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Secretion of Unphosphorylated and Phosphorylated Xyloside-induced Glycosaminoglycan Chains

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Secretion of *p*-nitrophenyl-β-xyloside-induced glycosaminoglycan chains in cultured fibroblasts is considered to involve transport through the endoplasmic reticulum and the Golgi apparatus. Purified glycosaminoglycans from fibroblast secretions contain small amounts of covalently bound [^{32}P]phosphate. However, exhaustive digestion with chondroitin AC and ABC lyases yields an unphosphorylated linkage region tetrasaccharide in the majority of all polysaccharide chains. The phosphate label is associated predominantly with material of the expected behavior of linkage region hexasaccharides. Thus, phosphorylation is not a prerequisite to secretion of xyloside-induced glycosaminoglycan chains.

Proteoglycans from human and bovine cartilage were shown to contain covalently bound phosphate residues [1-3]. Oegema *et al.* [4] first demonstrated that in addition to the phosphorylated protein moiety the proteoglycan from Swarm rat chondrosarcoma contained also phosphate ester groups on the C-2 atom of the glycosaminoglycan chain-initiating xylose. Phosphorylation of xylose residues appears now as a rather general proteoglycan modification reaction since it was found in heparan sulfate proteoglycans [5] and in a small dermatan sulfate proteoglycan from skin fibroblasts [6].

Since proteoglycans are transported from the rough endoplasmic reticulum through the smooth endoplasmic reticulum and the different cisternae of the Golgi apparatus to the extra-cellular space, phosphorylation could be considered as an intracellular traffic signal. It had been shown previously that phosphorylation of the small proteoglycan from cultured fibroblasts is an early post-translational event [6]. Treatment of fibroblasts with carbonyl cyanide *m*-chlorophenylhydrazone, an inhibitor of intracellular migration at the level of the endoplasmic reticulum [7] led to the accumulation of a core protein that carried substituted xylose residues [8]. These findings seem to be compatible with the suggested biological role of xylose phosphorylation. On the other hand, it had not been distinguished between the kinetics of formation of phosphorylated serine

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and xylose residues, respectively, nor had it been shown what the fate of an unphosphorylated proteoglycan would be.

In the present study, we took advantage from the fact that exogenously administered *p*-nitrophenyl-β-D-xyloside induces the synthesis of protein-free glycosaminoglycan chains [9], especially of galactosaminoglycans. It will be shown that the majority of these polysaccharide chains is devoid of phosphorylated xylose residues.

Experimental Procedures

Preparation of Radioactively-labeled Induced Glycosaminoglycan Chains

Human skin fibroblasts from apparently healthy juvenile and adult donors were maintained in culture as described previously [10]. One hour before labeling, Eagle's minimum essential medium was replaced by Waymouth MAB 87/3 medium ([11], as formulated in the catalogue of Gibco, Grand Island, NY, USA) that contained $MgCl_2$ instead of $MgSO_4$ and was supplemented with antibiotics and 4% dialyzed and heat-inactivated fetal calf serum (Boehringer-Mannheim, W. Germany). When subsequently labeling with [^{32}P]phosphate (carrier-free; Amersham-Buchler, Braunschweig, W. Germany), phosphate was omitted, but 1.5 mM sodium sulfate was added. For labeling with [^{35}S]sulfate (carrier-free; Amersham-Buchler) the medium did not contain streptomycin sesquisulfate; and the glucose concentration was reduced to 1.4 mM when labeling with D-[1-^{3}H]galactose (specific radioactivity 370-900 MBq/mmol; New England Nuclear, Dreieich, W. Germany). After pre-incubation, cells grown in a 75 cm^2 Falcon plastic flask received 5 ml and those in a 25 cm^2 flask 2 ml of radioactive medium that contained 1 mM *p*-nitrophenyl-β-D-xyloside (Koch-Light, Colnbrook, England).

After up to 16 h of incubation, medium was removed and made 70% saturated with $(NH_4)_2SO_4$ for precipitation of proteoglycans. The supernatant was dialyzed against 20 mM sodium phosphate, pH 7.4; 0.1% Triton X-100 (buffer A) and 0.15 NaCl. The retentate was loaded on a Dowex 1-X2, 200-400 mesh column (1 ml of resin/2.5 ml of culture medium) equilibrated with this buffer. The column was eluted stepwise with 1 vol of 0.15 M NaCl, 6 vol of 0.5 M NaCl and 3 vol of 3.0 M NaCl, all in buffer A. The fractions from the last step were pooled and unlabeled xyloside-induced glycosaminoglycan chains were added prior to dialysis against 0.1% Triton X-100 and lyophilization. The dried material was washed three times with chloroform/methanol, 2/1 by vol, and further purified by chromatography on a Sephadex G-100 column (48 cm × 1 cm) equilibrated and eluted with water. Fractions representing K_{av}-values between 0 and 0.8 were lyophilized, dissolved in 300 µl of 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 7.5 mM glucose-6-phosphate, and subjected to digestion with 1.1 Kunitz units of pancreatic ribonuclease (Sigma, Deisenhofen, W. Germany) and 0.4 U of deoxyribonuclease I (Boehringer-Mannheim) for 2 h at 37°C. The digest was then dialyzed against water and concentrated under reduced pressure.

For the preparation of unlabeled glycosaminoglycans confluent cultures in 75 cm^2 flasks were incubated for 48 h with 15 ml of medium containing 1 mM *p*-nitrophenyl-β-D-xyloside. Purification of induced chains was by ammonium sulfate precipitation and chromatography on Dowex 1-X2 only.

Small iduronic acid-rich proteoglycan was isolated for control purposes with the aid of a monospecific antiserum as described previously [6].

Characterization of Glycosaminoglycan Constituents

The molecular size of induced glycosaminoglycan chains was estimated by chromatography on a calibrated Sephacryl S-300 column (1 cm × 98 cm) equilibrated and eluted with 4 M guanidinium chloride in 50 mM sodium acetate buffer, pH 6.0, containing 0.1% Triton X-100.

p-Nitrophenol-linked oligosaccharides, unsaturated repeating disaccharides of the glycosaminoglycan chains, and the saturated chain termini were obtained by enzymatic degradation followed by a combination of size exclusion and hydrophobic interaction chromatography. For this purpose, the sample was dissolved in 300 μl of 50 mM Tris/HCl, 60 mM sodium acetate, 60 mM NaCl, 7.5 mM glucose-6-phosphate, and 0.01% bovine serum albumin, pH 8.0 [12], and digested for 2 h at 37°C with 100 mU chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan). Incubation continued for a further 2 h after addition of 100 mU chondroitin AC lyase (Seikagaku Kogyo). The digestion products were separated by chromatography on a 141 cm × 1 cm Ultrogel AcA 202 column (Pharmacia-LKB, Freiburg, W. Germany) equilibrated and eluted with 10% (v/v) ethanol at a flow rate of 1.4 ml/h.

p-Nitrophenol-linked oligosaccharides were further characterized by high voltage electrophoresis on Whatman No. 3MM paper, (Maidstone, England) in 0.08 M pyridine acetate, pH 5.8, for 1 h at 60 V/cm. ^{32}P-Radioactivity was detected by autoradiography at -70°C and ^{35}S-radioactivity by liquid scintillation counting.

The unsaturated disaccharides obtained after chondroitin lyase digestion were analyzed by high-performance liquid chromatography on a Partisil-PAC (10 μm, 25 cm × 4.6 mm, Whatman) column. The system was run at a flow rate of 1.2 ml/min with a solvent consisting of 52% acetonitrile, 12% methanol, and 36% aqueous buffer containing 0.5 M Tris base, 3.6 mM H_2SO_4 and 0.1 M boric acid, pH 8.0 [13].

Analytical Methods

Hexuronic acids [14] were quantified as described. Secondary ion mass spectra [15] were recorded from 1 mM (with respect to hexuronic acid) solutions that had been filtered through Millipore 0.45 μm filters. Radioactivity was determined with a Beckman 9000 liquid scintillation spectrophotometer using Unisolve (Packard) as scintillation cocktail. Settings were adjusted for determination of ^{32}P-radioactivity without interference by ^{35}S-radioactivity.

Results and Discussion

Cultured human skin fibroblasts synthesize and secrete greatly elevated quantities of glycosaminoglycans in the presence of *p*-nitrophenyl-β-xyloside. The intracellular routing of *p*-nitrophenyl xyloside-induced glycosaminoglycan chains has not been investigated previously. We have, therefore, comparatively studied the biosynthesis and secretion of induced glycosaminoglycans and of a small, iduronic acid-rich pro-

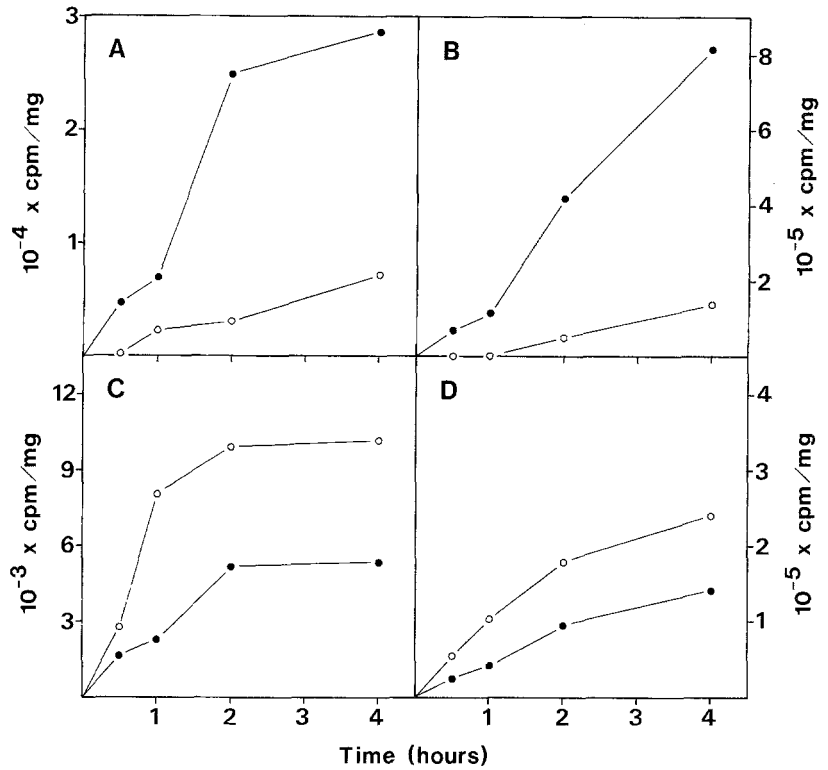

Figure 1. Influence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on synthesis and secretion of small proteoglycan and induced glycosaminoglycan chains. Fibroblasts were pre-incubated for 30 min with 1 mM *p*-nitrophenyl-β-xyloside in the absence (●) or presence (○) of 50 μM CCCP. Fresh medium was then added which contained additionally 90 kBq [^{35}S]sulfate/ml. A, secreted small proteoglycan; B, secreted glycosaminoglycan chains; C, cell-associated small proteoglycan; D, cell-associated glycosaminoglycan chains.

teoglycan under the influence of carbonyl cyanide *m*-chlorophenylhydrazone. This drug is a transport inhibitor at the level of the endoplasmic reticulum [7]. From the results presented in Fig. 1, however, it is evident that the drug causes also an intracellular accumulation of sulfated proteoglycan. Since sulfation is considered to occur in the *trans* cisternae of the Golgi apparatus [16, 17], an additional transport block at or beyond this subcompartment must be postulated. Accordingly, there is also an intracellular accumulation of protein-free sulfated polysaccharide. More important for the question of intracellular routing is the observation that carbonyl cyanide *m*-chlorphenylhydrazone led to a reduction of total sulfate incorporation into proteoglycan and induced chains by approximately 50%. Since in control experiments the incorporation of [^{3}H]leucine into proteoglycan core protein was unaffected by the drug, the reduced sulfate incorporation could best be explained by a delayed transport of glycosaminoglycan-free core protein through the endoplastmic reticulum, although reduced sulfate uptake or an altered metabolism of 3'-phosphoadenylyl sulfate cannot

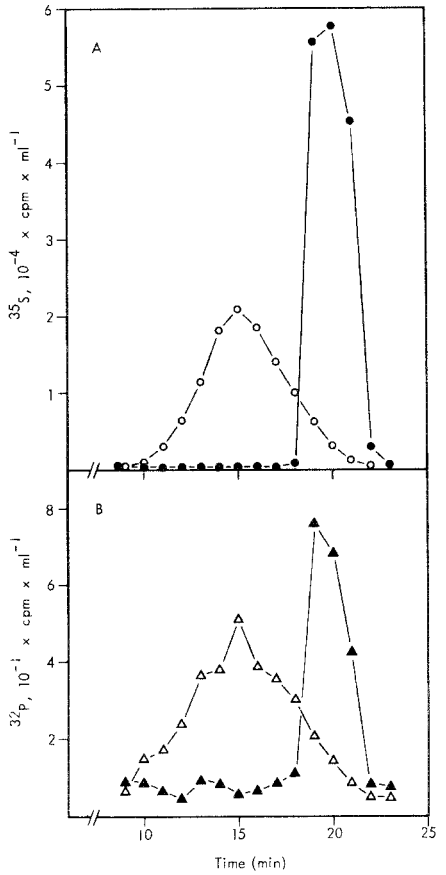

Figure 2. High-performance gel permeation chromatography of induced glycosaminoglycan chains. Induced chains were purified from the medium after incubating the cultures for 16 h in the presence of 1 mM *p*-nitrophenyl-β-xyloside and either 0.185 mBq [^{35}S]sulfate/ml or 3.3 MBq [^{32}P]phosphate/ml. Chromatography was performed on a TSK G 3000 SW column (7.5 × 500 mm) equilibrated with 50 mM sodium phosphate, pH 6.0, in 0.25 M Na_2SO_4, flow rate 1 ml/min, before (open symbols) and after (filled symbols) digestion with chondroitin ABC lyase.

be excluded. The analogous inhibition of the synthesis of induced glycosaminoglycans suggests that *p*-nitrophenyl xyloside is transported through the endoplasmic reticulum, too.

On the basis of this observation an attempt was made to investigate the phosphorylation of xyloside-induced glycosaminoglycan chains analogously to the study of the phosphorylation of small dermatan sulfate proteoglycan [6]. It had been ascertained that none of the enzymes used in the course of the preparation of glycosaminoglycans and of its degradation products contained measurable phosphatase activity towards *p*-nitrophenyl phosphate under the assay conditions described. Furthermore, the eluates from Dowex 1-X2 were also free of detectable phosphatase activity.

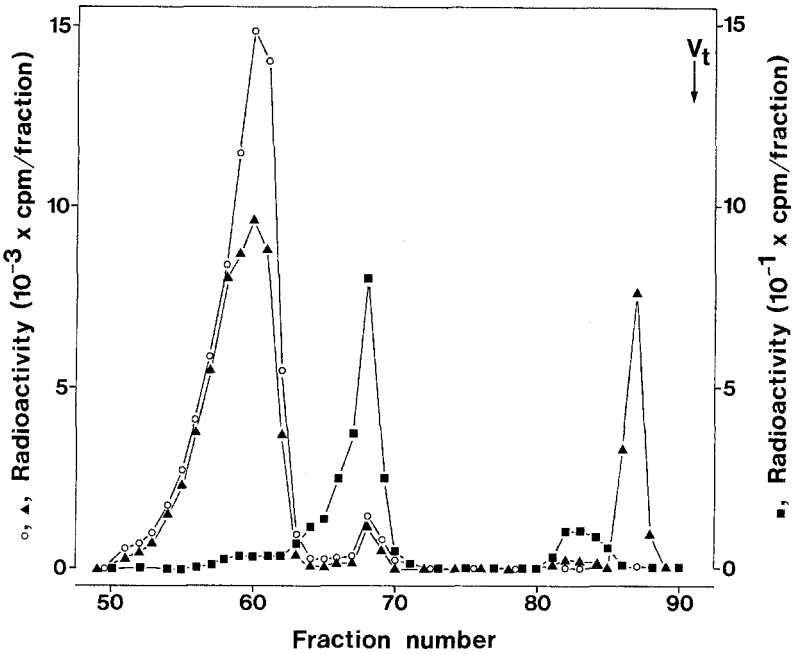

Figure 3. Gel chromatography on Ultrogel AcA 202 of chondroitin AC/ABC lyase digestion products. *p*-Nitrophenyl-β-xyloside-induced glycosaminoglycan chains were obtained after 12 h incubation of confluent cultures in 75 cm^2 flask with either [^{32}P]phosphate (■, 3.3 MBq/ml), [^{35}S]sulfate (○, 0.37 MBq/ml) or [^{14}C]galactose (▲, 1.5 mBq/ml). Combined digestions with the lyases were performed after adding an approximately 30-fold excess of unlabeled induced chains. The digest was chromatographed on an Ultrogel AcA 202 clumn equilibrated and eluted with 10% (v/v) of ethanol in water. The fraction weight was 0.78 g, the void volume 24 g (fraction 31). Radioactivities are given as obtained from one single culture flask.

Some pitfalls were encountered during the preparation of [^{32}P]phosphate-labeled glycosaminoglycan chains. Commercially available [^{32}P]phosphoric acid was found to be contaminated with [^{35}S]sulfate. The incubation of fibroblasts with such a precursor solution in sulfate-depleted medium resulted in an about two-fold greater incorporation of [^{35}S]sulfate than of [^{32}P]phosphate into induced glycosaminoglycans. This unwanted incorporation could be minimized by supplementing the incubation medium with 1.5 mM Na_2SO_4. Further difficulties were probably the results of radiation-induced cell damage which led to the appearance of phospholipids and nucleic acids in the culture medium. The following recoveries represent the result of a typical experiment where fibroblasts in 75 cm^2 flasks had been incubated with 3.3 MBq/ml of [^{32}P]phosphate and 1 mM *p*-nitrophenyl-β-xyloside for 16 h. After chromatography on Dowex 1-X2 and dialysis 5 100 cpm/flask (100%) were found; 4 100 cpm/flask (80%) remained after extraction with chloroform/methanol; after removal of low molecular weight compounds by chromatography on Sephadex G-100 the yield was 750 cpm/flask (15%); and after DNase and RNase digestion followed by dialysis only 330 cpm/flask (6%) could be recovered.

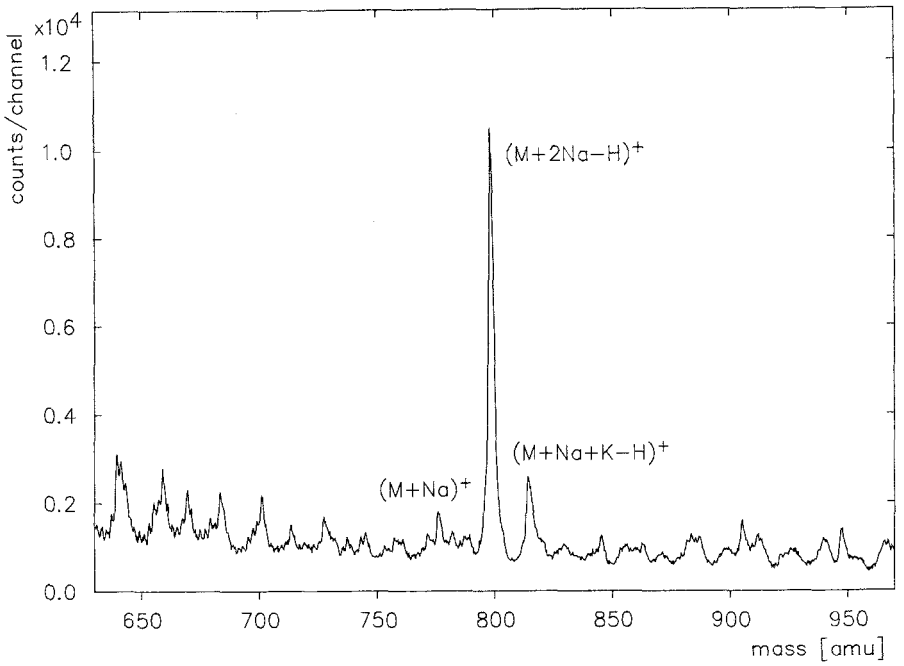

Figure 4. Mass spectrum of a linkage region saccharide. Material eluting with a mean k_{av}-value of 0.93 (peak fraction 87) from Ultrogel AcA 202 (see Fig. 3) was analyzed.

In spite of these difficulties, small amounts of ^{32}P-radioactivity were reproducibly found to be associated with induced glycosaminoglycan chains. A similar elution profile on high-performance gel chromatography was found for [^{32}P]phosphate- and [^{35}S]sulfate-labeled material. Chondroitin ABC lyase digestion yielded low molecular weight products (Fig. 2).

A more thorough analysis of the products obtained after c ombined chondroitin AC and ABC lyase degradation was performed on an Ultrogel AcA 202 column using 10% ethanol as eluant. Compounds containing *p*-nitrophenyl-residues behave atypically under these conditions due to interactions with the gel matrix. *p*-Nitrophenyl-β-xyloside, for example, is so strongly retarded that it elutes with a k_{av}-value of 1.72. To identify the degradation products of the chondroitin lyases, induced glycosaminoglycans obtained after incubation with either [^{35}S]sulfate, [^{3}H]galactose or [^{32}P]phosphate were, therefore, separately analyzed (Fig. 3). Unsaturated sulfated disaccharides were eluted with a k_{av}-value of 0.48 (peak fraction 60). A major peak containing [^{3}H]galactose but neither [^{35}S]sulfate nor [^{32}P]phosphate exhibited a k_{av}-value of 0.93 (peak fraction 87). The molecular mass of this material (753) was exactly the mass of the expected linkage region tetrasaccharide *O*-(4-enehexuronate)-(1-3)-*O*-(β-D-Gal)-(1-3)-*O*-(β-D-Gal)-(1-4)-*O*-(β-D-Xyl)-*O*-*p*-nitrophenol as determined by secondary ion mass spectroscopy (Fig. 4). [^{32}P]-Phosphate-labeled material was eluted with a k_{av}-value of 0.85 (peak fraction 82). This peak could contain a phosphorylated tetrasaccharide, but it contained only about 1% of the ^{3}H-radioactivity of the unphosphorylated tetrasaccharide and was

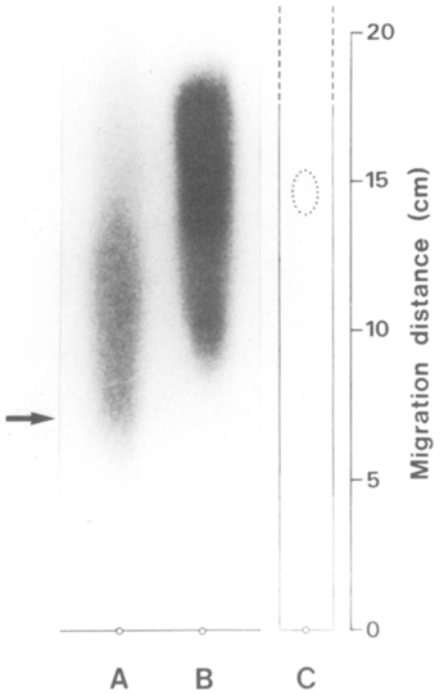

Figure 5. High voltage elecctrophoresis of phosphorylated linkage region saccharides. Aliquots of material eluting in peak fractions 82 (lane A) and 68 (lane B) from Ultrogel AcA 202 were subjected to high voltage electrophoresis at pH 5.8. Autoradiography was done for six weeks with the aid of a DuPont Cronex Lightning Plus intensifying screen. The mobility of peak fraction 68 from [^{35}S]sulfate-labeled material is shown in lane C. The arrow indicates the mobility of the unphosphorylated linkage region tetrasaccharide. The mobility of unsaturated monosulfated disaccharides was 25 cm.

therefore not investigated further. A further peak with a k_{av}-value of 0.62 (peak fraction 68) contained ^{32}P- and ^{35}S-radioactivity in addition to ^{3}H-radioactivity. The latter one accounted for about 17% of the radioactivity found in the unphosphorylated tetrasaccharide fraction. The elution position of this peak could be consistent with that of a phosphorylated and sulfated linkage region hexasaccharide. This conclusion was supported by the finding that small amounts of *p*-nitrophenolate could be detected after acid hydrolysis (4 h, 110°C, N_2) and addition of excess NaOH. The absorbance at 405 nm accounted for 28% of the absorbance of the similarly treated unphosphorylated tetrasaccharide fraction. High voltage electrophoresis at pH 5.8 followed by autoradiography revealed that the hexasaccharide peak was not homogenous (Fig. 5). Some of the material exhibited a slower electrophoretic mobility than the sulfated hexasaccharide species. It is suggested though not proved that it represents a phosphorylated but unsulfated hexasaccharide species. In this context it should be mentioned that degradation of a chondroitin sulfate proteoglycan by chondroitin AC lyase left attached to the core protein exclusively the linkage region tetrasaccharide. A hexasaccharide was present after treatment with chondroitin ABC lyase [18]. As we used

a combination of both lyases the appearance of phosphorylated linkage region hexasaccharides could indicate that phosphorylation of the linkage region interferes with the complete removal of the repetitive disaccharide units.

Taken together, our results demonstrate that phosphorylation is not a prerequisite for secretion of xyloside-induced glycosaminoglycan chains. From the incorporation of [^{3}H]-galactose it appears that at the most 15% of these chains contain phosphate ester groups. On the contrary, all heparan sulfate chains [5] and most likely all molecules of a chondrosarcoma proteoglycan [4] contained phosphorylated xylose residues. Sulfation of tyrosine residues of secretory proteins and sulfation of glycosaminoglycan chains (H. Greve and H. Kresse, unpublished result) also cannot be considered as signals for secretion. Export of proteoglycans and induced glycosaminoglycan chains occurs, therefore, most likely by unselective)bulk flow. It cannot be excluded, however, that the phosphate ester groups play a role during intracellular trafficking but are hydrolyzed prior to secretion.

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