A novel oligosaccharide, GlcA β 1-4Xyl β 1-(4-methylumbelliferone), synthesized by human cultured skin fibroblasts

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Human skin fibroblasts were cultured in the presence of 4-methylumbelliferyl- β -D-xyloside (Xyl-MU) using a mass-culture system with a microcarrier. The structures of Xyl-MU-induced sugars purified from the dialysable fraction of the incubation medium were investigated. In addition to glycosaminoglycans the elongation of which initiated by Xyl-MU has already been reported, and oligosaccharides similarly initiated by Xyl-MU, such as Gal-Gal-Xyl-MU, Gal-Xyl-MU and SA-Gal-Xyl-MU, a novel Xyl-MU-induced oligosaccharide was detected. This oligo-

saccharide was identified as GlcA β 1-4Xyl β 1-(4-methylumbelliferone) using sugar composition analysis, enzyme digestion, mass spectrometry and Smith degradation. Using this culture system, the amount of the new oligosaccharide produced increased with the incubation time, even after the production of glycosaminoglycan initiated by Xyl-MU and Gal-Xyl-MU had reached a plateau. These results suggest that this oligosaccharide may be involved in terminating the elongation of glycosaminoglycan chains that is initiated by Xyl-MU.

INTRODUCTION

The mechanisms involved in glycosaminoglycan (GAG) biosynthesis have yet to be elucidated fully. However, it has been demonstrated that GAG biosynthesis is initiated by the transfer of a xylose residue from UDP-xylose to a serine residue of the proteoglycan core protein, which is followed by synthesis of the linkage region (GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-Ser), catalysed by galactosyltransferases-I and -II and glucuronosyltransferase-I, and subsequent synthesis of repeating glucuronic acid and *N*-acetylhexosamine disaccharide units [1].

It has been reported that addition of a β -xyloside, such as *p*-nitrophenyl- β -D-xyloside, 4-methylumbelliferyl- β -D-xyloside (Xyl-MU) or benzyl- β -D-xyloside, to the cell culture medium induces elongation of the GAG chains, which is initiated by the β -xyloside acting as a primer [2–8]. Human cultured skin fibroblasts have also been reported to produce the same oligosaccharides, corresponding to the linkage-region structures between the core proteins and GAG chains, in medium containing *p*-nitrophenyl- β -D-xyloside [9]. We also observed that oligosaccharide synthesis by human skin fibroblasts cultured in medium containing Xyl-MU was initiated by Xyl-MU and resulted in production of Gal β 1-3Gal β 1-4Xyl-MU and Gal β 1-4Xyl-MU [10]. Freeze et al. also detected SA-Gal-Xyl-MU (where SA represents sialic acid), which had a G_{M3} ganglioside-like structure [11]. Further detailed studies of Xyl-MU-induced substances may prove helpful for the elucidation of the mechanisms involved in GAG biosynthesis.

In this study, human skin fibroblasts were mass-cultured in the presence of Xyl-MU using the microcarrier method, and Xyl-MU-induced substances obtained from the continuously collected culture medium were analysed. The results demonstrated

that, in addition to Xyl-MU-induced substances that have been reported already, a novel oligosaccharide, GlcA β 1-4Xyl β 1-MU, which has a structure not demonstrated in mammalian cells hitherto, was synthesized.

EXPERIMENTAL

Materials

Eagle's minimum essential medium (MEM), fetal bovine serum (FBS) and penicillin–streptomycin solution (5000 units/ml and 100 μ g/ml respectively) were purchased from Gibco (Grand Island, NY, U.S.A.), Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan) and Xyl-MU was from Nacalai Tesque Inc. (Kyoto, Japan). The 2-aminopyridine (PA) used was the same as that reported previously [12], saccharo-1,4-lactone was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Cytodex 2 and Sephadex G-15 were obtained from Pharmacia LKB (Uppsala, Sweden). Endo- β -xylosidase was purified from the scallop mid-gut gland, as described previously [12], and β -glucuronidase (exo-type) was purified from rabbit liver using *p*-nitrophenyl- β -D-glucuronide as a substrate [13]. All the other chemicals used were of the highest grade commercially available.

Large-scale cell culture

Human skin fibroblasts were cultured in Eagle's MEM supplemented with 10% (v/v) FBS and antibiotics in 5% (v/v) CO₂, 95% (v/v) air at 37 °C, as described previously [10]. When the cells had grown to confluence (in 20- or 100-mm diam. Petri dishes, five passages), the medium was removed, cell layers were washed with Dulbecco's calcium- and magnesium-free PBS

Abbreviations used: MU, 4-methylumbelliferone; Xyl-MU, 4-methylumbelliferyl- β -D-xyloside; Xyl, xylose; Gal, galactose; GlcA, glucuronic acid; SA, sialic acid; GAG, glycosaminoglycan; PA, 2-aminopyridine; MEM, minimum essential medium; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; CMF-PBS, Dulbecco's calcium- and magnesium-free PBS.

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(CMF-PBS) and 0.25 % (w/v) trypsin in CMF-PBS (3 ml/dish) was added. The cell layers were then incubated at 37 °C for 15 min, harvested by centrifugation at 700 rev./min for 5 min and suspended in DMEM containing 10 % (v/v) FBS.

Swollen Cytodex 2 (2 g dry weight) was added to 1.5 litres of DMEM containing 10 % (v/v) FBS, 1 % (v/v) penicillin-streptomycin solution and 3 g/l glucose in a 1.5 litre vessel, equilibrated and then placed in a cell mass-culture system equipped with an interactive four-gas pH/dissolved oxygen controller (CelliGen, New Brunswick Scientific Co., Edison, NJ, U.S.A.). The cell suspension described above was added to the vessel and the medium was incubated at 37 °C at pH 7.0 in 50 % (v/v) dissolved oxygen with the vessel's impeller rotating at 50 rev./min. Cytodex 2 was added, according to the cell growth rate, to a total amount of 15 g.

When the number of cells reached 2.8×10^8 , perfusion of the medium in the vessel with 0.5 mM Xyl-MU in DMEM was started using an automatic perfusion system at a rate sufficient to maintain the minimum glucose concentration in the medium at 0.75 g/l. The medium was recovered automatically in a drain bottle connected to the vessel.

Small-scale cell culture

Human skin fibroblasts were cultured in Eagle's MEM supplemented with 10 % (v/v) FBS and antibiotics at 37 °C in a humidified atmosphere of 5 % (v/v) CO₂ and 95 % (v/v) air [10]. The cells were plated at a density of 2×10^5 /100-mm diam. dish with 8 ml of medium and fed two or three times a week. Fibroblasts (obtained after four to six passages) were used for this study. Confluent cultured fibroblasts were incubated for 3 days with 1 mM Xyl-MU. The Xyl-MU-induced oligosaccharides were isolated as follows: the spent culture media were pooled, lyophilized and passed through a Sephadex G-25 column to remove high-molecular-mass materials. The fluorescent fractions retained by the column were collected, lyophilized and chromatographed on Sephadex G-15. Then those that were excluded by the column were collected, lyophilized and subjected to h.p.l.c. analysis.

H.p.l.c. analysis

An h.p.l.c. (L-6200, Hitachi, Tokyo, Japan) connected to a fluorescence spectrometer (F-1050, Hitachi) was used. The Xyl-MU-induced substances were detected by their fluorescence at excitation and emission wavelengths of 325 and 380 nm respectively, and the pyridylaminated (with PA) sugars were detected at excitation and emission wavelengths of 320 and 400 nm respectively.

Gel-filtration h.p.l.c. of the Xyl-MU-induced substances was performed using a Shodex OHPak KB-803 column (8 mm $\times$ 300 mm; Shoko Co., Tokyo, Japan) or a Shodex OHPak KB-2004 column (20 mm $\times$ 300 mm; Shoko) with 0.2 M NaCl as the eluent at a column temperature of 30 °C and flow rates of 1 or 3 ml/min respectively.

Reverse-phase h.p.l.c. was performed using a Shodex C18-10F column (20 mm $\times$ 250 mm; Shoko) with a linear gradient of water/acetonitrile. The PA-sugars were identified by analysis using an Ultrasphere ODS column (4.6 mm $\times$ 250 mm; Beckmann, Palo Alto, CA, U.S.A.) with 1 % (w/v) acetonitrile in 0.25 M sodium citrate buffer (pH 4.0) as the eluent [10].

Isolation of Xyl-MU-induced oligosaccharides

During incubation in DMEM medium containing Xyl-MU, the medium (9.5 litres) was collected continuously, pooled, concen-

trated with a lyophilizer and then dialysed against distilled water at 4 °C for 48 h using a Visking tube. The dialysable fraction was concentrated with a lyophilizer, then applied to a Sephadex G-15 column (4.2 cm $\times$ 132 cm), which was equilibrated and eluted with 0.2 M acetic acid at a flow rate of 50 ml/h. The fluorescence intensity of the eluate was monitored at excitation and emission wavelengths of 325 and 380 nm respectively. The fractions exhibiting fluorescence were recovered and subjected to preparative gel-filtration h.p.l.c. (Shodex OHPak KB-2004). The fractions containing the novel Xyl-MU-induced oligosaccharide, which was detected by its fluorescence, were recovered after h.p.l.c., desalted on a Sephadex G-15 column (1.0 cm $\times$ 120 cm) equilibrated with 0.2 M acetic acid and then lyophilized.

Enzyme digestion

Endo- β -xylosidase digestion of the Xyl-MU-induced oligosaccharide was performed in 0.1 M sodium acetate buffer (pH 4.0) at 37 °C overnight, as described previously [12], and β -glucuronidase (exo-type) digestion was performed in 0.1 M sodium acetate buffer (pH 4.5) at 37 °C overnight, as described previously [13].

Smith degradation

Smith degradation of the Xyl-MU-induced oligosaccharide and PA-oligosaccharide was performed using a modification of the method of Yamashita et al. [14]. Each sample was dissolved in 0.1 ml of 0.08 M NaIO₄ in 0.1 M sodium acetate buffer (pH 4.5) and allowed to stand at 4 °C for 12 h. Then 5 μ l of ethylene glycol was added, and the mixture was allowed to stand for 1 h. This was followed by addition of 0.5 ml of 0.25 M NaBH₄ in 0.1 M sodium borate buffer (pH 8.0), and the mixture was allowed to stand for a further 3 h. The pH was then adjusted to 4.0 by adding acetic acid and the solution was evaporated to dryness repeatedly in the presence of methanol under reduced pressure to remove the borate. The resulting reduced samples were mixed with 0.1 M HCl and allowed to stand at room temperature for 30 min, after which time each reaction was stopped by adding an equal volume of 0.1 M NaOH.

Analytical methods

In order to determine their sugar composition, samples were hydrolysed in 2 M HCl at 100 °C for 4 h and then pyridylaminated, as described previously [15]. The resulting PA-monosaccharides were identified by h.p.l.c. analysis on an Ultrasphere ODS column [15].

Mass spectra of the Xyl-MU-induced and PA-oligosaccharides were obtained using an ion-spray mass spectrometer (Sciex, API-III, Thornhill, Ontario, Canada) equipped with an atmospheric-pressure ionization source, as described previously [16]. Samples (0.1 nmol/ml) were dissolved in 0.5 mM ammonium acetate/acetonitrile (50:50, v/v) and injected at 2 μ l/min using a micro-h.p.l.c. syringe pump (pump 22, Harvard Apparatus Inc., MA, U.S.A.).

RESULTS

Production of Xyl-MU-induced oligosaccharide by human cultured skin fibroblasts

Human skin fibroblasts were mass-cultured in the presence of Xyl-MU using the microcarrier method. Aliquots of the culture medium were taken at various incubation times to monitor quantitative changes in the production of Xyl-MU-induced

Figure 1 Analysis by h.p.l.c. of the Xyl-MU-induced substances in human skin fibroblast culture medium after incubation for 71 (a), 89 (b), 100 (c), 114 (d) and 124 h (e)

The column used was a Shodex OHpak KB-803 (0.8 cm $\times$ 30 cm), which was eluted with 0.2 M NaCl at 30 $^{\circ}$ C at a flow rate of 1.0 ml/min. Fluorescence of the eluted fractions was monitored at excitation and emission wavelengths of 325 and 380 nm respectively. The arrows denote the Xyl-MU-induced GAG and oligosaccharides: 1, GAG-MU; 2, SA-Gal-Xyl-MU; 3, novel Xyl-MU-induced oligosaccharide; 4, Gal-Gal-Xyl-MU; 5, Gal-Xyl-MU.

substances using h.p.l.c. (Figure 1). The presence of GAG-MU (peak 1), Gal-Gal-Xyl-MU (peak 4) and Gal-Xyl-MU (peak 5), which have been demonstrated previously, was confirmed [10]. Peak 2 was a Xyl-MU-induced oligosaccharide, which had the same carbohydrate moiety as SA-Gal-Xyl-MU, reported previously by Freeze et al. [11]. Moreover, a novel Xyl-MU-induced substance was detected (peak 3). The amount of the oligosaccharide increased almost linearly with time during incubation for 71–124 h, even though the cell numbers remained constant during this period (Figure 2).

Isolation of the novel Xyl-MU-induced oligosaccharide

In order to purify the novel Xyl-MU-induced oligosaccharide (peak 3 in Figure 1), the human skin fibroblast culture medium was dialysed, the dialysable fraction was concentrated, subjected to gel-filtration on Sephadex G-15 (Figure 3) and an aliquot of each eluted fraction was subjected to gel-filtration h.p.l.c. (Shodex

Figure 2 Changes in the amounts of the Xyl-MU-induced oligosaccharides produced and cell numbers with incubation time

Incubated human cultured skin fibroblasts were perfused with DMEM containing 0.5 mM Xyl-MU. The medium collected at each required time point was analysed by h.p.l.c. on a Shodex OHpak KB-803 column (see Figure 1) and then the amounts of Xyl-MU-induced oligosaccharides produced were estimated according to their fluorescence intensities. $\square$, Gal-Gal-Xyl-MU; $\circ$, SA-Gal-Xyl-MU; $\bullet$, GlcA-Xyl-MU; $\blacksquare$, Gal-Xyl-MU. The cell numbers ($\triangle$) were counted after adding Trypan Blue to the cultures.

OHpak KB-803). As the peak indicated by the bar in Figure 3 was found to contain the novel Xyl-MU-induced oligosaccharide, these fractions were recovered and subjected to preparative gel-filtration h.p.l.c. (Shodex OHpak KB-2004), which yielded two major peaks. One of the peaks was found to correspond to the Xyl-MU-induced oligosaccharide, so this fraction was collected and desalted by gel-filtration chromatography on Sephadex G-15. The desalted fraction eluted as a single peak when subjected to reverse-phase h.p.l.c. on Shodex C18-10F (results not shown), and was used as the purified preparation of the novel Xyl-MU-induced oligosaccharide for structural analysis (described below).

Carbohydrate composition of the Xyl-MU-induced oligosaccharide

An aliquot of the purified Xyl-MU-Induced oligosaccharide was subjected to acid hydrolysis in 2 M HCl at 100 $^{\circ}$ C for 4 h and then pyridylaminated. The resulting PA-sugars were identified using an Ultrasphere ODS column. Only PA-glucuronic acid and PA-xylose were detected (Figure 4).

Figure 3 Gel-filtration chromatography on Sephadex G-15 of the dialysable fraction of the culture medium

Pooled culture media were dialysed against distilled water, the dialysable fraction was concentrated, applied to a Sephadex G-15 column (4.2 cm $\times$ 132 cm), which was equilibrated and eluted with 0.2 M acetic acid at a flow rate of 50 ml/min, and 12-ml fractions were collected. The eluate was monitored with a fluorimeter. The fractions indicated by the horizontal bar were collected and purified further.

Figure 4 Analysis by h.p.l.c. using an Ultrasphere ODS column of the pyridylaminated (PA) monosaccharides derived from the Xyl-MU-induced oligosaccharide hydrolysate

The Xyl-MU-induced oligosaccharide was hydrolysed in 2 M HCl at 100 °C for 4 h and the hydrolysate was evaporated to dryness and pyridylaminated. The PA-monosaccharides were analysed by h.p.l.c. using an Ultrasphere ODS column (0.6 cm $\times$ 25 cm), which was eluted with 0.25 M sodium citrate buffer (pH 4.0) containing 1% (w/v) acetonitrile at a flow rate of 0.5 ml/min. The PA-monosaccharides were detected at an excitation wavelength of 320 nm and an emission wavelength of 400 nm. The arrows denote the position of standard PA-monosaccharides: 1, PA-glucuronic acid; 2, PA-iduronic acid; 3, PA-galactose; 4, PA-xylose; 5, PA-*N*-acetylglucosamine; 6, PA-*N*-acetylgalactosamine.

β -Glucuronidase digestion of the Xyl-MU-induced oligosaccharide

An aliquot of the purified Xyl-MU-induced oligosaccharide was incubated with β -glucuronidase and then subjected to gel-filtration h.p.l.c. on a Shodex OHpak KB-804 column. The Xyl-MU-induced oligosaccharide shifted to a position corresponding to Xyl-MU after digestion (Figure 5b), but did not shift from the

Figure 5 Analysis by h.p.l.c. of the Xyl-MU-induced oligosaccharide before and after incubation with β -glucuronidase

The column used was a Shodex OHpak KB-804 (0.8 cm $\times$ 30 cm), which was eluted with 0.2 M NaCl at flow rate of 1 ml/min. (a) Before incubation with β -glucuronidase; (b) after incubation with the enzyme in the absence of 10 mM saccharo-1,4-lactone; (c) after incubation with the enzyme in the presence of 10 mM saccharo-1,4-lactone.

control position (Figure 5a) after digestion in the presence of the β -glucuronidase inhibitor saccharo-1,4-lactone (Figure 5c).

Mass spectrometry of the Xyl-MU-induced oligosaccharide

An aliquot of the purified Xyl-MU-induced oligosaccharide was subjected to ion-spray m.s. The m/z of the oligosaccharide was found to be 483 (Figure 6a), so this peak was analysed as the parent ion for fragmentation by m.s./m.s. analysis. Three daughter ion peaks with mass numbers of 175, 193 and 305 were obtained and identified as $[\text{MU} - \text{H}]^-$, $[\text{GlcA} - \text{H}]^-$ and $[(\text{Xyl-MU}) - \text{H}]^-$ respectively (Figure 6b). On the basis of these results, the parent ion peak at m/z 483 was concluded to be GlcA-Xyl-MU.

Smith degradation of the Xyl-MU-induced oligosaccharide

From the analytical results described above, the Xyl-MU-induced oligosaccharide appeared to be glucuronic acid- β -linked to Xyl-

Figure 6 Mass spectra (determined in negative mode) of the Xyl-MU-induced oligosaccharide

(a) MU-oligosaccharide; (b) daughter ions on m.s./m.s. analysis spectrum of Xyl-MU-induced oligosaccharide using m/z 483 as the parent ion (a).

MU. Therefore, its structure was most likely to be either GlcA β 1-4Xyl β 1-MU, GlcA β 1-3Xyl β 1-MU or GlcA β 1-2Xyl β 1-MU.

In order to examine the glucuronic acid to xylose linkage position, an aliquot of the purified Xyl-MU-induced oligosaccharide was subjected to endo- β -xylosidase digestion followed by pyridylation, and the resulting PA-oligosaccharide (GlcA-Xyl-PA) was subjected to mild Smith degradation. The degradation product was purified by reverse-phase h.p.l.c. on a Shodex C18-10F column, and the mass spectrum of this purified product showed a peak at m/z 139 (Figure 7a), fragmentation of which by m.s./m.s. analysis yielded a peak at m/z 94, which was a daughter ion of PA (Figure 7b).

These results showed that the structure of the Smith degradation product was that shown in Scheme 1. The glucuronic acid residue was found, therefore, to be linked at the C-4 position of xylose (Scheme 1).

Addition of saccharo-1,4-lactone or benzoic acid to the incubation medium

In order to elucidate the mechanism of transglucuronosylation to Xyl-MU, two different inhibitors were added to the incubation medium of the cultured fibroblasts in the 100-mm diam. dishes. Saccharo-1,4-lactone, a β -glucuronidase inhibitor, was added to determine whether the transglycosylation was catalysed by β -glucuronidase, but it did not affect GlcA-Xyl-MU production. Addition of benzoic acid, an inhibitor of glucuronosyltransferase involved in the detoxication system, also had no effect on GlcA-Xyl-MU production (results not shown).

Figure 7 Mass spectra (determined in positive mode) of the PA-oligosaccharide Smith degradation products

(a) PA-derivative; (b) daughter ions on m.s./m.s. analysis spectrum of the PA-derivative using m/z 139 as the parent ion (a).

Scheme 1 Flow diagram for the determination of the structure of the MU-oligosaccharide, GlcA β 1-4Xyl β 1-MU

DISCUSSION

It has been reported that when cells are cultured in medium containing a β -xyloside such as Xyl-MU or *p*-nitrophenyl- β -D-xyloside, GAG-chain elongation, initiated by Xyl-MU or *p*-nitrophenyl- β -D-xyloside acting as primers, is induced [2-7]. In addition to GAG-MU, elongation of some oligosaccharides, which are linkage-region structures between the core protein and GAG chain of proteoglycans, initiated by Xyl-MU or *p*-nitrophenyl- β -D-xyloside has been reported [10], resulting in production of Gal-Xyl-MU and Gal-Gal-Xyl-MU. Recently, Freeze et al. detected a novel oligosaccharide-MU, SA-Gal-Xyl-MU,

that was produced by human melanoma and Chinese hamster ovary cells [11] and showed that its structure was related not to the proteoglycan linkage region, but to a ganglioside. Therefore, it seems likely that analysis of such elongated oligosaccharides, produced using a β -xyloside as a primer, would yield information about the mechanisms involved in proteoglycan and glycolipid carbohydrate-chain biosynthesis.

In this study, large amounts of Xyl-MU-induced substances for structural analyses were prepared by mass-culture of human skin fibroblasts in the presence of Xyl-MU using the microcarrier method. Contrary to our expectations, only a very small amount of GAG-MU was synthesized using this system. Another oligosaccharide, GlcA-Gal-Gal-Xyl-MU, reported previously by Oegema and Kraft [17], was not found in the medium, and currently we are trying to find out why. However, we detected a novel Xyl-MU-induced oligosaccharide (Figure 1, peak 3), which was identified as GlcA β 1-4Xyl β 1-(4-methylumbelliferone), on the basis of the results of sugar analysis, enzyme digestion, mass spectrometry and Smith degradation. A structure corresponding to that of the carbohydrate moiety of this Xyl-MU-induced oligosaccharide, GlcA β 1-4Xyl, has not been found in animal tissue hitherto. However, the same carbohydrate moiety structure does occur as part of some plant polysaccharides, such as xyloglycan [18,19]. Moreover, a specific glucuronosyltransferase, which transfers glucuronic acid to the xylose residue of xyloglycan, has been found in the Golgi apparatus of the pea (*Pisum sativum* var. Alaska) [20].

The amount of the novel Xyl-MU-induced oligosaccharide produced increased with incubation time, whereas GAG-MU production showed no such increase. Freeze et al. reported that the carbohydrate-chain-containing part of the ganglioside structure, SA-Gal-Xyl-MU, was elongated by Xyl-MU acting as a primer, and that GAG-MU production ceased while the oligosaccharide was being produced [11]. Therefore, GlcA-Xyl-MU production may be related to regulation of GAG and glycolipid chain biosynthesis.

In order to elucidate the mechanism responsible for the transglucuronosylation of glucuronic acid to Xyl-MU, two different inhibitors were added to the incubation medium. The results obtained suggested that GlcA-Xyl-MU production was related neither to transglycosylation as a reverse β -glucuronidase reaction nor to the detoxification system. However, it is possible that the oligosaccharide may have resulted from an error by glucuronosyltransferase in the GAG biosynthesis system, although our results show clearly that GlcA-Xyl-MU was synthesized independently of the production of the oligosaccharide-MU series involved in GAG-chain elongation.

Glucuronic acid is a common constituent of GAGs. Recently, glucuronic acid residues were found to be constituents of polysaccharides from animal tissues, namely a glycoprotein from squid cartilage [21], cuticular collagen of the clam worm [22],

ganglioside from patients with neuropathy that reacts with the HNK-1 antibody and IgM paraprotein [23,24], and glucuronosyl-*N*-acetylglucosaminyl pyrophosphoryldolichol in simian virus 40-transformed human lung fibroblasts [25]. Therefore, it would appear that some, as yet undiscovered, substance containing glucuronic acid is present in mammalian tissues.

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