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Cellular Location of Enzymes Involved in Chondroitin Sulfate Breakdown by *Bacteroides thetaiotaomicron*

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Bacteroides thetaiotaomicron, a gram-negative anaerobe found in human colons, could utilize chondroitin sulfate, a tissue mucopolysaccharide, as its sole source of carbohydrate. The enzymes responsible for the breakdown of chondroitin sulfate by *B. thetaiotaomicron* were similar to those produced by *Proteus vulgaris* and *Flavobacterium heparinum* and included a lyase (EC 4.2.2.4), which degraded chondroitin sulfate into sulfated disaccharides, sulfatases (EC 3.1.6.4), which removed the sulfate residues, and a glucuronidase, which broke the unsulfated disaccharides into monosaccharide components. Chondroitin sulfate lyase, the first enzyme in the breakdown sequence, was not extracellular. It appeared to be located in the periplasmic space since lyase activity was released by treatment with ethylenediaminetetraacetate and lysozyme. Moreover, sodium polyanethole sulfonate, a high-molecular-weight inhibitor of chondroitin lyase, did not inhibit breakdown of chondroitin sulfate by intact bacteria. The sulfatase and glucuronidase appeared to be intracellular. None of these enzymes was strongly bound to membranes, and none of the steps in the breakdown of chondroitin sulfate was sensitive to oxygen.

Bacteroides is one of the major genera in the human colonic microflora, accounting for nearly 20% of all isolates (11). Members of this genus are gram negative and obligately anaerobic and require a fermentable carbohydrate for growth (2, 7, 22). In colons, most of the available carbohydrate is probably in the form of polysaccharides, and many strains of *Bacteroides* are able to utilize polysaccharides as sources of carbon and energy (20). Preliminary experiments with colonic *Bacteroides* strains have indicated that in at least some cases, the enzymes responsible for polysaccharide breakdown are not extracellular (16, 18, 19). However, no systematic investigation of the location of these enzymes has been made. In this paper we report the results of a study of the location of enzymes responsible for the breakdown by *Bacteroides thetaiotaomicron* of chondroitin sulfate, a mucopolysaccharide which is ubiquitous in tissue.

Chondroitin sulfate was chosen for this study because it is a linear, soluble, relatively well-characterized polysaccharide (8). In addition, mucopolysaccharides such as chondroitin sulfate may be an important source of carbohydrate for colon *Bacteroides* since the sloughing of epithelial cells into the intestinal tract provides a constant supply of these substances. Chondroitin sulfate-degrading activity is easily detectable in bacteria obtained from human feces by differential centrifugation (16). Since the enzymes responsible for chondroitin sulfate breakdown are not constitutive but are produced when bacteria are exposed to chondroitin sulfate (16, 17), this indicates that chondroitin sulfate is available in the colon and is utilized by some colonic bacteria.

Chondroitin sulfate consists of a repeating dimer containing D-glucuronic acid linked to *N*-acetyl-D-galactosamine (GalNAc) by a β (1 $\rightarrow$ 3) linkage. Each hexosamine residue contains a sulfate ester, either in the C-4 (chondroitin sulfate A) or C-6 (chondroitin sulfate C) position. In tissue, molecules of chondroitin sulfates A and C and of other similar mucopolysaccharides are covalently linked to proteins tbrough a bridge containing galactose, xylose, and serine residues (9). The molecular weight of chondroitin sulfate varies with the source, but it is usually about 10,000 to 20,000 (8, 23). Like most polysaccharides, chondroitin sulfates are polydisperse with respect to molecular weight.

Enzymes involved in chondroitin sulfate breakdown have been studied previously in *Proteus vulgaris* and *Flavobacterium heparinum* (26). The steps in chondroitin sulfate breakdown by these organisms are shown in Fig. 1. Chondroitin sulfate is first broken into unsaturated, sulfated disaccharides (ΔDi-4S in the case of chondroitin sulfate A and ΔDi-6S in the case of chondroitin sulfate C) by a β-eliminative cleavage of the bond next to the uronic acid residue (chondroitin sulfate lyase [EC 4.2.2.4]). The disaccharides are then desulfated (chondroitin 4-

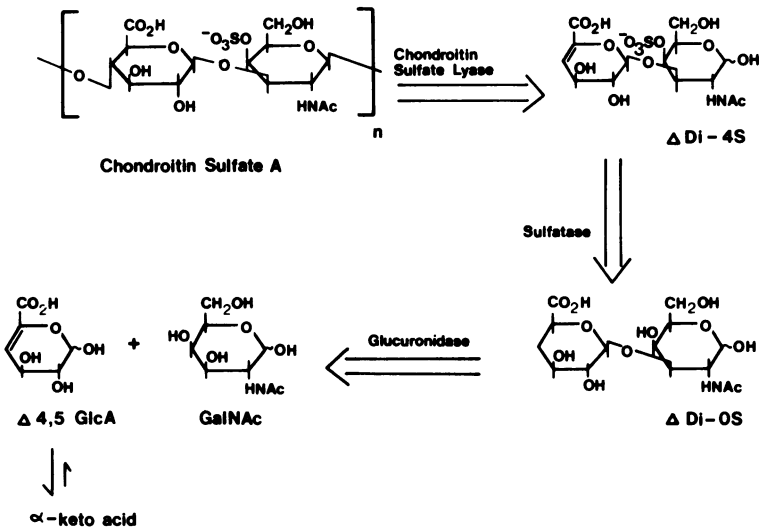

FIG. 1. *Enzymes involved in bacterial breakdown of chondroitin sulfate A (26). Breakdown of chondroitin sulfate C, which differs from chondroitin sulfate A by having a sulfate ester in the C-6 rather than the C-4 position of GalNAc, proceeds similarly, except that a 6-sulfated disaccharide (ΔDi-6S) is the product of lyase action.*

or 6-sulfate hydrolase [EC 3.1.6.4]) and finally hydrolyzed by a β-glucuronidase to produce GalNAc and Δ4,5 glucuronic acid. The latter compound is converted non-enzymatically into an α-keto acid. In the studies of the *P. vulgaris* and *F. heparinum* enzymes, no attempt was made to determine the location of the enzymes in the bacterial cells. However, the fact that the extract used for purification was taken from disrupted bacteria indicates that these enzymes were not extracellular.

MATERIALS AND METHODS

Organism and culture conditions. *B. thetaiotaomicron* VPI 5482A (NCTC 10852) was obtained from the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg. The growth medium was based on the defined medium of Varel and Bryant (22), except that 0.5% chondroitin sulfate replaced glucose as the carbon source and 0.05 M potassium phosphate buffer (pH 7.0) replaced the carbonate buffer. Chondroitin sulfate was added to the medium before autoclaving. This did not affect the chromatographic profile of chondroitin sulfate on Sephadex G-200, nor did it give rise to low-molecular-weight compounds detectable by paper chromatography (see below). The atmosphere was oxygen-free carbon dioxide. Growth of cultures was measured at 37°C by determining the absorbance at 650 nm (length of light path, 1 cm). Optical density (OD) was proportional to bacterial concentration. An OD at 650 nm (OD_{650}) of 1.0 corresponded to 3×10^9 colony-forming units per ml.

Location of enzymes. Extracellular fluid was obtained by centrifuging bacterial cultures at 21,000 × *g* for 20 min at 4°C. In some experiments, extracellular fluid was concentrated 20-fold by using a Minicon concentrator (Amicon Corp., Lexington, Mass.). In other experiments, extracellular fluid was collected anaerobically by centrifuging bacteria at 25 rather than 4°C in screw-capped Corex tubes which had been gassed out with oxygen-free carbon dioxide.

After removal of eetracellular fluid, bacteria were washed once and suspended in 0.05 M potassium phosphate buffer (pH 7.0). Bacteria were disrupted by 50% pulsed sonication for 2 min by using a Branson 200 sonifier equipped with a microtip (Branson Sonic Power Co., Danbury, Conn.). The power setting was 80 to 90 W. During sonication bacteria were kept in an ice water bath. After sonic disruption, undisrupted bacteria were removed by centrifugation at 15,000 × *g* for min at 4°C. The efficiency of sonication was estimated by comparing the concentration of protein in undisrupted bacteria with that in disrupted bacteria after centrifugation. Disrupted and undisrupted bacteria were each added to an equal volume of 1 N NaOH, and then the concentration of protein was determined by the method of Lowry et al. (10), using bovine serum albumin as a standard. The percent disruption was approximately 90%.

In some experiments, anaerobiosis was maintained during the disruption procedure by sonicating bacteria (which had been washed and suspended in prereduced basal medium containing no carbohydrate) under a stream of oxygen-free carbon dioxide. The criterion for anaerobiosis was that resazurin, the redox indicator in the medium, remained colorless after sonication.

Measurement of chondroitin sulfate breakdown. Breakdown of chondroitin sulfate by intact bacteria was measured by incubating bacteria which had been harvested at an OD_{650} of 0.8, washed once, and suspended in one-fifth volume of 0.05 M potassium

phosphate buffer (pH 7.0) with chondroitin sulfate A at 37°C. The final concentration of chondroitin sulfate A in the incubation mixture was 10 mg/ml. The concentration of cell protein in the incubation mixture was 2.4 to 2.6 mg/ml. In some experiments, anaerobiosis was maintained by washing and suspending bacteria in prereduced medium rather than in phosphate buffer and by carrying out all operations under oxygen-free carbon dioxide. At 10-min intervals, 0.5-ml portions of the incubation mixture were removed from the incubation mixture into 4.5 ml of ice-cold distilled water. After centrifugation at 21,000 × *g* for 10 min at 4°C to remove bacteria, the concentration of chondroitin sulfate in the supernatant fluid was determined by a cetylpyridinium chloride (CPC) precipitation assay (15) or by the carbazole assay for uronic acids (4). The CPC assay detects high-molecular-weight chondroitin sulfate A, but not oligomers of low molecular weight. The carbazole assay detects all fragments containing glucuronic acid, regardless of size. Breakdown by disrupted bacteria was determined similarly. In experiments involving sodium polyanethole sulfonate (SPS), the standard curve used in the CPC assay was generated with a combination of chondroitin sulfate A and SPS because SPS interferes with the CPC assay. SPS does not interfere with the carbazole assay. In all experiments, the initial concentration of chondroitin sulfate A as determined by the CPC assay agreed to within 10% with the concentration determined by the carbazole assay.

Chondroitin sulfate lyase activity was measured in disrupted bacteria or in extracellular fluid by measuring the increase in absorbance at 235 nm to the production of unsaturated disaccharides when chondroitin sulfate A (2 mg/ml in 0.05 M potassium phosphate buffer, pH 7.0) was incubated with enzyme at 37°C. Measurements were taken at 20-s intervals with a Gilford 250 recording spectrophotometer. Enzyme activity was linear throughout the assay period. One unit of enzyme activity was defined as an increase of 1.0 U of absorbance at 235 nm per min. An absorbance increase of 1.0 corresponded to 0.20 μmol of ΔDi-4S or 0.24 μmol of ΔDi-6S. Enzyme from disrupted bacteria degraded chondroitin sulfates A, B, and C, so the *Bacteroides* enzyme is probably a chondroitin ABC lyase. Breakdown of chondroitin sulfate A by intact bacteria could not be measured by this assay since the unsaturated disaccharides were taken into the cell and not released into the medium. All values for enzyme activities or for breakdown by intact bacteria were obtained by averaging the results of at least three separate experiments.

Sulfatase and glucuronidase activities were determined qualitatively by paper chromatography (see below) of incubation mixtures containing chondroitin sulfate A (2 mg/ml in 0.05 M potassium phosphate buffer), sulfatase-free chondroitin sulfate lyase (3 to 5 U), and enzyme. A partially purified sulfatase-free chondroitin lyase was obtained from *B. thetaiotaomicron* by a purification procedure based on the method of Yamagata et al. (26). The specific activity of this enzyme preparation was 130 μmol/min per mg of protein.

Chromatography of chondroitin sulfate and breakdown products. ΔDi-4S, ΔDi-6S, ΔDi-0S, and GalNAc were resolved by descending paper chromatography on Whatman no. 1 or 3 MM filter paper. Solvent system A consisted of glacial acetic acid, *n*-butanol, and 1 N NH_4OH (3:2:1, vol/vol) (26). Unsaturated disaccharides were visualized as absorbing spots under UV light. Unsaturated disaccharides, saturated disaccharides, and GalNAc could be visualized by spraying with *p*-anisidine in phthalic acid (21) and heating at 0°C for 10 min. Compounds were identified tentatively by comparing their migration distances, UV absorptions, and colors after anisidine-phthalate staining with those of authentic standards. For quantitation, known amounts of sample were streaked onto strips of Whatman 3 MM filter paper (1 by 20 inches [2.54 by 50.8 cm]) and chromatographed for 18 h in solvent system A. After the chromatograms were dried, segments corresponding to regions which reacted with *p*-anisidine on a parallel strip were cut from the chromatogram. These segments were eluted with 1 to 2 ml of distilled water. Absorbance at 235 nm, uronic acid concentration (4), and GalNAc concentration (14) were determined. Since solvent system A did not move oligomers larger than tetrasaccharides far enough from the origin for resolution even after 36 h, a second solvent system was used to resolve higher oligomers. This solvent system (solvent system B) contained glacial acetic acid, *n*-butanol, and 1 M NH_4OH (6:4:4, vol/vol), and chromatograms were developed for at least 36 h. Under these conditions, oligomers as large as octasaccharides could be resolved.

To detect changes in the molecular weight of chondroitin sulfate due to enzyme action, chromatography on Sephadex G-200 (23) with 1 M NaCl as the eluant was used. The column dimensions were 2.5 by 80 cm, the flow rate was 20 ml/h, and fractions of 5 ml were collected. The void volume was 180 ml, and the total included volume was 525 ml. Since the Sephadex G-200 did not resolve low-molecular-weight breakdown products very well, chromatography on Sephadex G-50 (fine) with 1 M NaCl as the eluant was used to resolve oligomers 2 to 16 monosaccharides long (DP2 through DP16). The column dimensions were 2.5 by 95 cm, the flow rate was 20 ml/h, and fractions of 4 ml were collected. Chondroitin sulfate A eluted in the void volume (180 ml). The total included volume was 460 ml.

Release of periplasmic enzymes. Periplasmic enzymes were released by treating bacteria with lysozyme and EDTA. The method of Witholt et al. (25) was used, except that 0.2 M glycylglycine-hydrochloride buffer (pH 8.0) replaced the Tris-hydrochloride buffer. The OD_{650} of the culture was 0.9 to 1.0; i.e., bacteria were in late logarithmic phase. Alkaline phosphatase, an enzyme which is periplasmic in *Escherichia coli*, was assumed to be a marker enzyme for the periplasmic space. Phosphoglucose isomerase was used as a cytoplasmic marker. Alkaline pbosphatase activity was determined by using *p*-nitrophenyl phosphate as the substrate (6). Phosphoglucose isomerase activity was determined by using standard assay procedures (12). After treatment with lysozyme and EDTA and using sucrose as an osmotic stabilizer, bacteria were harvested by centrifugation, and the supernatant fluid was removed. Bacteria were then

resuspended in the same EDTA-lysozyme-sucrose mixture and disrupted by sonication.

Cell debris was removed by centrifugation. The chondroitin lyase, alkaline phosphatase, and phosphoglucose isomerase activities in the supernatant and in the disrupted cell extract were measured after the addition of $MgCl_2$. Since sucrose and EDTA interfered with the determination of sulfatase and glucuronidase activities, both the supernatant fluid and the disrupted cell extract were dialyzed overnight against two changes of 0.05 M potassium phosphate buffer (pH 7.0) before the assays for these enzymes. Dialyzed enzyme preparations were incubated with chondroitin sulfate A (2 mg/ml in 0.05 M potassium phosphate buffer, pH 7.0). Since most of the lyase had been removed from the treated cells, sulfatase-free chondroitin lyase (from *B. thetaiotaomicron*) was added to the incubation mixture containing the extract from disrupted bacteria. After 2 h at 37°C, 0.1-ml portions of the incubation mixture were streaked onto strips of Whatman 3 MM filter paper. The chromatography conditions and the procedures for elution and quantitation of products were as described above. The variation in these determinations was 5 to %, and the limit of detection was 0.01 μmol.

To determine whether any of the chondroitin sulfate-degrading enzymes were associated with membranes, a crude membrane fraction was obtained and assayed for lyase and sulfatase activities. Bacteria (OD_{650}, 0.8) were harvested, washed once, and suspended in various buffers. Potassium phosphate (0.05 M, pH 7.0), glycylglycine hydrochloride (0.05 M, pH 8.0), and Tris-hydrochloride (0.05 M, pH 8.0) were tried in separate experiments. Suspended bacteria were disrupted with a French pressure cell (2,000 lb/in^2) and centrifuged at 21,000 × *g* for 20 min at 4°C to remove cell debris. The supernatant fluid was centrifuged at 100,000 × *g* for 1 h to pellet the membranes. Both the 100,000-×-*g* supernatant fluid and the suspended membranes were tested for chondroitin lyase and sulfatase activities. Phosphoglucose isomerase, a cytoplasmic enzyme, was used as an indicator of the amount of soluble enzyme activity which was trapped in the membrane pellet rather than bound to the membrane.

Chemicals. Chondroitin sulfate A (Sigma Chemical Co.) was used for the enzyme assays and breakdown experiments. Exhaustive digestion of this preparation with sulfatase-free chondroitin sulfate lyase from *B. thetaiotaomicron*, followed by paper chromatographic separation of the products, revealed that the ratio of ΔDi-4S to ΔDi-6S was 3:1. Since ΔDi-6S is characteristic of chondroitin sulfate C, another isomer of chondroitin sulfate, this indicates that the chondroitin sulfate A used in these experiments contained as much as 25% chondroitin sulfate C. The presence of chondroitin sulfate C did not affect the experiments described here because both isomers were degraded by *B. thetaiotaomicron*. Moreover, since recent reports indicate that some polymers of chondroitin sulfate may contain both 4-sulfated and 6-sulfated disaccharides (5), it may not be possible to obtain a pure preparation of chondroitin sulfate A. In the growth medium, a cruder preparation of chondroitin sulfate, chondroitin sulfate type II (Sigma Chemical Co.), was used. This grade of chondroitin sulfate contains isomers A and C, traces of other mucopolysaccharides, and protein (ca. 10%). Authentic standards of ΔDi-4S, ΔDi-6S, and the desulfated disaccharide (ΔDi-0S) were obtained from Miles Laboratories, Elkhart, Ind.

RESULTS

Absence of extracellular enzyme. Growth of *B. thetaiotaomicron* in medium containing chondroitin sulfate as the sole carbohydrate source was accompanied by rapid disappearance of chondroitin sulfate from the medium (Table 1). Accordingly, if an extracellular chondroitin sulfate-degrading enzyme were being produced, it should have been detectable at some point during the logarithmic phase. No chondroitin sulfate lyase activity was detected in the extracellular fluid at any time during the logarithmic phase, even when this fluid was concentrated 20-fold. However, chondroitin sulfate lyase activity was easily detectable in disrupted bacteria at all stages of growth (Table 1). Chondroitin sulfate lyase from disrupted bacteria was not sensitive to oxygen. It was not destroyed by the method used to concentrate the extracellular fluid, nor was it inhibited by spent media. Moreover, levels of lyase activity sufficient to account for the disappearance of chondroitin sulfate from the medium were well within the limits of detection. These results indicate that chondroitin sulfate lyase is not an extracellular enzyme but rather is associated with the bacterial cell.

Although the data in Table 1 demonstrate that chondroitin sulfate lyase is not extracellular, they do not rule out the possibility that some other extracellular enzyme exists, which is not detected by the assay of absorbance at 235 nm or which, unlike the lyase, is sensitive to oxygen. To determine whether such an enzyme was being produced, we collected extracellular fluid from bacteria harvested in early exponential phase (OD_{650}, 0.4) or in late exponential phase (OD_{650}, 0.9). This fluid was incubated with chondroitin sulfate A (final concentration, 10 mg/ml) for 2 h at 37°C under a carbon dioxide atmosphere. At the beginning of the incubation and after 2 h, portions of the mixture were heated at 90°C for 10 min to stop any enzyme action and then placed on a Sephadex G-200 column. Any breakdown of chondroitin sulfate should have been detectable as a shift in the chondroitin sulfate peak or as an increase in low-molecular-weight material. Figure 2 shows the results of the incubation when extracellular fluid from cells in late exponential phase was used. No change in molecular weight distribution was observed after 2 h of incubation. The low-molecular-weight peak (eluant volume, 400 to 500 ml)

TABLE 1. *Location and level of chondroitin sulfate lyase during growth of B. thetaiotaomicron 5482A on chondroitin sulfate*[a]

Incubation time (h)	OD_{650}	Concn of chondroitin sulfate remaining in medium (mg/ml)	Chondroitin sulfate lyase activity		
			Extracellular (U/ml of culture fluid)[b]	Cell associated	
				U/ml of culture fluid[b]	U/mg of cell protein[b]
0	0.1	5.0			
2.5	0.2	4.0	<0.005	0.04	1.1
4.2	0.4	3.3	<0.005	0.16	2.2
5.8	0.7	2.0	<0.005	0.45	2.6
6.5	0.9	0.3	<0.005	0.75	2.6
8.0	1.1	0.1	<0.005	0.94	2.7

[a] A 2-ml sample of bacteria which were grown overnight in medium containing chondroitin sulfate was inoculated into 100 ml of chondroitin sulfate medium. At intervals, bacteria were collected and harvested by centrifugation. The chondroitin sulfate concentration in the extracellular fluid was determined by the CPC assay (15). The chondroitin sulfate lyase activity in concentrated extracellular fluid and in sonically disrupted bacteria was determined as described in the text. Enzyme activities in disrupted bacteria were corrected for efficiency of sonication.

[b] One unit of activity was equivalent to an absorbance change at 235 nm of 1 U/min at 37°C. A change of 1 absorbance unit at 235 nm was equivalent to 0.20 μmol of ΔDi-4S or 0.25 μmol of ΔDi-6S. Both ΔDi-4S and ΔDi-6S were released during lyase digestion since the chondroitin sulfate A used in these experiments contained about 25% chondroitin sulfate C. The limit of detection for lyase activity was 0.005 U/ml.

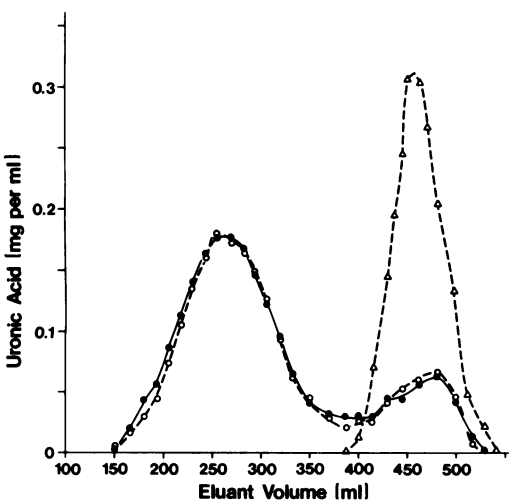

FIG. 2. *Incubation of chondroitin sulfate A (10 mg/ml) with extracellular fluid collected anaerobically from cells in late exponential phase (OD_{650}, 0.9) growing on chondroitin sulfate. The molecular weight distribution on Sephadex G-200 of chondroitin sulfate after a 2-h incubation (●) was identical to the distribution before incubation (○). The width of the chondroitin sulfate peak (eluant volume, 180 to 350 ml) was due to the range of molecular weights and not to the amount of sample loaded onto the column. The low-molecular-weight peak (at an eluant volume of 400 to 500 ml) was present in the extracellular fluid before the addition of chondroitin sulfate A. It was not present in uninoculated medium. Under identical conditions, an extract from sonically disrupted bacteria completely degraded the chondroitin sulfate after 1 h (△).*

which appeared in both the initial (zero time) and final (2-h) incubations was in the medium before the addition of chondroitin sulfate and was probably due to undigested fragments of chondroitin sulfate. It was not present in uninoculated medium. By contrast, disrupted bacteria incubated under identical conditions completely degraded chondroitin sulfate A to low-molecular-weight compounds within 1 h (Fig. 2). Similar results (data not shown) were obtained with the bacteria harvested in early exponential phase (OD_{650}, 0.4). These results indicate that no extracellular chondroitin sulfate-degrading enzyme is produced by *B. thetaiotaomicron*.

Breakdown by intact bacteria. Bacteria which had been washed and suspended in phosphate buffer were able to break down chondroitin sulfate (Fig. 3). No chondroitin sulfate lyase activity was detectable in the extracellular fluid at any time during this incubation, so breakdown was not due to release of this enzyme into the medium. There was a corresponding decrease in chondroitin sulfate concentration, whether it was measured by the CPC assay (for intact chondroitin sulfate) or by the carbazole assay (for total uronic acid). This indicates that products of chondroitin sulfate breakdown were taken into the cells as chondroitin sulfate was degraded. When longer incubation times were used, after the concentration of intact chondroitin had dropped to zero, there was still uronic acid in the medium. This may have been due to accumulation in the medium of some low-molecular-weight fragments of chondroitin sulfate

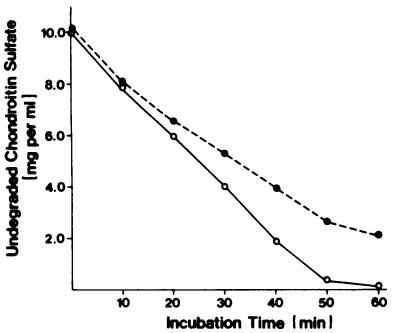

FIG. 3. *Breakdown of chondroitin sulfate by intact bacteria. Bacteria grown on chondroitin sulfate to an OD_{650} of 0.9 were harvested, washed, and suspended in 0.05 M potassium phosphate buffer (pH 7). Chondroitin sulfate was added to a final concentration of 10 mg/ml. The final concentration of bacteria was five times that in the original culture (2.4 to 2.6 mg of cell protein per ml of reaction mixture). At intervals, portions of the incubation mixture were removed and diluted, and the bacteria were removed by centrifugation. The concentration of high-molecular-weight chondroitin sulfate in the supernatant fluid (○) was determined by the CPC assay (15). The total concentration of chondroitin sulfate plus low-molecular-weight oligomers (●) was determined by the carbazole assay (4).*

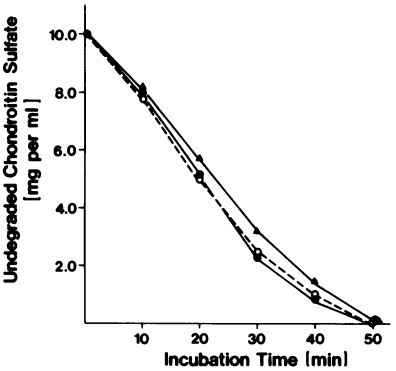

FIG. 4. *Effect of oxygen on breakdown of chondroitin sulfate by intact bacteria. Conditions were described in the legend to Fig. 3, except that all procedures were done anaerobically and bacteria were washed and suspended in prereduced medium (no carbohydrate). The incubation mixture was bubbled gently with carbon dioxide (●) or air (○) for 5 min; then chondroitin sulfate was added, and bubbling was continued throughout the incubation (37°C). The chondroitin sulfate concentrations in the extracellular fluid were measured by the CPC assay (15). The breakdown of chondroitin sulfate by bacteria which had been washed and suspended in 0.05 M phosphate buffer (pH 7.0) is shown for comparison (△).*

(either undegraded portions of the original molecule or products of enzymic breakdown which had leaked back into the medium).

The process of chondroitin sulfate breakdown by intact bacteria was not sensitive to oxygen. Breakdown by bacteria which were exposed to bubbling air was as rapid as breakdown by bacteria which were incubated anaerobically (Fig. 4). Bacteria which were suspended in phosphate buffer broke down chondroitin sulfate somewhat more slowly than bacteria which were suspended in basal medium, but this difference was probably not significant given the experimental error of 5 to 10%.

Breakdown of chondroitin sulfate by disrupted bacteria was much more rapid than breakdown by intact bacteria (Fig. 5), indicating that there is a barrier between the degradative enzymes and the extracellular fluid. Further evidence that the initial enzyme responsible for the breakdown of chondroitin sulfate is not exposed to the medium comes from the finding that SPS did not inhibit breakdown of chondroitin sulfate by intact bacteria, even though it inhibited breakdown by disrupted bacteria (Fig. 5). SPS is a high-molecular-weight inhibitor of chondroitin sulfate lyase (1). Because of its size and negative charge, SPS should not be able to diffuse into the periplasmic space, but should inhibit a chondroitin sulfate-degrading enzyme only if its active site is exposed on the outside of the outer membrane.

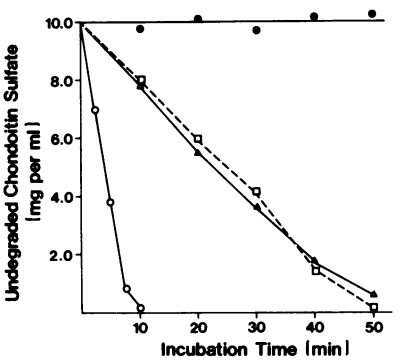

FIG. 5. *Effect of SPS, a chondroitin sulfate lyase inhibitor, on breakdown of chondroitin sulfate A by intact bacteria. Bacteria were prepared as described in the legend to Fig. 3 and then incubated either with (□) or without (△) added SPS (final concentration, 1 mg/ml). To confirm that this concentration of SPS inhibited lyase activity, a portion of the suspended cells was disrupted and then incubated with (●) or without (○) added SPS. The final concentration of cell protein in the disrupted cell incubation mixtures was the same as that in the intact cell incubation mixtures (2.4 mg/ml). The efficiency of sonication was 90%.*

Nature of the degradative enzymes. Incubation of extracts from disrupted bacteria

with chondroitin sulfate A resulted in products which had the same migration rate on paper chromatograms, the same absorbance at 235 nm, and the same uronic acid content as authentic standards of ΔDi-6S and ΔDi-4S. ΔDi-0S, the product of sulfatase action on sulfated disaccharides, and GalNAc, the product of glucuronidase action on ΔDi-0S, were also detected. This indicates that *B. thetaiotaomicron*, like *P. vulgaris* and *F. heparinum*, produces a sulfatase and a glucuronidase in addition to chondroitin sulfate lyase. To determine whether *B. thetaiotaomicron* might produce additional enzymes which degrade chondroitin sulfate into pieces larger than disaccharides, disrupted bacteria were incubated with chondroitin sulfate A. All procedures were carried out anaerobically in case there might be an oxygen-sensitive enzyme. At intervals, portions of the incubation mixture were removed and analyzed on a Sephadex G-50 column. This column could resolve oligosaccharides up to 14 to 16 disaccharides long (Fig. 6). An enzyme which degraded chondroitin sulfate A into pieces larger than di- or tetrasaccharides should have produced an oligosaccharide peak early in the incubation. However, larger oligosaccharides were observed only after longer incubation times. This is consistent with action by an enzyme which degrades chondroitin sulfate by removing disaccharides sequentially. Such an enzyme eventually produces a range of oligomers. The absence of oligosaccharides at early incubation times was confirmed by paper chromatography of the samples shown in Fig. 6 under conditions which permitted resolution of oligomers as large as octasaccharides. Thus, it appears that the only enzymes involved in chondroitin sulfate breakdown by *B. thetaiotaomicron* are a chondroitin sulfate lyase, a sulfatase, and a glucuronidase.

Release of periplasmic enzymes. To determine whether any of these enzyme activities were periplasmic, bacteria were treated with EDTA and lysozyme, using 20% sucrose as the osmotic stabilizer. The method which worked best was the method of Witholt et al. (25), except that 0.2 M glycylglycine hydrochloride replaced Tris-hydrochloride as the buffer and bacteria in late exponential phase (OD_{650}, 0.9 to 1.0) were used. This treatment released 65 to 70% of the chondroitin sulfate lyase and 55 to 65% of the alkaline phosphatase but less than 14% of the intracellular enzyme phosphoglucose isomerase. Methods which used Tris-hydrochloride as the buffer and required bacteria in early or midexponential phase (13, 24) were unsatisfactory because of the high percentage (40 to 60%) of phosphoglucose isomerase released into the medium. This seems to have been due mainly to the Tris buffer rather than to the EDTA or the other components of the spheroplasting mixture since suspending cells in Tris alone resulted in the loss of 40 to 60% of the intracellular enzyme. Low concentrations of Tris (0.01 M) released more intracellular enzyme than high concentrations (0.1 M), presumably because the higher concentrations provided some osmotic stabilization. Microscopically, the bacteria appeared to be normal, so the effect of Tris may have been

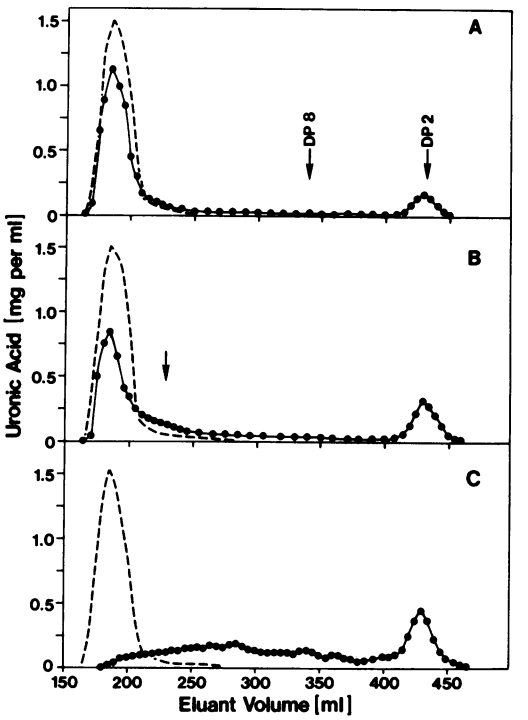

FIG. 6. *Breakdown of chondroitin sulfate by disrupted bacteria. Bacteria grown to an OD_{650} of 0.8 on chondroitin sulfate were harvested, washed, and suspended anaerobically in an equal volume of prereduced medium containing no carbohydrate. After disruption by sonication, chondroitin sulfate was added to a final concentration of 5 mg/ml, and the mixture was incubated at 37°C. Anaerobiosis was maintained throughout. At intervals, 20-ml portions of the mixture were removed, concentrated fourfold by flash evaporation, and chromatographed on Sephadex G-50. The arrows in (A) indicate the elution volumes of authentic samples of disaccharide (DP2) and octasaccharide (DP8). The void volume was 180 ml, and the fully included volume was 456 ml. The dashed lines indicate the profile of chondroitin sulfate before digestion. After 5 min (A), disaccharides (DP2) but no larger oligomers were detected. After 15 min (B), small amounts of oligomers larger than DP2 were detected (arrow). Only after 30 min, when depolymerization of high-molecular-weight chondroitin sulfate was complete, were other oligomers between DP2 and DP16 detected (C).*

to impair the integrity of the cell membranes enough to make them leaky but not enough to disrupt the bacteria.

Under conditions which released chondroitin sulfate lyase from the cells, the sulfatase and glucuronidase activities remained with the cell. Incubation of the periplasmic fraction with chondroitin sulfate A produced ΔDi-4S and ΔDi-6S, the products of lyase activity, but no ΔDi-0S or GalNAc (Table 2), whereas incubation of the disrupted cell extract with chondroitin sulfate A produced all of these compounds.

Association with membranes. When bacteria were disrupted by a French pressure cell and membranes were pelleted by high-speed centrifugation, approximately 10% of the chondroitin sulfate lyase co-precipitated with the membranes. Since 9% of the phosphoglucose isomerase, a soluble enzyme, also remained with the membranes and since washing with buffer (0.05 M glycylglycine hydrochloride, pH 8.0, or 0.05 M potassium phosphate, pH 7.0) removed all of the lyase activity, the lyase is probably not tightly bound to membranes. No sulfatase activity was detected in the suspended membranes. When bacteria were disrupted and washed in 0.02 M Tris buffer (pH 8.0), 4 to 5% of the lyase activity stayed with the membranes even after four washes, a treatment which removed all of the phosphoglucose isomerase with the first wash. This lyase activity could be removed by washing with 1 M NaCl.

DISCUSSION

The breakdown of chondroitin sulfate by *B. thetaiotaomicron* involves the following three types of enzyme activity: a lyase, which breaks chondroitin sulfate into unsaturated, sulfated disaccharides; a sulfatase, which removes the sulfates from the sulfated disaccharides; and a glucuronidase, which cleaves the unsulfated disaccharides into their monosaccharide components. There is no evidence of any other type of enzyme activity, such as an enzyme which degrades chondroitin sulfate into oligomers larger than disaccharides. The enzymes produced by *B. thetaiotaomicron* appear to be similar in their actions to the enzymes produced by *P. vulgaris* and *F. heparinum* (26). Further work is needed to determine whether the *B. thetaiotaomicron* enzymes are identical to those produced by *P. vulgaris* and *F. heparinum* and whether there is more than one lyase (i.e., a chondroitin sulfate A lyase and a chondroitin sulfate C lyase) or more than one sulfatase (i.e., chondroitin 4-sulfatase and chondroitin 6-sulfatase).

TABLE 2. *Location of sulfatase and glucuronidase activities*

Enzyme source	Amt (μmol) of the following products of enzyme activity:			
	ΔDi-6S[a]	ΔDi-4S[b]	ΔDi-0S[c]	GalNAc[d]
EDTA-lysozme wash	0.07	0.30	<0.01[e]	<0.01
Bacteria disrupted after treatment with EDTA and lysozyme	0.03	0.15	0.02	0.18

[a] Product of chondroitin sulfate lyase action on chondroitin sulfate C.
[b] Product of chondroitin sulfate lyase action on chondroitin sulfate A.
[c] Product of chondroitin 4-sulfatase action on ΔDi-4S or of chondroitin 6-sulfatase on ΔDi-6S.
[d] Product of glucuronidase action on ΔDi-0S.
[e] The limit of detection was 0.01 μmol.

Despite the high molecular weight of chondroitin sulfate, none of the enzymes responsible for its breakdown by *B. thetaiotaomicron* was extracellular. The possibility that chondroitin sulfate lyase, the first enzyme in the breakdown sequence, is located on the outside of the outer membrane seems to be ruled out by the finding that SPS, a high-molecular-weight inhibitor of the lyase, did not inhibit breakdown of chondroitin sulfate by intact bacteria. Of course, the failure of SPS to inhibit activity in intact bacteria could have been due to a distortion of the active site which resulted from an embedding of the enzyme in the outer membrane. However, the lyase did not sediment with cell membranes during high-speed centrifugation and thus does not appear to be an integral membrane protein.

A periplasmic location for the lyase is supported by two lines of evidence. First, the breakdown of chondroitin sulfate by intact bacteria was severalfold slower than the breakdown by disrupted bacteria, indicating that there is a barrier between enzyme and substrate in intact bacteria. Second, treatment of bacteria with lysozyme and EDTA released more than 60% of the lyase activity but less than 14% of the intracellular marker phosphoglucose isomerase. Since the lyase was not strongly associated with cell membranes, this indicates that the lyase is free in the periplasmic space or loosely associated with the cytoplasmic membrane or the cell wall-outer membrane complex. Because of its size and negative charge at physiological pH, it is not likely that chondroitin sulfate can enter the periplasmic space by diffusion through pores. Accordingly, there is probably an outer membrane receptor which binds chondroitin sulfate and brings it into contact with the lyase. Indirect evidence for such a receptor comes from the observation that treatment of *B. thetaio-*

taomicron with iodoacetate abolishes the ability of intact bacteria to degrade chondroitin sulfate but does not inactivate the chondroitin sulfate lyase (Salyers, unpublished data). Further investigation of this possibility is currently under way in our laboratory.

The sulfatase, which removes sulfate from the disaccharides produced by the lyase, was not released by the same treatment which released chondroitin sulfate lyase and alkaline phosphatase. Since sulfatase activity was not associated with the bacterial membranes, this indicates that the sulfatase is an intracellular enzyme and that the sulfated disaccharides must be transported into the cell before the sulfates are removed and the disaccharides are cleaved into monosaccharides by a glucuronidase. None of the steps involved in utilization of chondroitin sulfate by *B. thetaiotaomicron* is sensitive to oxygen. Accordingly, the adverse effects of oxygen on these obligate anaerobes must occur at some other level of cellular metabolism.

Polysaccharide-degrading enzyme systems which are associated with bacterial cells rather than being extracellular may be widespread in nature, especially in highly competitive ecosystems, such as human colons. In such environments, production of extracellular enzymes is not feasible if the products of enzymic breakdown are readily utilized by other bacteria. Laminarinases produced by some colonic *Bacteroides* species appear to be cell associated rather than extracellular (18). Similarly, some hemicellulases produced by bacteria from the bovine rumen have been reported to be associated with bacterial cells (3).

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