Carbohydrate Research, 142 (1985) 277–284
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

INTERACTION OF DEOXYHALOSUCROSE DERIVATIVES WITH DEXTRANSUCRASE*

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(Received September 13th, 1984; accepted for publication, March 1st, 1985)

ABSTRACT

Members of a series of deoxyhalosucrose analogs substituted at one, two, or three primary carbon atoms with bromine or chlorine were prepared. Dextransucrase isolated from *Streptococcus sanguis* was separately treated with 6-bromo-6-deoxysucrose, 6,6′-dibromo-6,6′-dideoxysucrose, 6,1′,6′-tribromotrideoxysucrose, and 6,6′-dichlorodideoxysucrose, in order to determine if they were inactivators. Variation in time of exposure, and in the concentration of the sucrose analogs, did not yield significant irreversible inactivation. In supplementary studies, it was found that the compounds serve as weak, reversible inhibitors.

INTRODUCTION

Dextransucrase (EC 2.4.1.5) has been shown to play an important role in the development of dental caries[1–4]. The enzyme catalyzes the formation of dextran by transfer of the D-glucosyl groups of sucrose to a growing chain[5,6], and yields a chain that is primarily α-D-(1→6)-linked. A detailed understanding of how the enzyme functions is essential. The characteristics of the active site of the enzyme are poorly understood, although a number of investigators have attempted to identify catalytically important functional groups. Some studies have suggested that protonation of a functionality is important for activity[7–9]. Inoue and Smith[7] reported that partially oxidized dextran is a potent inhibitor of dextransucrase, and proposed that the inhibition results from the interaction of dialdehydes with reactive groups that are close to the dextran binding site. Photochemical oxidation, in the presence of Methylene Blue or Rose Bengal, has been shown to inactivate the enzyme[8], and this has been attributed to the modification of L-histidyl residues at the active site.

Several groups[10–13] have shown that α-D-glucosyl fluoride is an ideal analog of sucrose, as it also serves as a D-glucosyl donor, and has K_m and V_{max} values comparable to those of sucrose[14]. Such sucrose analogs as 6-azido-6-deoxysucrose have been reported to be inactivators[15]. Mono- and di-aminodeoxysucroses have

*Supported by U.S.P.H.S. Grant No. DE 03731.

0008-6215/85/$ 03.30

been reported to be inhibitors, and the suggestion has been made that inhibition by them is due to perturbation of basic groups at the active site. Another class of inhibitory sucrose analogs is that of the deoxyhalo derivatives. In a patent[16], it was indicated that 6,6′-dichloro-6,6′-dideoxysucrose and the 6,6′-dibromo analog could inactivate the enzyme. These halo derivatives appeared to have the requisite characteristics of a compound that might cause modifications at the active site. We therefore decided to examine some of these more carefully, prepared several halo derivatives of sucrose, and now report the results.

RESULTS

Halosucrose derivatives were synthesized as described in the Experimental section, using the procedure of Anisuzzaman and Whistler[17], which involves the reaction of sucrose with the appropriate carbon tetrahalide and triphenylphosphine in pyridine solution. Following purification, the products were characterized by ^{13}C- and ^{1}H-n.m.r. spectroscopy, or by mass spectral analysis of the peracetates. [It is important to point out that the spectra were free from signals caused by con-

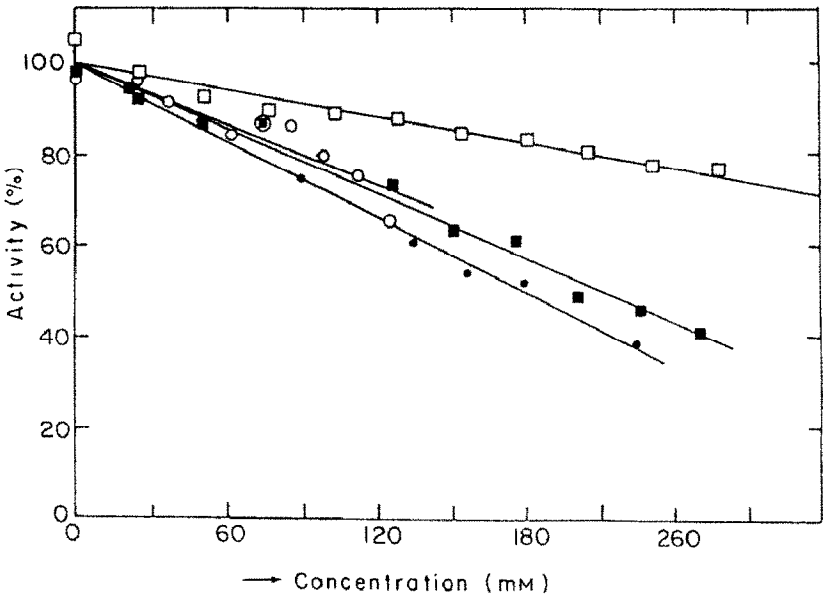

Fig. 1. Inactivation of dextransucrase as a function of concentration. Dextransucrase (0.05 unit) was incubated with the compound indicated, namely, 6-bromo-6-deoxysucrose (●——●), 6,6′-dibromo-6,6′-dideoxysucrose (■——■), 6,1′,6′-tribromo-6,1′,6′-trideoxysucrose (○——○), and 6,6′-dichloro-6,6′-dideoxysucrose (□——□) at the indicated concentration in the presence of 0.1M phosphate buffer, pH 6.0, in a final volume of 30 μL, for 60 min at 37°. It was then treated with [^{14}C]sucrose (final concentration, 50mM, 1.5×10^5 d.p.m.) in the presence of 25mM Dextran T-10 and 0.1M phosphate buffer, pH 6.0, for 5 min at 37°. Reaction was stopped by heating for 3 min at 100°; 10 μL from each was spotted on Whatman No. 1 MM paper, chromatographed, and counted as described under Methods.

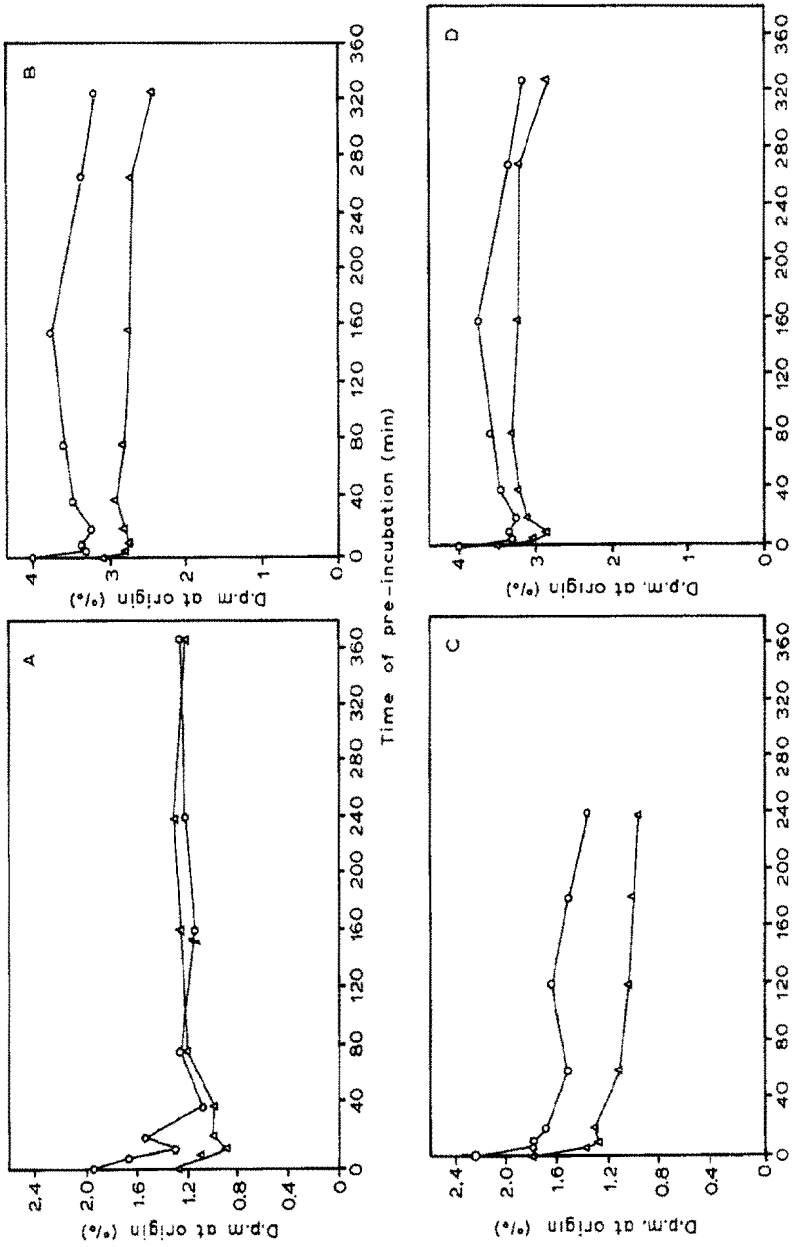

Fig. 2. Inactivation of dextransucrase as a function of time. Dextransucrase (0.5 unit) was incubated with: A, 6-bromo-6-deoxysucrose (170mM); B, 6,6'-dibromo-6,6'-dideoxysucrose (160mM); C, 6,1',6'-tribromo-6,1',6'-trideoxysucrose (90mM); and D, 6,6'-dichloro-6,6'-dideoxysucrose (190mM). Incubations were conducted at 37° in the presence of 0.1M phosphate buffer, pH 6.0, in a total volume of 0.2 mL. Aliquots (20 μL) were removed at the indicated times, and treated with [^{14}C]sucrose (final concentration, 67mM, 1.5×10^5 d.p.m.) in the presence of 33mM Dextran T-10 and 0.1M phosphate buffer, pH 6.0, for 5 min at 37°. Reactions were stopped by heating for 3 min at 100°; 10 μL from each was spotted on Whatman No. 1 MM paper, chromatographed, and counted as described under Methods. Reactions were conducted in the presence (△——△) and absence (○——○) of the halosucrose derivatives.

taminants, especially such by-products of oxidation of triphenylphosphine as (halomethyl)triphenylphosphonium halide. We observed that (chloromethyl)triphenylphosphonium chloride is a good inhibitor of dextransucrase (50% inhibition at 8mM)]. In this way, the following compounds were prepared: 6-bromo-6-deoxysucrose, 6,6′-dibromo-6,6′-dideoxysucrose, 6,1′,6′-tribromo-6,1′,6′-trideoxysucrose; and 6,6′-dichloro-6,6′-dideoxysucrose.

The ability of the sucrose analogs to inactivate dextransucrase was evaluated in two types of experiment. Under one protocol, the enzyme was pre-incubated

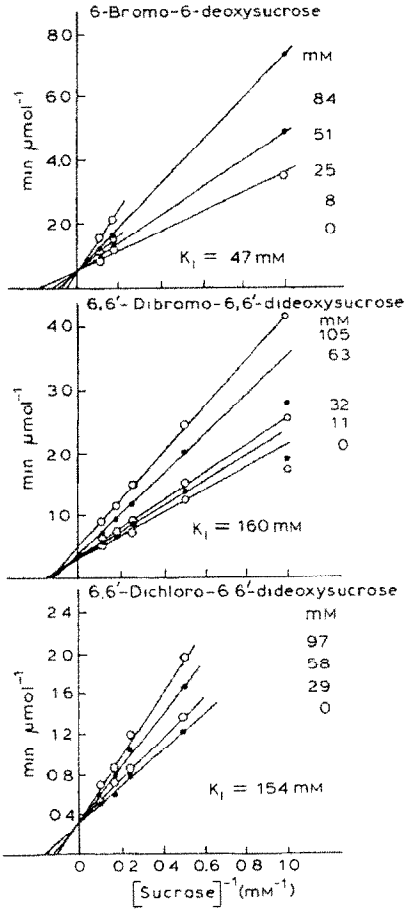

Fig. 3. Kinetics of dextransucrase inhibition. Dextransucrase (0.05 unit in the case of 6,6′-dibromo-6,6′-dideoxysucrose and 6,6′-dichloro-6,6′-dideoxysucrose, and 0.025 unit in the case of 6-bromo-6-deoxysucrose) was treated with the indicated concentrations of [^{14}C]sucrose (1.5×10^6 d.p.m. in each reaction) in the presence of the indicated concentrations of the inhibitors and 25mM Dextran T-10 in 0.1M phosphate buffer, pH 6.0, in a total volume of 0.04 mL, for 2 min at 37°. Reactions were stopped by heating for 3 min at 100°, and aliquots (10 μL) were spotted on Whatman No. 1 MM paper, chromatographed, and counted as described under Methods. The percentage of the applied counts that remained at the origin was determined, and plotted as the reciprocal of the rate against the reciprocal of the sucrose concentration.

with various concentrations of the compounds for 60 min, and then assayed for activity. (Control pre-incubations were performed under each set of conditions, wherein the enzyme was kept in buffer for the prescribed period.) The second involved pre-incubation for various times, at fixed concentrations of the reagents. The results of the studies using the first protocol are shown in Fig. 1. It appeared that all of the compounds inactivated the enzyme. Because the concentrations required for 50% inactivation ranged between 180mM for the 6-bromo derivative and 482mM for the 6,6′-dichloro compound, it did not appear that any of these were exceptionally effective inhibitors. This was substantiated in studies conducted under the second protocol (see Fig. 2), in which only small differences between the control and experimental reactions were seen in 6-h reactions. Furthermore, where differences were observed, it appears that the shapes of the curves are very similar, and the zero time points reflect the changes. Were these halo derivatives covalently modifying the enzyme in such a way as to cause inactivation, much greater differences would have been expected.

The apparent lack of active-site modification left unanswered the question as to the mode by which these compounds serve to inhibit dextransucrase. It was therefore decided to determine if they act as reversible inhibitors. A study of the kinetics of the reaction, as a function of sucrose concentration in the presence of fixed amounts of the analogs, was performed, but, because insufficient amounts of 6,1′,6′-tribromo-6,1′,6′-trideoxysucrose were available to conduct this type of analysis, only the three other compounds were examined. Double-reciprocal plots of the results, shown in Fig. 3, indicated that 6,6′-dichloro-6,6′-dideoxysucrose, and 6-bromo-6-deoxysucrose are competitive inhibitors, whereas the 6,6′-dibromo derivative displays a mixture of competitive and noncompetitive inhibition. Inhibitor constants for these compounds were determined, and are given in Fig. 3; it may be seen that none of the analogs is a very active inhibitor, as the K_i values vary between 47 and 160mM, compared with a K_m for sucrose of 5mM under the reaction conditions used[18].

DISCUSSION

The limited availability of sucrose analogs has deterred the development of our understanding of the substrate specificity of dextransucrase. Previous studies by Grier and Mayer[13] attempted to evaluate substrate specificity by exploiting the observation that α-D-glucosyl fluoride serves as an effective donor substrate, and a competitive inhibitor. Members of a series of α-D-glucosyl fluorides that were D-glucose epimers or derivatives were studied, and most of these also proved to be weakly competitive inhibitors. Of specific interest to the present study were α-D-glucosyl fluoride, 6-deoxy-α-D-glucosyl fluoride, and α-D-xylosyl fluoride, which have K_i values of 9.3, 5.3, and 4.2mM, respectively[13]. Thus, it appears that, when the 6-hydroxyl group is replaced by a less bulky group, the binding of the analog increases. Based on these observations, the increase in the K_i values for the 6-halo-substituted sucrose derivatives is not surprising.

TABLE I

COMPARISON OF THE INHIBITORY PROPERTIES OF DEOXYHALOSUCROSE DERIVATIVES

Compound	*Concentration (mM)*	*Inhibition (%)*	K_i *(mM)*
6-Bromo-6-deoxysucrose	240	62	47
6,6′-Dibromo-6,6′-dideoxysucrose	250	57	160
6,1′,6′-Tribromo-6,1′,6′-trideoxysucrose	240	50	—
6,6′-Dichloro-6,6′-dideoxysucrose	240	23	154

Because α-D-glucosyl fluoride acts as a donor substrae, with a K_m similar to that of sucrose, it may be speculated that the D-fructosyl moiety may not play a significant role in binding to the enzyme. However, our studies indicate that bulky substituents on C-6′ diminish binding even more (compare 6-bromo-6-deoxysucrose with 6,6′-dibromo- and 6,6′-dichloro-6,6′-dideoxysucrose). This may be an indication that although the enzyme does not bind the D-fructosyl group tightly, there is only a limited spatial tolerance around it.

In our studies, we were unable to demonstrate that any of the four halogenated derivatives of sucrose tested could irreversibly inactivate the enzyme. This was seen in experiments in which the enzyme was pre-incubated with the compounds. Significant inactivation could not be observed, either as a function of time, or as a function of the concentration of the analogs. The degree of inactivation at 240mM is shown in Table I. 6-Bromo-6-deoxysucrose produced 62% inactivation, and was the most effective of the four compounds tested. These results contrast to the report in a patent by Robyt and Zikopoulos[16], who indicated that 6,6′-dibromo-6,6′-dideoxysucrose and 6,6′-dichloro-6,6′-dideoxysucrose form "dead-end" complexes with the enzyme at much lower concentrations. For example, 150mM concentrations of 6,6′-dibromo-6,6′-dideoxysucrose or 6,6′-dichloro-6,6′-dideoxysucrose were reported to cause 100 and 80% inactivation, respectively, within 10 min. The evaluation of the effectiveness of their analogs involved pre-incubation of the enzyme with the test compounds, and was, therefore, similar to the method employed in our analysis. We are unable to explain the differences in the results; however, in our preliminary investigations, significant inactivation was observed, which was eventually shown to be due to the presence of small proportions of (halomethyl)triphenylphosphonium halide, a by-product of the synthetic reaction. Once appropriate care was exercised to insure its removal, inactivation was no longer seen.

The marginal inactivation observed in the pre-incubation studies may be a reflection of the fact that these compounds are weak, reversible inhibitors (see Table I), and were present during the measurement of enzyme activity. The reversible inhibition may also explain the fact that several chlorodeoxysucrose derivatives were found to decrease acid production and adherence of *S. mutans*[19].

EXPERIMENTAL

Materials. Highly purified dextransucrase (EC 2.4.1.5) from *Streptococcus sanguis* ATCC 10558 was obtained by the method of Grahame and Mayer[20]. Reagent-grade chemicals utilized in this study were obtained from the following sources: [U^{14}C]sucrose from New England Nuclear (Boston, MA), carbon tetrabromide and (chloromethyl)triphenylphosphonium chloride from Aldrich Chemical Co. (Milwaukee, WI), and Silica Gel 60 precoated t.l.c. plates, Silica Gel G 60, silicic acid, and triphenylphosphine from MCB (Gibbstown, NJ). All other reagents were of reagent grade, and commercially available from common chemical suppliers.

Methods. — Chromatographic procedures. Descending paper-chromatography and thin-layer and column chromatography were conducted in one of the following solvents: (*I*) 45:5:3 (v/v) ethyl acetate–ethanol–water, (*II*) 75:25:1 (v/v) acetonitrile–water–NH_4OH, and (*III*) 6:1:3 (v/v) 1-propanol–ethyl acetate–water. Thin-layer chromatograms were developed in solvent *I*, and made visible by spraying with 1:3 H_2SO_4–methanol and charring at 100°. Aromatic compounds on plastic t.l.c. sheets impregnated with a fluorescent indicator were detected by u.v. light. L.c. separations were performed in a Varian 5000 liquid chromatograph having a Partisil M9, 10/25 PAC column (Whatman) by using solvent *II*. Eluates were monitored by means of a refractive-index detector (Varian Series RI-3). Column chromatography was conducted on a column (3 × 85 cm) of silicic acid, using solvent *I* as the eluant. Aliquots of the collected fractions were serially spotted on t.l.c. plates, which were developed and visualized as described.

Spectroscopic analyses. N.m.r. spectra ^{1}H and ^{13}C were recorded with a Bruker WP-200 Fourier-transform spectrometer. Sugars were dissolved in deuterium oxide (99.9%) with Me_4Si as the external and acetone as the internal reference standard. Mass-spectral analyses were performed with a KRATOS MS 30 spectrometer, the data were analyzed, by the DS-55 Mass Spectrometry Data System, at the OSU Campus Chemical Instrumentation Center.

Radioisotopic analyses. Radioactive samples were counted in a Packard 460 C liquid scintillation spectrometer. Radioactive, paper-chromatogram strips (1 × 2.54 cm) were counted in 10 mL of scintillation fluid (4.0 g of PPO and 0.1 g of POPOP per L of toluene).

Synthetic procedures. In order to synthesize the primary halo derivatives of sucrose, a modification of the selective halogenation procedure of Anisuzzaman and Whistler[17] was used under the reaction conditions they described.

With the molar ratios of reagents kept constant, the time and temperature of the reactions were varied in an attempt to alter the product ratios. Reaction with CBr_4 for 5 h at 50° produced 6,6′-dibromo-6,6′-dideoxysucrose (R_F 0.29) as the major product, along with minor proportions of 6-bromo-6-deoxysucrose (R_F 0.09), 6′-bromo-6′-deoxysucrose (R_F 0.09), and 6,1′,6′-tribromo-6,1′,6′-trideoxysucrose (R_F 0.58), as well as some unidentified sugars. In 1 h at 40°, 6-bromo-6-deoxy- and

6′-bromo-6′-deoxy-sucrose were virtually the only products observed; however, >50% of the sucrose remained unreacted. When the reaction was conducted for 45 min at 85°, the proportion of 6,1′,6′-tribromo-6,1′,6′-trideoxysucrose increased substantially, although the 6,6′-dibromo derivative was still the preponderant product. The brominated sugars were purified by chromatography on sililic acid, as already described, but the separation did not resolve the 6-bromo and the 6′-bromo analogs, and left them contaminated with (bromomethyl)triphenylphosphonium bromide; They were separated by l.c., which did not completely resolve the 6-bromo and 6′-bromo deoxysucrose. In order to resolve them, the leading edge of the peak (6′-bromo) and the trailing edge of the peak (6-bromo) were each rechromatographed. In this way, 6-bromo-6-deoxysucrose was obtained in pure form (final yield, 10%); however, the 6′-bromo-6′-deoxysucrose remained contaminated. Following column chromatography, the 6,1′,6′-tribromo derivative was further purified by preparative, thin-layer chromatography, to give a final yield of ~5%.

Each of the isolated products was characterized by its ^{1}H- and ^{13}C-n.m.r. spectra, and by its mass spectrum.

REFERENCES

1 H. W. SCHERP, *Science*, 173 (1971) 1199–1205.
2 A. M. CHLUDZINSKY, G. R. GERMAINE, AND C. F. SCHACHTELE, *J. Bacteriol.*, 118 (1974) 1–7.
3 H. MUKASA AND H. D. SLADE, *Infect. Immun.*, 10 (1974) 1135–1145.
4 W. R. SCALES, L. W. LONG, AND J. R. EDWARDS, *Carbohydr. Res.*, 42 (1975) 325–338.
5 R. L. SIDEBOTHAM, *Adv. Carbohydr. Chem. Biochem.*, 30 (1974) 371–444.
6 A. T. ARNETT AND R. M. MAYER, *Carbohydr. Res.*, 42 (1975) 339–345.
7 M. INOUE AND E. E. SMITH, *Carbohydr. Res.*, 80 (1980) 163–177.
8 T. KOGA AND M. INOUE, *Carbohydr. Res.*, 93 (1981) 125–133.
9 K. ONO, D. W. NUESSLE, AND E. E. SMITH, *Carbohydr. Res.*, 88 (1981) 119–134.
10 D. S. GENGHOFF AND E. J. HEHRE, *Proc. Soc. Exp. Biol. Med.*, 140 (1972) 1298–1301.
11 W. R. FIGURES AND J. R. EDWARDS, *Carbohydr. Res.*, 48 (1976) 245–253.
12 S. M. JUNG AND R. M. MAYER, *Arch. Biochem. Biophys.*, 208 (1981) 288–295.
13 T. J. GRIER AND R. M. MAYER, *Arch. Biochem. Biophys.*, 212 (1981) 651–659.
14 S. SINGH, C. M. MAYNARD, F.-T. LIN, K. G. TAYLOR, AND R. J. DOYLE, in R. J. DOYLE AND J. E. CIARDI (Eds.), *Glucosyltransferases, Glucans, Sucrose and Dental Caries*, IRL Press, Arlington, VA, 1983, pp. 155–160.
15 S. THANIYAVARN, S. SINGH, K. G. TAYLOR, AND R. J. DOYLE in ref. 14, p. 161.
16 J. F. ROBYT AND J. N. ZIKOPOULOS, U. S. Pat. 4,335,100.
17 A. K. ANISUZZAMAN AND R. L. WHISTLER, *Carbohydr. Res.*, 61 (1978) 511–518.
18 R. M. MAYER, M. M. MATHEWS, C. L. FUTERMAN, V. K. PARNAIK, AND S. M. JUNG, *Arch. Biochem. Biophys.*, 208 (1981) 227–287.
19 D. B. DRUCKER, *Arch. Oral Biol.*, 28 (1983) 833–837.
20 D. A. GRAHAME AND R. M. MAYER, *Carbohydr. Res.*, 142 (1985) 285–298.