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BIODEGRADATION OF OXIDIZED REGENERATED CELLULOSE

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ABSTRACT

The *in vitro* solubilization and degradation of regenerated cellulose was studied under conditions which approximate those found *in vivo*, when the material is used as an adhesion barrier to assist normal wound repair. Factors affecting solubilization which were examined included the effects of serum or plasma, and the presence of hydrolytic enzymes. Products of the solubilization and degradation processes were examined by high performance liquid chromatography coupled with pulsed amperometric detection. The oxidized polymer readily undergoes chain shortening to give oligomers which, in the presence of plasma or serum, are further hydrolyzed to smaller fragments, including glucuronic acid and glucose. Proposed mechanisms of degradation are discussed.

INTRODUCTION

INTERCEED®, TC7, a form of oxidized regenerated cellulose (ORC), is a knitted fabric which has been successfully used in wound healing to prevent post-surgical adhesions[1]. When ORC is surgically implanted *in vivo* to form a physical barrier between adjacent tissues, it is observed to be bioabsorbable. During this process, the ORC undergoes gradual physical changes, first hydrating, swelling to become a gel-like material, and eventually dissolving and disappearing from the site of implantation.

ORC is produced by oxidation of cellulose with nitrogen dioxide, and this process has been studied in detail[2–9]. The oxidation is known to occur at the primary carbon atoms (C-6) and, in the product used in this study, the content of carboxyl groups is typically 12–18%, corresponding to ~72% conversion of the D-glucose units into D-glucuronic acid residues. A secondary oxidative process, which generates carbonyl functions at either C-2 or C-3, is also believed to occur, and to modify further the structure of the polymer[10–13]. Thus, ~15% of the total carbonyl groups

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result from this secondary oxidation, and are ketonic. Assuming random distribution these carbonyl groups are present in one in ten residues, some of which are therefore doubly oxidized. As the C-3 and C-6 atoms of neighboring chains are involved in fibril formation *via* inter-chain hydrogen bonding[14], it is believed that the minor NO_2 oxidation favors the 2-hydroxyl group.

The purpose of this study was to examine the *in vitro* behavior of ORC under conditions which approach those of the *in vivo* environment. Of particular interest were the responses of the molecule to solubilization, and the products resulting from the biodegradation processes. In the past, such studies have been complex, due to the difficulty of detecting and resolving small amounts of carbohydrates in multi-component mixtures. Recent introduction of pulsed amperometric detection (p.a.d.), coupled with high performance liquid chromatography (h.p.l.c.), has facilitated examination of mixtures of diverse underivatized carbohydrate structures. Therefore, the use of such new methodology was ideally suited for this study.

RESULTS

Solubilization. — When ORC is suspended in water, no appreciable solvation takes place, even after several weeks at 37°, but a fall in pH to ~2.5 is observed. Upon addition of mild bases such as sodium carbonate, pyridine, or sodium hydroxide, the material behaves as a polyanion, requiring continuous titration of the mixture in order to maintain a neutral pH. When neutrality is maintained, the ORC begins to curl, loses its fibrous nature, and then gels; continued titration and incubation produces a clear solution which becomes slightly yellow when transient alkalinity is generated. The solubilization and physical appearance of these changes closely resemble those observed[15] in animals implanted with ORC. Within 24 h post-implantation, the material becomes completely gelatinous, and within 48 h only small fragments of it remain. *In vivo* studies have also shown a rapid drop in the pH of the fluid surrounding the site of implantation of the ORC. Subsequent, time-dependent restoration of physiological pH was found concomitant with the gel formation and solubilization of the material.

Fig. 1 shows a comparison of the p.a.d.–h.p.l.c. profiles of ORC, cellulose (rayon) and alginic acid solubilized by incubation in 0.12M NaOH, for 24 h at 37°. There is essentially no solubilization and degradation of unoxidized cellulose under these conditions. Alginic acid, a naturally occurring heteropolymer consisting of mannuronic and guluronic acid residues, shows an increased susceptibility to chain shortening, as indicated by the formation of oligomers of different sizes. Being more extensively oxidized and thus structurally more heterogeneous than alginic acid, ORC presents a more complex profile of increased degradation to oligomeric products (see Table I).

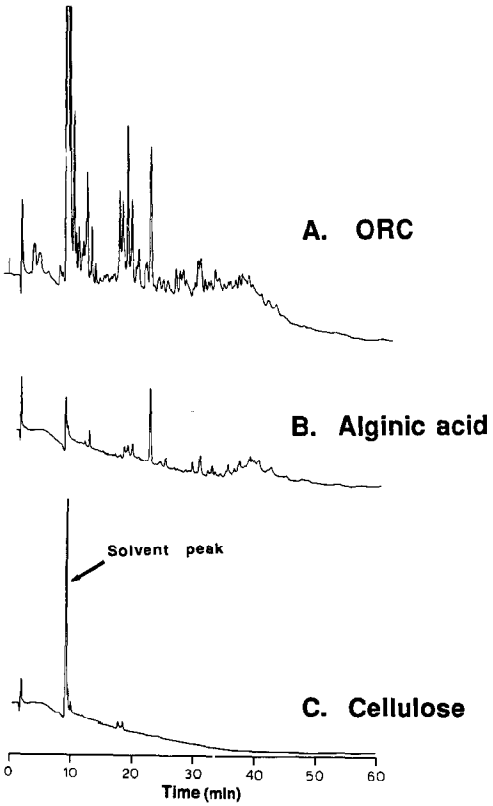


Fig. 1. P.a.d.–h.p.l.c. profiles of structurally related polysaccharides solubilized in base as described in the experimental section. (A) Solubilized ORC, (B) solubilized alginic acid, and (C) solubilized cellulose (rayon).

TABLE I

RESOLUTION AND ANALYSIS OF DEGRADATION PRODUCTS OF ORC BY p.a.d.–h.p.l.c.

Elution time (min)	*Degree of polymerization*		*Composition (%) after solubilization*[a]			
	Dextran oligomers	D-*Galacturonic acid oligomers*	*Pyridine*[b]	*NaOH*[c]	*PBS*[d]	*Serum*[e]
0–2	1[f]	—	0	0	0	55
2–13	2–4	0–1	28	13	9	36
13–36	4–20	2–10	48	45	69	10
36–43	20–24	>10	19	33	13	3
>43	>24	—	4	16	9	0

[a]Values are from integration of areas under chromatographic elution profiles. [b]D_2O–pyridine-d_5 solubilized TC7 (14 mg/mL). [c]0.12M NaOH for 24 h at 37°. [d]Phosphate buffered saline, pH 7.4 at 37°. [e]Serum, pH 7.4, for 24 h at 37°. [f]Elution times for glucose, 2.8 min; cellobiose, 3.2 min; glucuronic acid, 12.8 min.

In order to simulate better the *in vivo* conditions of solubilization and degradation, ORC was dialyzed (mol. wt. cut-off 3500) against phosphate-buffered saline (pH 7.4); this resembles some aspects of the *in vivo* situation in which biological fluids are constantly replaced, and more rapidly restore the pH to neutrality or slight alkalinity. Under these conditions, ~90% of the material (by weight) was solubilized within 21 d and was recovered as the white, powdery sodium salt of polyglycuronic acid. P.a.d.–h.p.l.c. profiles of this material, and of ORC solubilized in aqueous pyridine (pH 6.5), showed that, even under these mild conditions, some degradation of ORC takes place (see Fig. 2).

Characterization of oligomeric fractions. — Well defined oligomers characterized by fast atom bombardment–mass spectrometry were used as standards to characterize the region of the h.p.l.c. profile up to a degree of polymerization (d.p.) of 10 (see Table I). These were (a) β-(1→4)-polymannuronic acid pentamer and

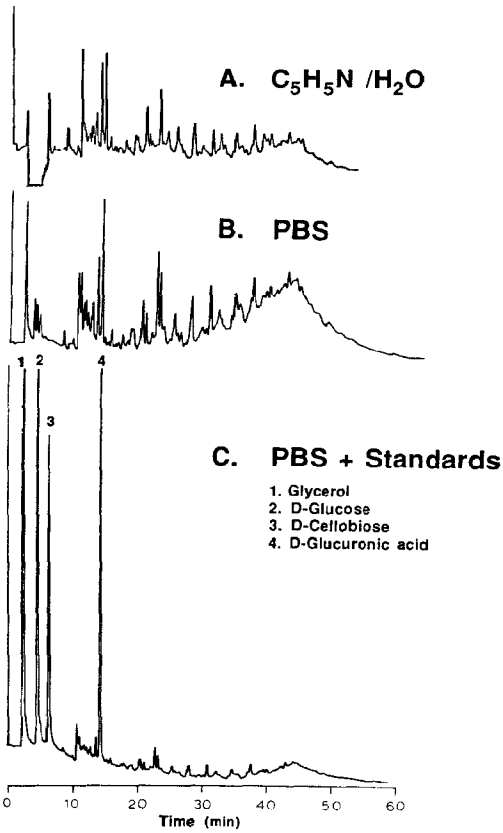


Fig. 2. P.a.d.–h.p.l.c. profiles of ORC. (A) Solubilized in aqueous pyridine, (B) solubilized in PBS, and (C) (B) "spiked" with monosaccharide standards; glycerol was the preservative present in the filtration membranes.

trimer in which the terminal, nonreducing end unit was 4,5-unsaturated; (b) α-(1→4)-polygalacturonic acid dimer, trimer, tetamer and pentamer, also bearing the 4,5-unsaturated terminal unit; and (c) α-(1→4)-polygalacturonic acids of d.p. 2, 4, and 6.

Effects of plasma or serum. — The maximum amount of ORC which dissolved completely in plasma or serum in 24 h at 37° without causing appreciable changes in pH was found to be 3.0 $mg.mL^{-1}$. When a further 3.0 mg of ORC per mL was added to these serum–plasma solutions, incubation for an additional 3 d was needed for complete dissolution. P.a.d.–h.p.l.c. analysis of the solubilized material is summarized in Table I. When ORC was incubated in serum, a considerably higher proportion of the degradation products was eluted in the 1–13-min period, corresponding to standards of low degree of polymerization (*i.e.*, monomers to tetramers; *cf.* Table I).

Enzymic degradation. — Incubation of ORC with cellulase at its pH optimum (5.0) in 50mM acetate buffer led to rapid and complete solubilization of the material. We had already established that, under mild, non-enzymic conditions,

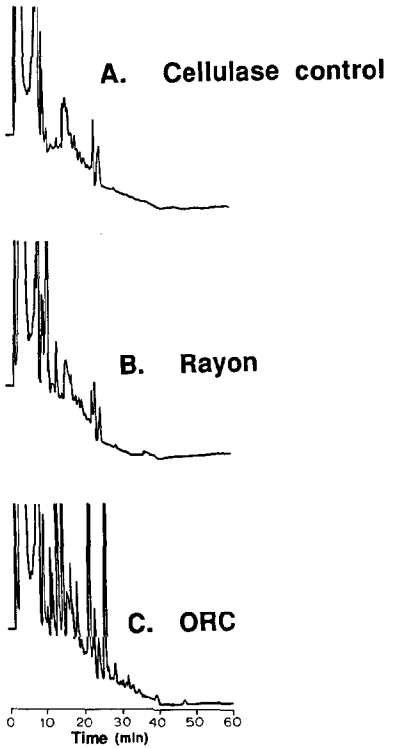


Fig. 3. P.a.d.–h.p.l.c. profiles following cellulolytic activity. (A) Control showing carbohydrate background in commercial cellulase, (B) cellulose (rayon) partially solubilized with cellulase, and (C) ORC solubilized with cellulase.

solubilization of ORC is a slow process. P.a.d.-h.p.l.c. analysis of cellulase hydrolysate clearly showed that enzymic degradation of the polymer had occurred (see Fig. 3). Under identical conditions, hydrolysis of cellulose was much slower, and, in fact, complete solubilization was not observed. Product inhibition and poor hydration are frequently suggested to explain the low rates observed for cellulase hydrolysis. Clearly, the oxidative structural modifications of ORC do not inhibit its cellulolytic degradation. Since the polymer is largely composed of D-glucuronic acid residues, ORC may be more easily hydrated than cellulose, and thus more susceptible to the β-D-glucanase activity of cellulase.

Treatment of ORC with β-D-glucosiduronase and β-D-glucosidase, which, *in vivo,* act upon glycosylated macromolecules, did not result in significant degradation. However, the (soluble) sodium salt of ORC, obtained by dialysis against PBS, did undergo some breakdown, but only in the presence of β-D-glucosidase. Again, the concept of hydration of the substrate and its availability to these enzymes seems to be an important factor. Furthermore, since some chain shortening by β-elimination occurs even during dialysis of ORC, the resulting oligomers may be substrates of a size more appropriate for hydrolysis by β-D-glycosidases. Since both β-D-glucosidase and β-D-glucosiduronase possess *exo* enzymic activity, it might be expected that D-glucose and D-glucuronic acid would be generated in substantial proportions and be detected by p.a.d.–h.p.l.c. Although there is a slight general shift in oligomeric profile, only β-D-glucosidase appeared to cause significant hydrolysis producing D-glucose, cellobiose, and D-glucuronic acid.

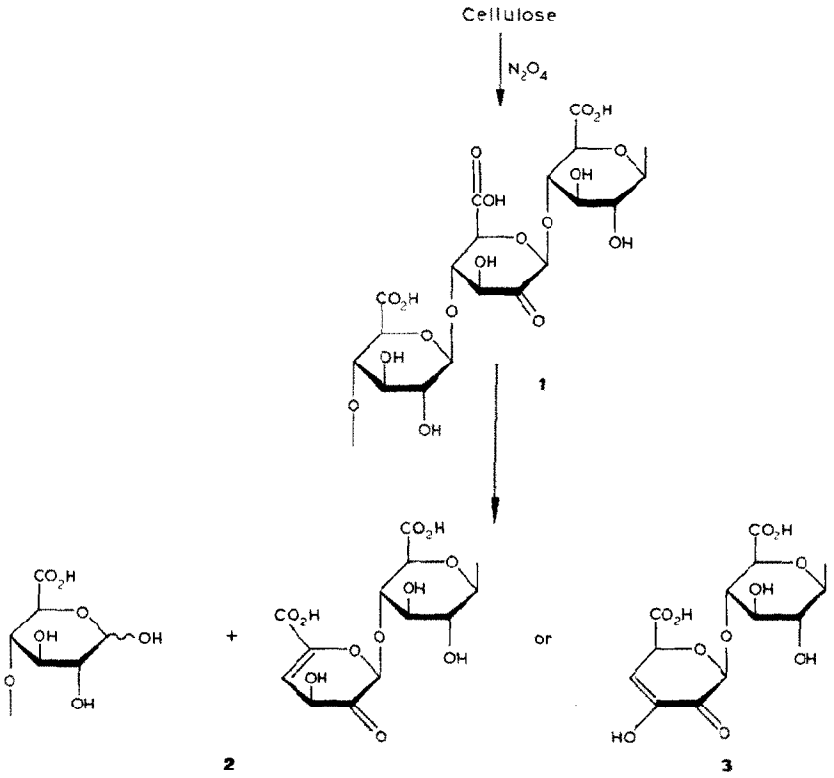
DISCUSSION

Modified celluloses are beginning to be utilized in the biomedical field for a variety of purposes, in part because of their potential ease of degradation to nontoxic materials. Although cellulose and its derivatives are indeed substrates for cellulases, these enzymes are primarily found in microorganisms and are not involved in biodegradation processes in humans. Thus, the degradation of modified celluloses must rely largely on the inherent lability of the polymer resulting from the modified structure and, perhaps, the catalytic activity of endogenous glycosidases.

Structural changes arising from exposure of cellulose to various oxidative conditions have been studied extensively, and it is generally accepted that NO_2 oxidation proceeds without D-glucosyl ring opening, and may be represented as shown in formulas **1–3**.

Biodegradation of periodate-oxidized cellulose, in which ring opening between C-2 and C-3 has occurred, has been studied and the instability of the product reported[16]. Since ORC may be knitted into a relatively stable fabric, some of the mechanical integrity of the polymer in the solid state is preserved. Introduction of carbonyl groups, which are better proton acceptors, may therefore lead to macro structural stabilization differing from that found in cellulose. In solution, the

combined influences of the carboxyl carbonyl group at C-6 and the ring-oxygen atom increases the lability of the intersaccharide linkage at C-4 (**1**), particularly under basic conditions favoring depolymerization. The endocyclic carbonyl at C-2 (C-3) further destabilizes the β-(1→4) linkage, thus facilitating glycosidic cleavage by β-elimination[17]. The presence of both the exocyclic and endocyclic carbonyl group in D-glucosyl residues which have undergone double oxidation may have a profound influence and further lower the pH requirements for β-elimination. This



is supported by the generation of oligomeric fragments *in vitro* under essentially neutral conditions. Both the 4,5-ene derivative (**2**) and the α,β-unsaturated ketone (**3**) would be expected to undergo further cleavage, even under the prevealing mild conditions. Thus, the terminal residues of oligomers generated by chain shortening–depolymerization would be cleaved, to give two- or three-carbon fragments. The p.a.d.–h.p.l.c. analysis revealed monomeric units of two and three carbon atoms, which could not be identified because of interfering solvent peaks, as well as the expected large quantities of glucuronic acid and glucose. Thus, ORC is readily solubilized and partially degraded *in vitro* when the simulated *in vivo* conditions are maintained (*i.e.*, neutral pH at 37°). The oligomer fragments which

accompany solubilization could be readily demonstrated by using p.a.d.-h.p.l.c. methods. When the ORC is placed in plasma or serum, the solubilization process results in more-rapid generation of smaller fragments in the monomer-to-tetramer size range. The only monomers identified were those expected from a poly-glucosiduronic structure containing a random distribution of glucose and doubly oxidized glucose residues.

EXPERIMENTAL

Materials. — ORC, designated INTERCEED® (TC7) by Johnson & Johnson Patient Care, and unoxidized cellulose (rayon), were obtained from Johnson & Johnson Patient Care. Alginic acid, cellobiose, microcrystalline cellulose, cellulase, β-D-glucosidase and β-glucosiduronase were from Sigma Chemical Co. All chromatographic equipment and supplies were from Dionex Corporation.

Solubilization. — Typically, ORC was suspended at a concentration of 10 $mg.mL^{-1}$ of fluid, and incubated at 37° until complete dissolution occurred. Incubations in PBS, plasma, and serum (freshly drawn from human volunteers) were carried out under sterile conditions. Solubilization by dialysis was conducted by suspending ORC (800 mg) in a dialysis bag (mol. wt. cut-off, 3500) containing 100 mL of PBS and dialyzing against 1500 mL of PBS at room temperature, with frequent changes of the dialysis solution. After complete dissolution, followed by a brief dialysis against distilled water, the solution within the dialysis bag was filtered, and the filtrate lyophilized, to yield the sodium salt of ORC.

Alkaline treatment. — Alginic acid, ORC, and rayon at a concentration of 10 $mg.mL^{-1}$ were suspended in 0.12M NaOH, and incubated for 24 h at 37 and 80° in vacuum-sealed tubes. The resulting suspensions were filtered through nylon G-6 membranes (0.22-μm pore size). The products were then examined by using p.a.d.–h.p.l.c. under monomeric and oligomeric separation modes, respectively.

Enzymic digestion. — A commercial cellulase (grade I, from *Aspergillus niger*, or Type VI from *Penicillium funiculosum*) was partially purified by passing 200 mL of solution (5 $mg.mL^{-1}$ of 50mM sodium acetate buffer, pH 5.0) through an Amicon pm 30 membrane (mol. wt. cut-off, 30,000) at 4° until the volume was decreased to 25 mL. A 4.0-mL aliquot of this solution was added to 1.0 g of substrate in 200 mL of 50mM sodium acetate buffer, pH 5.0, and incubated for 6 h at 37° with periodic shaking. The resulting suspension was filtered through Whatman AF/6 micro glass-fiber filters, and the filtrate lyophilized. The resulting powder was resuspended in distilled water (250 $mg.mL^{-1}$) and the material deproteinized by the addition of an equal volume of 0.3M $ZnSO_4$. The excess of $ZnSO_4$ was neutralized with a saturated solution of $Ba(OH)_2$, the resulting precipitate removed by centrifugation at 1500*g*, and the supernatant liquor filtered through nylon G-6 membranes (0.22-μm pore size). The digest was then analyzed by p.a.d.–h.p.l.c. Similar procedures were used to "clean up" the glycosidases by dissolving them in their respective optimal-pH buffers. β-D-Glycosidases were then suspended in PBS,

pH 7.4, at 1 mg.mL^{-1} and the sodium salt of TC7 was added at a concentration of 10 mg.mL^{-1}; these were incubated for 24 h at 37°, and then deproteinized as already described.

Chromatographic analyses. — All separations were carried out on a Dionex AS6 pellicular anion-exchange column (4.6 × 250 mm) fitted with an AG6 guard column. Samples (50 μL) were eluted by one of two different elution methods, optimized for the separation either of oligomers or monomers of the saccharide units of the original materials. Oligomers were eluted with a linear gradient of 5mM NaOH–10mM sodium acetate to 10% 5mM NaOH–90%M sodium acetate in 35 min, followed by isocratic elution for an additional 25 min. Monomers were resolved with a linear gradient of 100% of 5mM NaOH to an eluant 150mM in NaOH and 500mM in sodium acetate in 30 min, and then eluted isocratically for an additional 15 min. Flow rates were 1.0 mL.min^{-1}, and all analyses were followed by a wash of 700mM NaOH at a flow rate of 0.5 mL.min^{-1} in order to improve the base-line stability. Detection was accomplished with a Dionex PAD 2 detector with a gold working electrode operated in the triphasic mode (E1, 0.5 V for 360 ms, E2, 0.80 V for 120 ms and E3, 60 V for 420 ms) at 300 nA full scale. Assignment of d.p. was based on chromatographic analysis of commercially available standards and well characterized (f.a.b.-m.s.) oligomers obtained by enzymic hydrolysis of alginic acid and poly(D-galacturonic acid). The latter were kindly provided by Dr. James F. Preston III, Department of Microbiology and Cell Science, the University of Florida, Gainesville, FL. Data collection, manipulations, and base-line corrections were carried out by using a Macintosh SE microcomputer and Dynamax Complete Data and Control Software.

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