ABNORMAL PROTEODERMATAN SULFATE IN THREE PATIENTS WITH COFFIN-LOWRY SYNDROME

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SUMMARY

The properties of [^{35}S]sulfate-labeled proteoglycans secreted by normal human skin fibroblasts were compared with those synthesized by fibroblasts from three patients with Coffin-Lowry syndrome. 60-80% of secreted radioactive macromolecules from normal fibroblasts were eluted from a Sepharose CL-4B column with a mean K_{AV}-value of 0.56 (pool 2); 3-10% of the radioactivity appeared in the exclusion volume of the column (pool 1). In contrast, 17-60% of the proteoglycans from the patients were found in the void volume. The bulk of remaining material was eluted with a mean K_{AV}-value of 0.47. Pool 2 glycan chains from two patients exhibited an increased hydrodynamic size.

Pool 1 from normal cells contained predominantly a glucuronic acid-rich proteodermatan sulfate, iduronic acid amounting for approximately 20% of glucuronic acid. In the respective proteodermatan sulfate from the patients, the relative iduronic acid content was at least 33% of that of glucuronic acid. Pool 2 material of all cell lines was characterized predominantly as iduronic acid-rich proteodermatan sulfate. In the proteoglycans from two patients the content of chondroitin 4-sulfate-derived disaccharides was increased at the expense of 6-sulfated chondroitin disaccharides. Native proteoglycans from the patients were less efficiently endocytosed by fibroblasts than their normal counterparts.

Coffin-Lowry fibroblasts had a normal capability to synthesize glycosaminoglycan chains on an arteficial acceptor, p-nitrophenyl-β-D-xyloside. They were also normal in 3'-phosphoadenylylsulfate: chondroitin 4- and 6-sulfotransferase activities.

INTRODUCTION

The Coffin-Lowry syndrome is a rare genetically transmitted disorder that is marked by mental retardation, thick, soft skin, tapering fingers, a characteristic facies and skeletal abnormalities (5, 8, 9, 12). Males are more severely affected than females, suggesting X-linked semi-dominant transmission.

The primary defect of the Coffin-Lowry syndrome is completely unknown. Coffin et al. (5) found a drastically reduced number of elastic fibers in the skin of one patient, but this observation could not be confirmed in other cases. Temtamy et al. (12) considered the appearance of inclusion bodies with fine fibrillogranular content as pathognomonic, but the quantity of membrane-bound material is not such that it suggests a storage disease (8). To our knowledge, no search for the accumulation of other substances has been undertaken.

When we first were confronted with a patient with Coffin-Lowry syndrome, we considered the possibility that the appearance of the skin could be caused by an excessive extracellular accumulation of a structural abnormal proteoglycan. Extracellularly located proteoglycans reenter the cells by receptor-mediated endocytosis before complete degradation within the lysosomes takes place. In case of proteodermatan sulfate the recognition marker required for this uptake process resides in its protein moiety (13), so that a structural anomaly of the protein core could possibly result in its extracellular accumulation.

Proteodermatan sulfate is of greatest quantitative importance among the proteoglycans secreted by skin fibroblasts. Proteodermatan sulfate from embryonic human skin fibroblasts could be separated into two types, a large proteoglycan with glucuronic acid-rich and a smaller proteoglycan with iduronic acid-rich dermatan sulfate chains (4). Differences in the carbohydrate structure of cell-associated and secreted dermatan sulfate have been described (3, 4, 11).

We will report here that structural and functional anomalies can be found in the proteodermatan sulfate synthesized and secreted by cultured fibroblasts from patients with Coffin-Lowry syndrome.

PATIENTS

Patient 1 (J.B.) is a 19-y-old boy who was diagnosed by Dr. U. Feldmann of this university. The patient exhibits the full clinical picture of the Coffin-Lowry syndrome and especially suffers from spine deformities. The mother and two sisters of the patient showed no obvious sign of the disease. Patient 2 (C.S.) is a 4-y-old boy, also diagnosed by Dr. Feldmann. He has an affected elder brother, and his mother seems to be mildly affected. These patients will be described in detail elsewhere. From patient 3 (G.) who was diagnosed by Dr. J. Gehler, University of Mainz, clinical data were not available to us.

METHODS

Fibroblast cultures from the patients and from normal individuals (age 3-30 y) were obtained from skin biopsies and further propagated in modified Eagle's Minimum Essential Medium as described previously (2). From patient 1 a biopsy was taken at two different occasions. The cultures were used between the fourth and sixteenth passage. In each set of experiments cultures from patients and controls had undergone a similar number of subcultures. There was no obvious difference in the growth characteristics of the fibroblasts from the patients and controls.

[^{35}S]O_4-labeled proteoglycans were prepared in the following manner: Fibroblasts were grown to confluency in a 75 cm^2 Falcon plastic flask. The labeling medium, 10 ml, was the same medium but deficient in streptomycin sesquisulfate and supplemented with 150 µCi $Na_2(^{35}S)O_4$ (carrier-free); Amersham-Buchler, Braunschweig, Germany). In some experiments, $Na_2(^{35}S)O_4$ was replaced by 150 µCi [6-^{3}H]glucosamine hydrochloride (sp. radioactivity 38 Ci/mmole; Amersham-Buchler); in others, both isotopes were used. At the end of the incubation period, usually after 48 h, medium was made 70% saturated with $(NH_2)_2SO_4$. The precipitate was collected by centrifugation and dissolved in the original volume of 20 mM Tris/HCl buffer, pH 7.5, containing 0.1 M 6-aminohexanoic acid, 10 mM EDTA, 5 mM benzamidine, 10 mM N-ethylmaleimide, 0.1% (v/v) Triton X-100 (buffer A), and 0.15 M NaCl. After dialysis overnight against this buffer, the retentate was loaded on a 0.7 x 13 cm DEAE-Trisacryl (LKB Instruments, Gräfelfing, Germany) column, equilibrated with this buffer. The column was eluted stepwise with 15 ml each of 0.15 M NaCl, 0.3 NaCl, and 1.0 M NaCl, all in buffer A. Appropriate fractions from the last step were pooled and dialyzed against 0.1% Triton X-100. The detergent was used to minimize unspecific adsorption of proteoglycans to the surface of glas ware and dialysis bags.

For gel chromatography the samples were dried under reduced pressure and then dissolved in 50 mM sodium acetate buffer, pH 6.0, containing 4 M guanidinium hydrochloride and the protease inhibitor cocktail mentioned above. The material was loaded onto a 1.2 x 100 cm Sepharose CL-4B (Pharmacia, Freiburg, Germany) column equilibrated with the same buffer, and chromatographed at a flow rate of 6 ml/h, fraction volume 1.0 ml. Appropriate fractions were pooled, dialyzed against 0.1% Triton X-100, and concentrated in vacuo. Liberation of glycosaminoglycan chains by β-elimination was done as described (13). The samples were concentrated again, salt was removed by washing with methanol, and the samples were digested in parallel with 20 mU chondroitin AC lyase (EC 4.2.2.5; Sigma, Munich, Germany) and 20 mU chondroitin ABC lyase (EC 4.2.2.4; Sigma), respectively, for 4 h at 37°C, final volume 20 µl (15). The digest was spotted on Schleicher and Schüll paper 2043b (Dassel, Germany) and subjected to descending paper chromatography in butan-1-ol/1 M NH_3/acetic acid (2:1:3, by vol.). The paper was cut into 1-cm segments which were eluted in scintillation vials with 1 ml H_2O before liquid scintillation counting. The amount of heparan sulfate was calculated from the proportion of radioactivity that was resistant towards chondroitin ABC lyase. The amount of dermatan sulfate was obtained from the difference of radioactivity degraded by chondroitin AC lyase and chondroitin ABC lyase.

For treatment of proteoglycans with hyaluronate lyase (EC 4.2.2.1) (Calbiochem-Behring, Giessen, Germany), Sepharose CL-4B fractions were concentrated after dialysis, Triton X-100 was removed by washing with methanol, and the sample was dissolved in 120 µl 50 mM sodium phosphate buffer, pH 6.0, containing 100 turbidity reducing units of enzyme, 0.33 mM pepstatin, and the protease inhibitors mentioned above. After 4 h at 37°C, the digest was rechromatographed under dissociative conditions.

The hydrodynamic size of glycosaminoglycan chains was determined by chromatography on a 1.2 x 90 cm Sephacryl S-300 (Pharmacia) column which had been equilibrated with 50 mM sodium acetate buffer, pH 6.0, containing 4 M guanidinium chloride. The flow rate was 6 ml/h. Molecular weight standards were kindly provided by Dr. Wasteson, Uppsala, Sweden (14).

Studies on the metabolism of exogenously added [^{35}S]O_4-labeled proteoglycans were performed as described (13), except that the proteoglycans were purified by chromatography on DEAE-Trisacryl as mentioned above. Triton X-100 was omitted from all buffers. Measurements of glycosaminoglycans biosynthesis in the presence or absence of p-nitrophenyl-β-D-xyloside were done as quoted earlier (13).

Chondroitin sulfotransferase activity (EC 2.8.2.?) was determined in the following manner: 100 µg chondroitin (kindly provided by D. Göhler of this institute), approx. 150 µg of cell protein and 2.2 nmole 3'-phosphoadenylyl-[^{35}S]sulfate (sp. radioactivity 50 Ci/mole, prepared as in 10) were incubated for 24 h at 22°C in 50 mM sodium phsophate buffer, pH 7.0, containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM KF, 80 mM NaCl, 0.02% NaN_3 and 0.05% Triton X-100, final volume 105 µl. Sulfated product was recovered by high pressure liquid chromatography on a TSK G 2000 SW column (Varian, Darmstadt, Germany) before dialysis and degradation with chondroitin ABC lyase.

RESULTS

After biosynthesis, proteoglycans are distributed into different compartments. In case of cultured skin fibroblasts, the bulk of material is secreted into the culture medium. A minor part remains inside the cell and is preferentially transferred to lysosomes. Coffin-Lowry fibroblasts behave like normal cells with regard to the rate of biosynthesis of proteoglycans and their distribution into these pools (Fig 1). The turnover of intracellular glycosaminoglycans is also normal (result not shown). Addition of p-nitrophenyl-β-D-xyloside to the culture medium leads to an increased incorporation of [^{35}S]sulfate into protein-free glycosaminoglycan chains. Glycosaminoglycan biosynthesis of Coffin-Lowry fibroblasts was stimulated to a similar extent as that of normal cells (Fig 1).

Gel chromatography of secreted proteoglycans from the patients, however, revealed an abnormality of their hydrodynamic size. As shown in Fig 2D, 3-10% ($\underline{n}$ = 5) of radioactive proteoglycans from normal cells (age of the donors was 3-12 y) were eluted in the exclusion volume of a Sepharose CL-4B column when the column was operated under dissociative conditions. These proteoglycans will be referred to as pool 1 material. In the patients, 17-60% of the label were found in the void volume (Fig 2A-C). It should be noted that there was considerable variability even within one cell line. The proportion of large size proteoglycans of patient 1 who was most intensively studied, varied between 30-60% in four separate experiments, in which fibroblasts grown from two different skin biopsies were used.

The bulk of secreted proteoglycans from normal cells was eluted from the Sepharose CL-4B column with a mean Kav-value of 0.56 (pool 2). The corresponding proteoglycan peak from the patients represented larger material because its Kav-value was 0.47. The increased hydrodynamic size was ascertained by cochromatography of [^{35}S]sulfate-labeled proteoglycans from patient 1, and control material obtained after incubation in the

presence of [^{3}H]glucosamine (Fig 2A). Differences in the elution profiles of [^{35}S]- and [^{3}H]-labeled material are mainly due to labeling of hyaluronate in the latter case.

Excessive amounts of pool 1 proteoglycans were most likely not generated by the formation of abnormally stable aggregates of pool 2 proteoglycans with hyaluronate. Upon digestion of [35S]- and [^{3}H]-labeled pool 1 proteoglycans from patient 1 with hyaluronate lyase and rechromatography on Sepharose CL-4B, no pool 2 proteoglycans could be recovered though the molecular size was diminished (Fig 3). It could not be decided whether this was due to proteolytic degradation by a non-inhibitable protease, or whether indeed hyaluronate-proteoglycan complexes were degraded.

An increased size of the patients'proteoglycans from pool 2 could be caused by an increased number of glycan chains and/or an increased chain length. Protein-free carbohydrate chains were obtained by β-elimination and chromatographed on a Sephacryl S-300 column. Figure 4 shows that pool 2 proteoglycans from patients 1 and 2 contained glycan chains of larger size than those from controls. Differences in the chain length of pool 1 proteoglycans were not observed.

Glycosaminoglycan distribution pattern. The composition of glycosaminoglycan chains of the proteoglycans from pool 1 and 2 was analyzed after specific enzymatic degradation. As shown in Table 1, pool 1 from normal cells contained predominantly a glucuronic acid-rich proteodermatan sulfate. Iduronic acid amounted for approximately 20% of glucuronic acid. In the respective proteodermatan sulfate from the patients, the relative iduronic acid content varied between 33-65% of that of glucuronic acid. The bulk of pool 2 proteoglycans was characterized as iduronic acid-rich proteodermatan sulfate, regardless of the cell line tested. In each pool from patients 1 und 3 there was an increase of chondroitin 4-sulfate disaccharides at the expense of 6-sulfated chondroitin disaccharides. Such an abnormality was not observed in the proteoglycans from patient 2.

Chondroitin sulfotransferase activity. It is shown in Table 2 that fibroblasts from patients 1 and 2 did neither differ significantly from normal cells in their chondroitin sulfotransferase activity nor in the position of the sulfate ester linkages formed. Thus, the different ratio of 4- and 6-sulfated chondroitin disaccharides found in native proteoglycans from both patients (see above) cannot be reproduced when a protein-free substrate of sulfotransferase is used as acceptor under in vitro conditions.

Endocytosis of proteoglycans. Proteoglycans from the patients showed also a metabolic abnormality because they were less efficiently endocytosed by fibroblasts than their normal counterpart. A typical example is given in Table 3. It is evident that Coffin-Lowry fibroblasts are able to internalize normal proteoglycans at a normal rate, whereas the rate of endocytosis of Coffin-Lowry proteoglycans was reduced regardless of whether normal or Coffin-Lowry fibroblasts were used as recipients.

DISCUSSION

Several qualitative and quantitative abnormalities of proteodermatan sulfate secreted by Coffin-Lowry fibroblasts have been found in the present investigation: (1) an increased proportion of the large glucuronic acid-rich proteodermatan sulfate at the expense of the smaller iduronic acid-rich proteodermatan sulfate; (2) an increased proportion of the iduronic acid content of the large proteoglycan; (3) an increased size of the smaller proteodermatan sulfate; (4) a reduced capability of secreted proteoglycans for being endocytosed. Furthermore, in two of three patients an altered ratio of 4- and 6-sulfated chondroitin sulfate disaccharides within the glycosaminoglycan chains was found.

Carlstedt et al. (3) reported that the core protein of the large proteodermatan sulfate species secreted by embryonic skin fibroblasts has an M_r of over 400,000, whereas that of the smaller species has an M_r = 47,000. This could suggest that both core proteins are genetically distinct. In preliminary experiments we have not yet been able to detect a M_r 400,000 core protein in the secretions of normal or Coffin-Lowry fibroblasts. We cannot therefore answer the question whether or not Coffin-Lowry fibroblasts express a core protein found as yet only in embryonic cells.

Alternatively, pool 1 proteoglycans could represent aggregates of pool 2 proteoglycans. Proteodermatan sulfate shows the phenomenon of self-association and may form aggregates with hyaluronic acid. Hyaluronate is present in pool 1; however, degradation of this polymer by a specific enzyme does not result in the conversion of pool 1 proteoglycans into pool 2 proteoglycans (Fig 3). Likewise, aggregation due to self-association seems unlikely because self-association is abolished in the presence of 4 M guanidinium chloride (6). On the other hand, interactions of proteoglycans with components not yet fully characterized may be resistant to such treatment (7). If pool 1 material from the patients would arise in part by the formation of abnormally stable aggregates of pool 2 proteoglycans, one could explain the increased iduronic acid content of pool 1 proteoglycans. It could then be discussed whether aggregate formation is caused by the production of an aggregating component or by the formation of abnormal proteodermatan sulfate.

During and after biosynthesis of the core proteins, a variety of posttranslational modifications occurs: synthesis, modification and sulfation of glycan chains, attachment of N- and O-glycosidically linked oligosaccharides and, most likely, proteolytic processing. It has not yet been established in case of proteoglycans as and to what extent the amino acid sequence of the protein cores has an influence on these secondary processes.

The three Coffin-Lowry cell lines investigated had some, but not all, abnormalities of proteodermatan sulfate in common. Because the disease is genetically lethal in case of males, the frequency of new mutations must be high, and several allelic mutations should exist. As we studied secondary consequences of an unknown primary defect, it is not surprising that a certain variability was found. It is our present hypothesis that the primary defect of the Coffin-Lowry syndrome may concern expression, biosynthesis and/or posttranslational processing of a proteodermatan sulfate core protein. The functionally altered proteoglycan cannot be endocytosed at a normal rate, and therefore accumulates extracellularly. Studies are in progress to analyze proteodermatan sulfate core proteins by a more direct approach. Though the present results cannot be interpreted unequivocally, they may be of help in establishing a method for a biochemical diagnosis of the disorder.

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1. Relative distribution pattern of [35S]glycosaminoglycans of pool 1 and pool 2

Glycosaminoglycan type	Normal individuals (n = 3)		Patient 1		Patient 2		Patient 3	
	Pool 1	Pool 2	Pool 1	Pool 2	Pool 1	Pool 2	Pool 1	Pool 2
Chondroitin 4-sulfate	19-22	3-10	39	17	20	3	23	8
Chondroitin 6-sulfate	48-52	3-12	19	4	49	3	29	5
Dermatan sulfate	13-15	76-81	38	69	23	87	29	75
Heparan sulfate	8-16	4-13	3	12	8	7	19	11

Pool 1 was obtained from the proteoglycans eluting in the exclusion volume of a Sepharose CL-4B column. Pool 2 was prepared from proteoglycans eluting with a mean Kav-value of 0.56 (normal individuals) and of 0.47 (patients), respectively, from the same column. The sum of the radioactive glycosaminoglycans isolated from the different pools was taken as 100%.

The ratio of chondroitin 6-sulfate/chondroitin 4-sulfate was 1 in each individual cell line.

2. Chondroitin sulfotransferase activity of cultured fibroblasts

Enzyme source	pmole/24 h and mg cell protein	chondroitin 4-sulfate / chondroitin 6-sulfate
Patient 1	140	1.33
Patient 2	120	1.05
Controls	130	1.11
	280	0.85
	150	1.28

3. Uptake and adsorption of [35S]proteoglycans

Source of proteoglycan	10^{-3} Dose x cpm	Recipient: Patient Uptake % of added amount	Recipient: Patient Adsorption % of added amount	Recipient: Father Uptake % of added amount	Recipient: Father Adsorption % of added amount
Father of patient 1	60	17.8	4.1	19.4	3.2
	120	14.8	3.9	16.4	3.1
Patient 1	60	7.8	5.2	7.4	4.3
	120	7.2	4.8	7.5	4.8

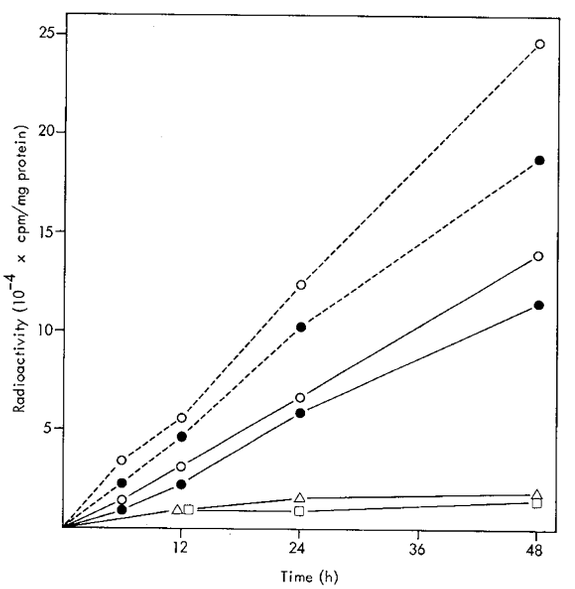

Figure 1:

Time-dependent incorporation of [^{35}S]sulfate into sulfated glycosaminoglycans.

(□) intracellular and (●) extracellular glycosaminoglycans of patient 1; (△) intracellular and (○) extracellular glycosaminoglycans of a normal control; dashed lines represent incorporation in the presence of 1 mM p-nitrophenyl-β-D-xyloside.

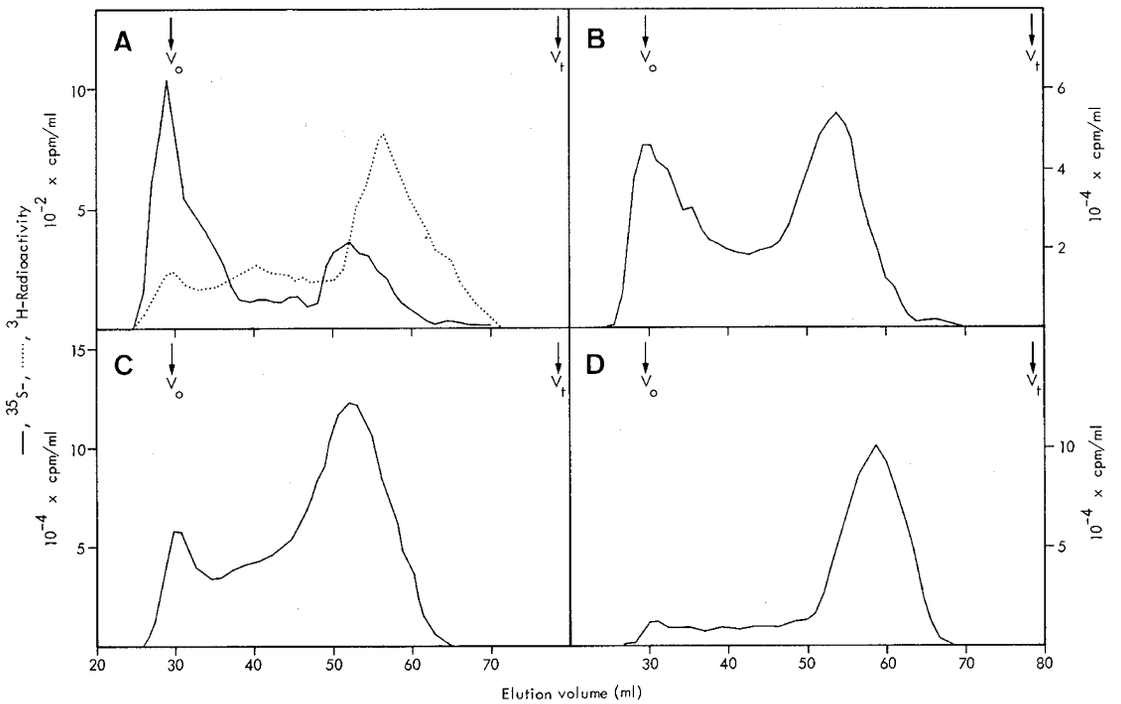

Figure 2:

Gel chromatography on Sepharose CL-4B of [^{35}S]proteoglycans (solid lines) from patient 1 (A), 2 (B) and 3 (C) and from a normal control (D).

Proteoglycans from patient 1 were cochromatographed with a similar quantity of ^{3}H-labeled proteoglycans (dashed line) from a normal control.

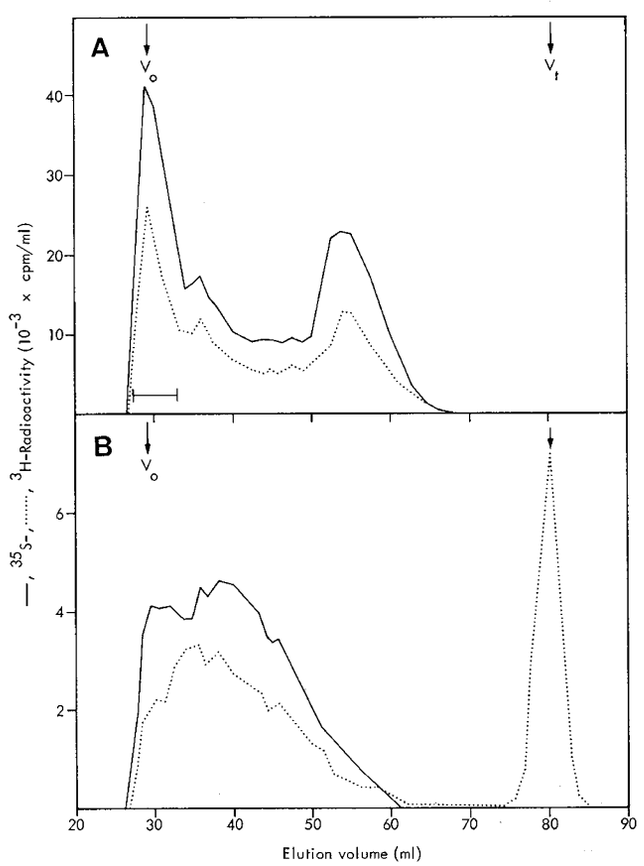

Figure 3:

Gel chromatography on Sepharose CL-4B of ^{35}S- and ^{3}H-labeled proteoglycans from patient 1 (A).

Fractions indicated by the bar were digested with hyaluronate lyase and rechormatographed (B).

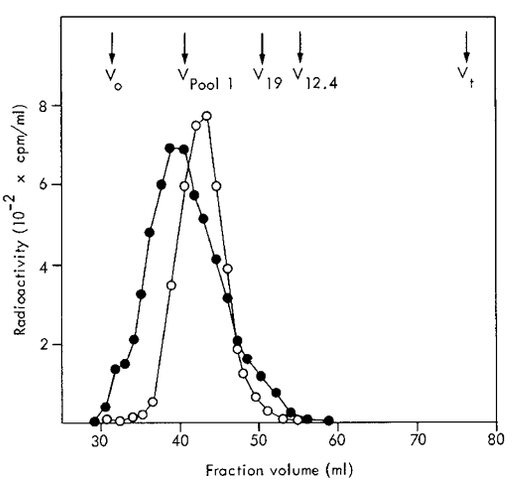

Figure 4:

Gel chromatography on Sephacryl S-300 of [^{35}S]glycosaminoglycan chains.

(●) pool 2 glycosaminoglycans from patient 1 (those from patient 2 behaved similarly); (○) pool 2 glycosaminoglycans from a normal control (those from patient 3 behaved similarly). The arrows denote the elution maxima of pool 1 glycosaminoglycans (patients and control) and of glycosaminoglycans with M_r = 19,000 and M_r = 12,400, respectively.

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