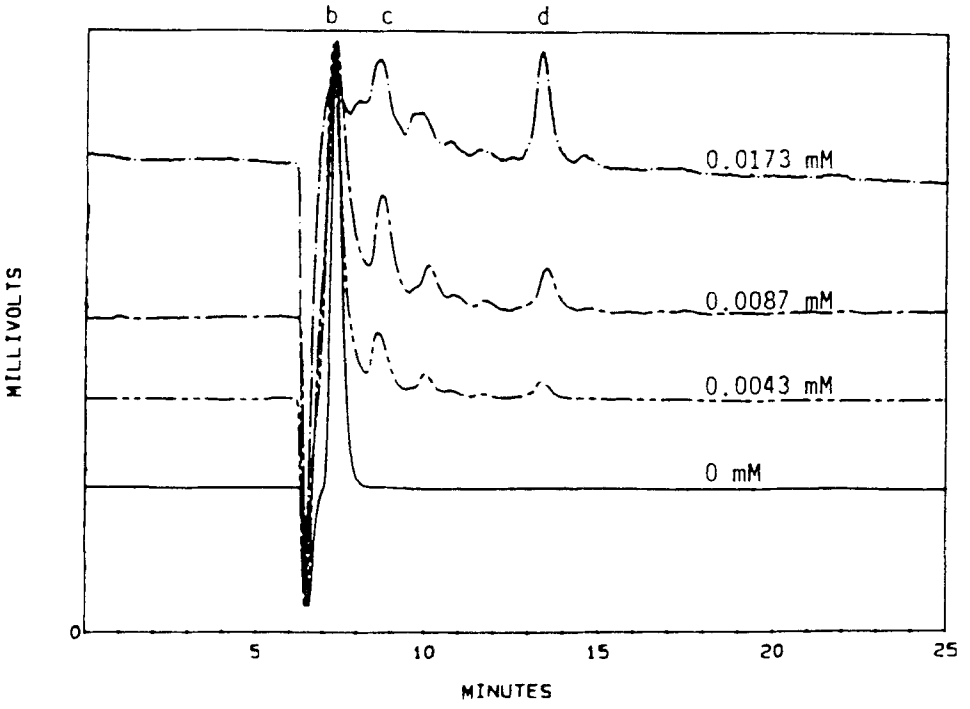


Figure 3. HPLC chromatogram (RI) of cellobiose (7.8 mM) reacted with varying amounts of [FAS] in the absence of added H_2O_2 under UV light exposure for 168 h. [FAS] = 0 (_____), 0.0043 mM (___ _ _ ___), 0.0087 mM (___ _ ___), 0.0173 mM (___ . ___). Peak **b** is cellobiose (and co-eluates), **c** is D-glucose (and co-eluates), **d** is formic acid.

H_2O_2 or FAS alone is sufficient to cause cellobiose degradation to occur under UV light.

To determine if FAS alone was sufficient to cause cellobiose degradation under UV exposed conditions, a series of cellobiose solutions was made in which H_2O_2 was absent and [FAS] was varied at 0, 0.0043, 0.0087, 0.0173, 0.0347 and 0.0694 mM. The significance of these concentrations is that 0.0043 mM FAS in a solution that is 8.8 mM in cellobiose is equivalent to 80 parts Fe^{+2} per million parts cellobiose. A recent study of weathered cotton fiber samples has revealed total iron contents as high as 95 ppm.[23] Solutions of varying [FAS] were allowed to react for 168 h with UV irradiation. The RI chromatograms of the reaction mixtures containing 0, 0.0043, 0.0087 and 0.0173 mM FAS are shown in **Fig. 3**. Notable degradation

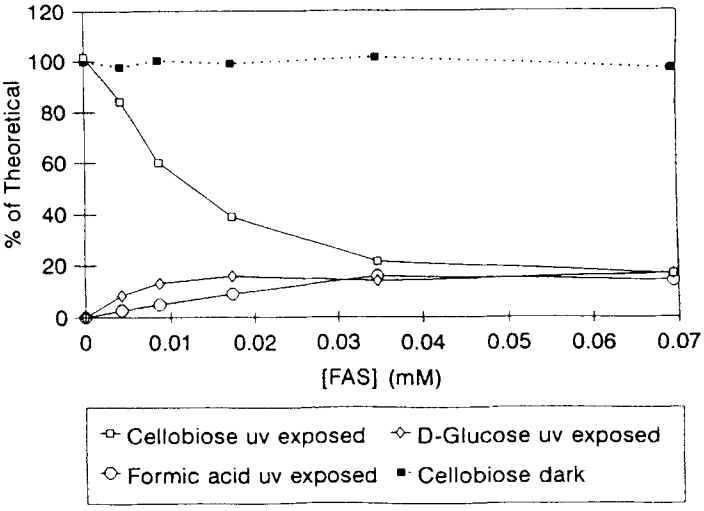


Figure 4. Degradation of cellobiose (7.8 mM) under exposure to UV light (168 h) with concomitant formation of D-glucose and formic acid in the presence of varying amounts of FAS (mM) (0, 0.0043, 0.0087, 0.0173, 0.0347 and 0.0694) and in the absence of added H_2O_2. No D-glucose or formic acid were found in the samples left in the dark at all FAS concentrations tested.

has occurred even in the lowest [FAS] (0.0043 mM); considerable degradation was observed in the more concentrated samples. Comparison with standards showed that the first major peak after cellobiose (9.0 min) had a retention time identical to that of D-glucose and D-glucono-δ-lactone standards, the second major peak (10.2 min) co-eluted with D-arabinose, while the third major peak (13.5 min) co-eluted with formic acid. While significant degradation occurred for all of the UV exposed samples containing FAS, there was no degradation in the sample in which both FAS and H_2O_2 were absent. Replicate samples left in the dark for 168 h were not degraded. Cellobiose degradation at these FAS concentrations was monitored by HPLC and the results are plotted in **Fig. 4**. Percent of theoretical represents the percentage of the maximum amount of D-glucose or formic acid theoretically obtained if all of the cellobiose in the starting mixture was converted to either product (2 moles of D-glucose and 12 moles of formic acid per mole of cellobiose).

Also addressed was the question of whether H_2O_2 alone was sufficient to cause degradation of cellobiose under UV exposed

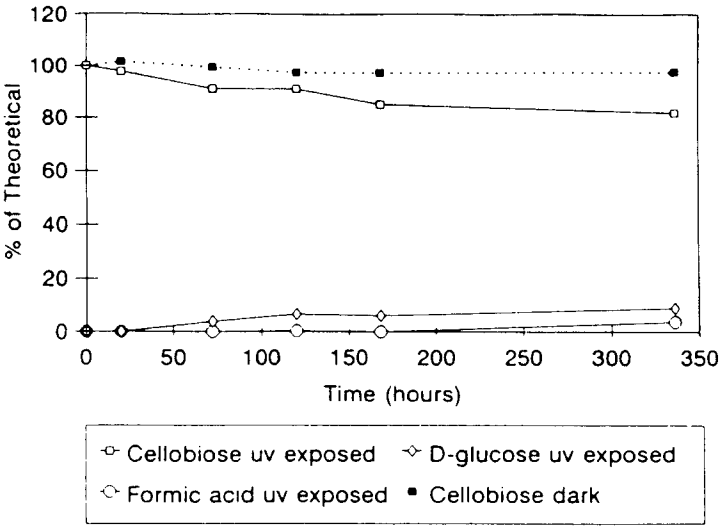


Figure 5. Degradation of cellobiose (7.8 mM) under exposure to UV light for varying time periods (20, 72, 120, 168 and 336 h) with concomitant formation of D-glucose and formic acid in the presence of H_2O_2 (2.2 mM) and in the absence of FAS. No D-glucose or formic acid were found in the samples left in the dark at all times tested.

conditions. A series of cellobiose solutions was made in which FAS was absent and H_2O_2 was varied at 2.2, 22 and 73 mM. These solutions were either left in the dark or exposed to UV light for 20, 72, 120, 168 and 336 h. For the 2.2 mM sample ([FAS] = 0) small amounts of degradation were seen at 72 h under UV light. At 336 h over 80% of the original cellobiose remained and the D-glucose and formic acid contents were 8.9% and 3.7% of their theoretical maxima, respectively (**Fig. 5**). Samples left in the dark at this concentration did not degrade.

A ten-fold increase in $[H_2O_2]$ to 22 mM ([FAS] = 0) was enough to cause significant degradation at all times monitored. Only 44.3% cellobiose and co-eluates remained when this higher concentration of peroxide was included for this time interval (**Fig. 6**), whereas, at $[H_2O_2]$ of 2.2 mM no degradation had occurred after 20 h of UV exposure. From 44.3% it dropped steadily to 19.3% by 336 h. During the same time period, the amount of formic acid generated rose steadily from 4.3% to 17.2%. For samples in the dark, only very

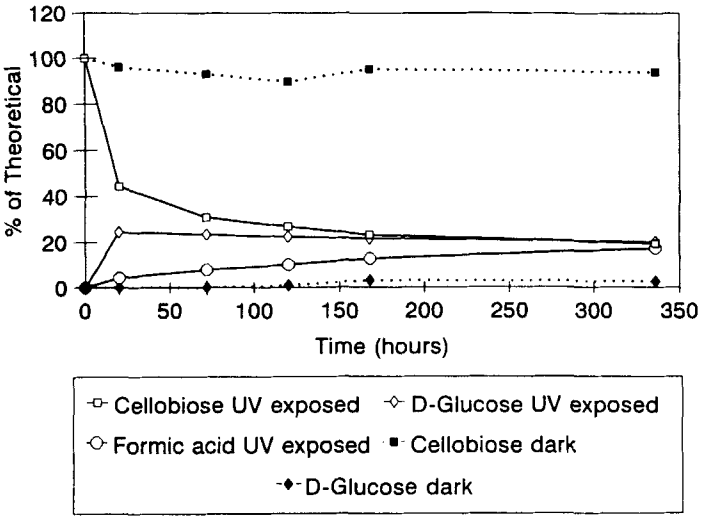


Figure 6. Degradation of cellobiose (7.8 mM) under exposure to UV light for varying time periods (20, 72, 120, 168 and 336 h) with concomitant formation of D-glucose and formic acid in the presence of H_2O_2 (22 mM) and in the absence of FAS. No formic acid was found in the samples left in the dark at all times tested.

slight degradation was observed at this concentration at 120, 168 and 336 h. No degradation was observed after 20 or 72 h.

At the highest H_2O_2 concentration (73 mM, [FAS] = 0) studied in this series, significant degradation had occurred within 20 h under UV exposure, and by 120 h, very little cellobiose and its co-eluates remained (6.1%). After 336 h, both cellobiose and D-glucose were below 5% (**Fig. 7**). For samples left in the dark, only very slight degradation was observed at this [H_2O_2] at 72, 120, 168 and 336 h. No degradation was evident after 20 h.

The chromatograms of the 336 h UV exposed reaction mixtures at [FAS] = 0 and [H_2O_2] = 0, 2.2, 22 and 73 mM are shown in **Figs. 8-a** (RI) and **8-b** (UV at 210 nm). Apparent from **Fig. 8-a** is the rise in D-glucose and co-eluate concentration in going from the 2.2 mM sample to the 22 mM sample, and its fall in going from the 22 mM sample to the 73 mM sample, indicating that D-glucose is likely an intermediate degradation product. From **Fig. 8-b** it is apparent that several UV absorbing compounds were formed from the degradation of cellobiose. In addition to formic acid (RT = 13.3 min), two that

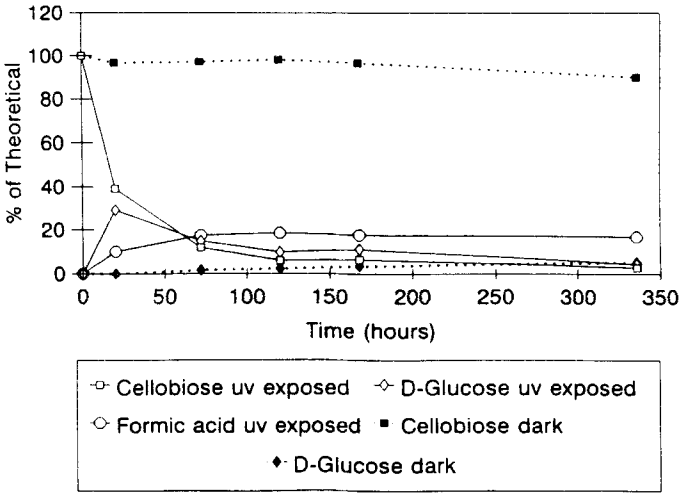


Figure 7. Degradation of cellobiose (7.8 mM) under exposure to UV light for varying time periods (20, 72, 120, 168 and 336 h) with concomitant formation of D-glucose and formic acid in the presence of H_2O_2 (73 mM) and in the absence of FAS. No formic acid was found in the samples left in the dark at all times tested.

were obvious from comparison to standards were oxalic acid (RT = 7.1 min) and glycolic acid (RT = 11.6 min). Oxalic acid co-eluted with cellobiose and, in the RI chromatogram (**Fig. 8-a**) its influence can be seen by the broadening effect it had on the cellobiose peak (RT *ca.* 7.2 min), most notably in the 22 mM sample. Oxalic acid has been reported as a product of the Fenton reaction in the presence of D-glucose.[26-29] It is likely that oxalic acid is not the only compound co-eluting with cellobiose, as indicated by the shoulder peak on the tail side of the oxalic peak in the UV-210 nm chromatogram. Considering that maltobionic and lactobionic acids have been reported among the reaction products from maltose and lactose,[30] cellobionic acid and/or *cellobiono-δ*-lactone are quite possibly degradation products of cellobiose. Also possible are D-glucopyranosyl-β-(1→3)-arabinose and D-glucopyranosyl-β-(1→2)-erythrose and their aldonic acid forms as degradation products, as are the aldosulose and aldosonic acid forms of cellobionic acid, since D-arabinonic acid, D-*arabino*-hexulosonic acid and D-*arabino*-hexosulose have been reported from the treatment of D-glucose with Fenton reagent.[31]

Isbell *et al.* proposed a mechanism for the degradation of reducing disaccharides in alkaline H_2O_2 in which peroxide adds to the aldehydic carbon atom of the acyclic form of the aldohexose residue followed by decomposition to the next lower disaccharide and formic acid.[8] The process would continue stepwise until interrupted by the glycosidic linkage (Scheme 2). Evidence that the mechanism proceeds via the acyclic form comes from an earlier study by Isbell *et al.*, in which it was found that the rate of oxidative degradation of aldoses with alkaline hydrogen peroxide correlates with the stability of the acyclic form of the sugar.[32]

Isbell and Naves proposed that under alkaline H_2O_2 conditions, Scheme 2 could operate by either ionic or free radical mechanisms. At lower pH's and with added ferrous ion, or UV light to generate •OH, a free radical mechanism would be expected to dominate. This mechanism proposed the conversion of **5** to a peroxy intermediate **6**, which, after basic hydrolysis of the ester **7** yielded an aldohexose and glyceraldehyde by way of hemiacetal **8**. Under the acidic conditions of our study (pH varies from near 5 to < 3 as the reaction proceeds, depending upon the original FAS concentration), acid catalyzed hydrolysis could account for formation of the aldohexose D-glucose. While hydroxyl radicals can add directly to the aldehydic function as occurs in Scheme 2, they can also abstract carbon-bound hydrogen atoms from the carbohydrate.[33] If this occurred at the anomeric carbon of the non-reducing glucopyranosyl unit of cellobiose, hydrolysis could occur at the glycosidic linkage (Scheme 3). The rate of hydrolysis is enhanced if the bond to be hydrolyzed is next to a radical site.[34]

The effect of UV light on cellobiose solutions containing either FAS or H_2O_2 but not both was profound. While significant degradation occurred for all FAS tested in the absence of H_2O_2 with UV light for 168 h, no degradation was observed for any of the corresponding samples in the dark. For samples containing H_2O_2 but devoid of FAS, only those with $[H_2O_2] \geq 2.2$ mM experienced any degradation in the dark, and in each case the amount of degradation was quite small in comparison and only occurred after long periods of time. In the dark, metallic catalysts such as the Fenton reagent are required to produce hydroxyl radicals from H_2O_2. Such catalysts are not needed in the presence of UV light; the action of the light itself can generate •OH. Hydrogen peroxide is known to absorb at

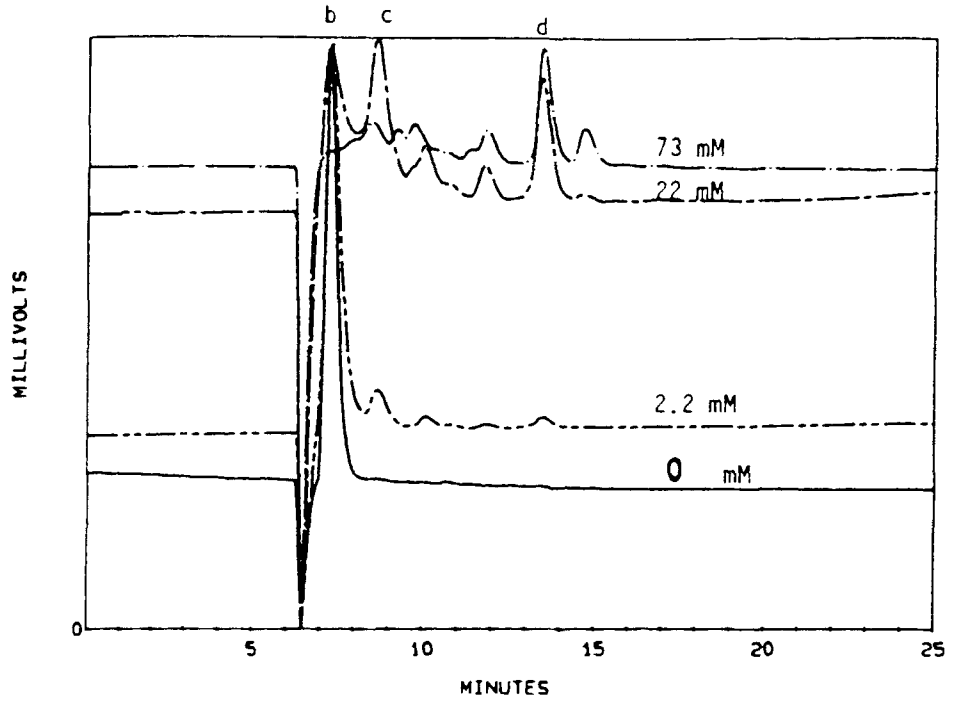


Figure 8-a. HPLC chromatogram (RI) of cellobiose (7.8 mM) reacted with varying concentrations of H_2O_2 in the absence of FAS under UV light exposure for 336 h. [H_2O_2] = 0 mM (_____), 2.2 mM (___ _ _ ___), 22 mM (___ _ ___), 73 mM (___ ___). Peak **b** is cellobiose (and co-eluates), **c** is D-glucose (and co-eluates), **d** is formic acid.

wavelengths > 400 nm and use has often been made of this property to induce oxidation of compounds that are not attacked by peroxide in the dark.[35]

For UV-irradiated cellobiose solutions containing FAS but devoid of H_2O_2, it is apparent that the photochemical process must involve FAS in some manner, since UV-irradiated solutions that contain neither FAS nor H_2O_2 experience no degradation. The principal photochemical process for Fe^{+2} is

$$Fe^{+2} + h\nu \rightarrow Fe^{+3} + e^{-}(aq)$$

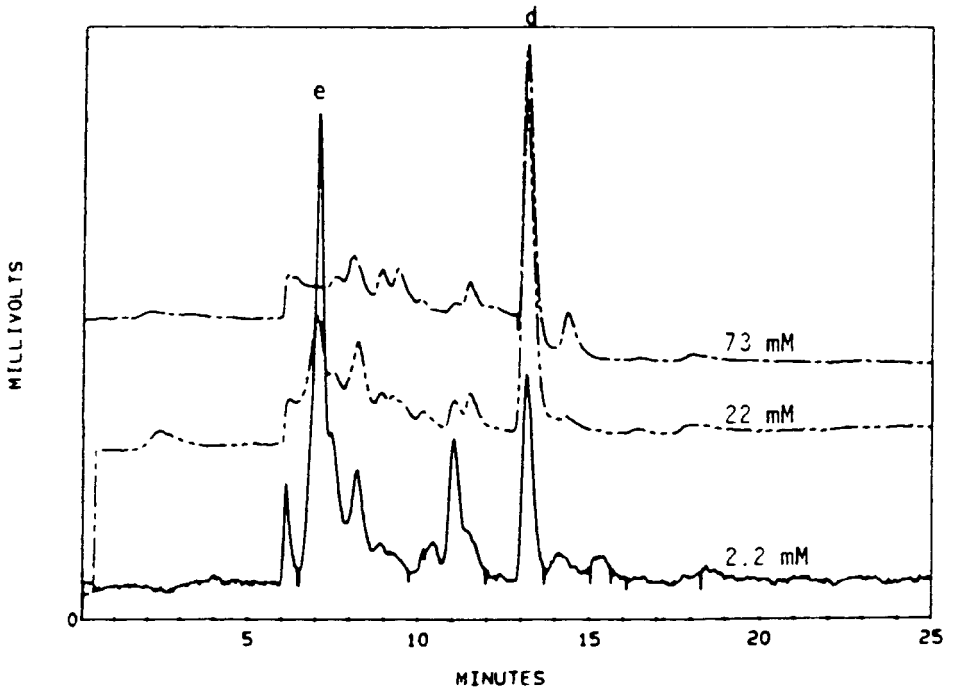


Figure 8-b. HPLC chromatogram (UV at 210 nm) of cellobiose (7.8 mM) reacted with varying concentrations of H_2O_2 in the absence of FAS under UV light exposure for 336 h. [H_2O_2] = 2.2 mM (_____), 22 mM (___ _ _ ___), 73 mM (___ _ ___). Peak **d** is formic acid and peak **e** is oxalic acid. Glycolic acid appears at RT 11.5 min as a shoulder peak.

with a λ_{max} of 254 nm.[36] The reduction of molecular oxygen yields superoxide ion as the primary product.[37-39]

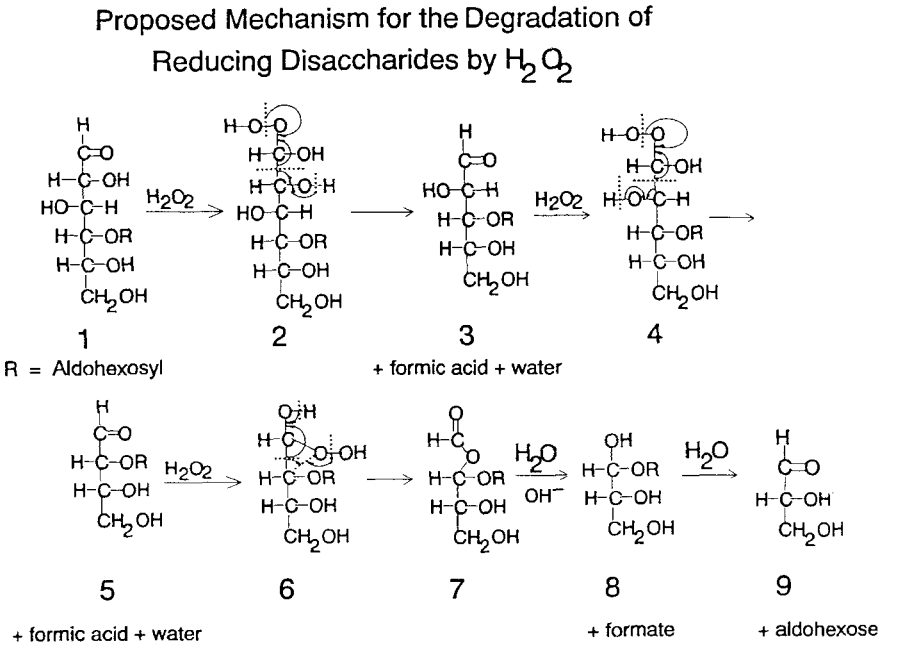
$$O_2 + e^- \rightleftharpoons O_2^- \quad Eo' = -0.16 \text{ V vs. NHE}$$

When this process occurs in water, the product rapidly hydrolyzes and disproportionates.[40]

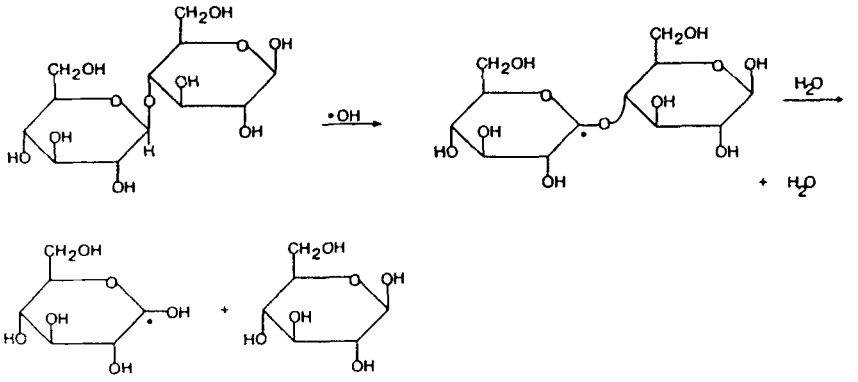
$$O_2^- + H_2O \rightarrow HO_2\bullet + OH^-$$

$$HO_2\bullet + O_2^- + H_2O \rightarrow H_2O_2 + O_2 + OH^- \qquad k = 1 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$$

$$HO_2\bullet + HO_2\bullet \rightarrow H_2O_2 + O_2 \qquad k = 8.5 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$$



Scheme 2. Isbell's mechanism for the degradation of reducing disaccharides in alkaline H_2O_2.[8]



Scheme 3. Radical formation by hydrogen abstraction at the anomeric carbon of cellobiose and subsequent hydrolysis to D-glucose.

Under these conditions the Fenton reaction can proceed via generated H_2O_2.

Here it should be noted that H_2O_2 is not necessarily the only oxidizing species. No attempt was made to exclude atmospheric O_2, and O_2 is produced in both the Fenton reaction and during the destruction of H_2O_2 by catalase. Since no degradation was observed for those samples in which both FAS and H_2O_2 were excluded, O_2 by itself is not causing oxidation. Its participation in the oxidative process after the Fenton reaction has begun cannot be ruled out, however.

In summary, this study has determined that cellobiose is degraded to D-glucose, D-arabinose, and organic acids under Fenton conditions, with the extent of degradation increasing with increasing concentrations of Fe^{+2} or H_2O_2 and with increasing reaction time. Reaction mixtures exposed to UV light experienced a higher degree of degradation than those not exposed. In the dark, both Fe^{+2} and H_2O_2 were necessary in order to cause degradation, whereas under UV exposure, either Fe^{+2} or H_2O_2 alone sufficed. When exposed to UV light, cellobiose can be degraded in the presence of trace amounts of Fe^{+2} at concentrations found in cotton fibers.

EXPERIMENTAL

Reagents. Carbohydrates and organic acids used as standards were obtained commercially and used without further purification. Standard solutions were made to a concentration of 2.67 mg/mL. The following reference standards were used: D-arabinose (Sigma),[41] D-cellobiose (Aldrich), formaldehyde (Baker, sold as 37% solution), formic acid (Baker, sold as 88% solution), D-*glucono*-δ-lactone (ICN Pharmaceuticals), D-glucose (Baker), glycolic acid (Aldrich), glyoxylic acid monohydrate (Aldrich) and oxalic acid (Fisher). The following reagents were used: ferrous ammonium sulfate hexahydrate (FAS) (Baker), Chelex chelating resin (Sigma), hydrogen peroxide (30%, Sigma), sulfuric acid (Mallinckrodt), catalase (9300 units/mg, Sigma). Stock solutions of FAS and hydrogen peroxide were used freshly prepared. Catalase solutions were stored at 4 °C. Water was purified through a Millipore MilliQ system (specific conductance 18 $\mu\Omega$ cm).

Sample Preparation and Reaction Procedures. Reaction mixture solutions were prepared to proper concentrations in 20 mL Pyrex

test tubes using the appropriate amounts of cellobiose (7.8 mM throughout), FAS and H_2O_2 stock solutions. Concentrations given are original concentrations; some precipitation of Fe^{+2} and/or Fe^{+3} hydroxides and/or oxides was observed in the reaction mixtures of high [FAS] as the reactions proceeded, and this precipitation could reduce the amount of Fe^{+2} ion available for reaction. The solutions were mixed by vortex action and the test tubes were capped and placed under UV light or in the dark for a specified time period. Solutions exposed to UV were placed in a revolving carousel in the reactor chamber of a Rayonet™ photochemical reactor. Encircling the inner wall of the cylindrical compartment of the photochemical reactor chamber were 16 tubular lamps of 75W each, mounted vertically. The wavelength of maximum output for the lamps was 350 nm. The intensity reading at the center of the reactor is 9200 $\mu W/cm^2$ (manufacturer's data). As the carousel rotated, the test tubes came within approximately 2 cm of the lamps. A fan operating at the bottom of the chamber provided ventilation, keeping the temperature below 45 °C. After the allotted time period, the photochemical reactor was turned off, the test tubes were removed from the reactor, and catalase (0.5 mL, 0.2 mg/mL) was added. By observation of the disappearance of the HPLC peak (UV-210 nm detector) for H_2O_2, catalase was shown to be effective at destroying excess peroxide at pH's encountered in all reaction mixtures except those of the highest [FAS] (pH of reaction mixture with [FAS] = 5.1 mM was 2.7, all others above 3.4). Reaction mixtures with [FAS] $\geq$ 0.127 mM were eluted twice through Chelex beds (2 mL) prepared in Pasteur pipettes to remove FAS. By spectrophotometric analysis at 508 nm to detect the ferrous 1,10-*o*-phenanthroline complex, it was found that these elutions completely removed 5.1 X 10^{-3} FAS from the eluate, at least to the detection limits of the method. The solutions were prepared for HPLC analysis by filtration through Millex GV (Millipore) 0.22 μM filters using a commercially available vacuum extraction apparatus (Model 10 SPE, Baker) directly into sample vials placed inside the extraction system rack.

Chromatographic Procedure. The chromatographic procedure was based on one developed previously by Timpa and Burke.[42] A Beckman Model 100-A dual-piston pump was used with an Altex 210 manual injector (20 μL injection loop). The refractive index (RI) detector was a Knauer differential refractometer and the UV-visible detector

was a Beckman Model 165 with a microprocessor-based, programmable system controller (Model 421). The mobile phase was 0.013N sulfuric acid, that was filtered through a 0.45 μm filter (type HA, Millipore) and degassed by sonication before use. The column (Aminex HPX-87H, BioRad) was a cross-linked polystyrene ion-exchange resin in the hydrogen form and fitted with a guard column of similar material (Micro Guard, ion exchange, BioRad). The column was maintained at 40 °C by a column heater (Model LC23, Bioanalytical Systems). The flow rate of the mobile phase through the system was maintained at 0.6 mL/min. Detection with the UV-vis detector was conducted at a wavelength of 210 nm. Data was collected by a Waters 840 Data and Chromatography Control Station interfaced with a Waters System Interface Module. Peak areas were measured using manual baseline to baseline integration and compared to those of external standards. Data points are the average of four replicates.

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41. Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U. S. Department of Agriculture over others not mentioned.

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