

Natural History of Alcoholic Hepatitis

IV. GLYCOSAMINOGLYCURONANS AND COLLAGEN IN THE HEPATIC CONNECTIVE TISSUE

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ABSTRACT The extractable and nonextractable collagen and glycosaminoglycuronans (GAG) were estimated and characterized in 32 dried, defatted human livers obtained at necropsy. 10 had normal livers. 22 of the 32 livers were from patients who drank in excess: 5 had fatty livers, 7 had alcoholic hepatitis, and 10 had cirrhosis. Livers with alcoholic hepatitis or cirrhosis had significantly increased total and 1 N NaCl-extractable collagen. Only alcoholic hepatitis livers had significantly increased Tris-buffer-extractable GAG, but the amino acid composition of these GAG (proteoglycans) was no different from that of normal livers. The major fraction of these GAG had isoelectric pH (pI) ≤ 3.1 in all livers. Livers with alcoholic hepatitis or cirrhosis had significantly increased nonextractable GAG. The major GAG fraction of all livers was chondroitin-4 or -6-SO₄. Alcoholic hepatitis livers had a significant increase of hyaluronic acid and an unidentified hyaluronidase-resistant GAG. Fatty livers showed no differences from normal ones.

The data indicates that alcoholic hepatitis is associated with a significantly increased fibroblast activity, but fatty livers of alcoholics are not. The changes in histologically "inactive" micronodular cirrhosis of alcoholic patients indicate continued activity of fibroblasts in the connective tissue of these cirrhotic livers.

INTRODUCTION

The pathogenesis of cirrhosis in alcoholics was thought to have been related to fatty changes in the liver, but recently alcoholic hepatitis has been recognized as a precursor of cirrhosis (1). Only a proportion of ex-

tensive drinkers develop cirrhosis. The reason for the progression of ethanol-induced liver injury to cirrhosis may be related to the response of hepatic fibroblasts to injury (2). This suggestion is supported by the demonstrated differences of growth characteristics of hepatic fibroblasts in tissue culture grown from biopsies of alcoholic hepatitis livers as opposed to normal or fatty livers (3). Recent observations suggest that the development of cirrhosis may be related to direct induction of hepatic fibroblasts by ethanol (4) or to their induction by ethanol-induced parenchymal injury. Increased activity of hepatic fibroblasts is manifested by formation of collagen fibers and extracellular ground substance. An intimate relationship between glycosaminoglycuronans (GAG)¹ of the ground substance and fiber formation has been described (5, 6). The induced fibroblast may produce excessive amounts of GAG, which may or may not be hydrolyzed by lysosomal hyaluronidase (5, 7). The present study was undertaken to determine the collagen and GAG concentrations and compositions in normal, fatty, alcoholic hepatitis, and cirrhotic livers.

METHODS

Livers were obtained at autopsy from 10 nonalcoholic patients who had no liver disease and from 22 alcoholic patients with 3 types of liver injury: 5 had fatty liver, 7 had alcoholic hepatitis and 10 had cirrhosis. Patients with fatty liver died from causes unrelated to liver disease: three with trauma, one with pneumonia, and one with alcoholic delirium. Patients with alcoholic hepatitis had progressive and severe parenchymal hepatic failure. Patients with cirrhosis died of complications of their disease: three had

¹ *Abbreviations used in this paper:* CPC, cetylpyridinium chloride; DDL, dried defatted human liver powder; GAG, glycosaminoglycuronans; HO-Pr, hydroxyproline; pI, isoelectric pH; PMN, polymorphonuclear.

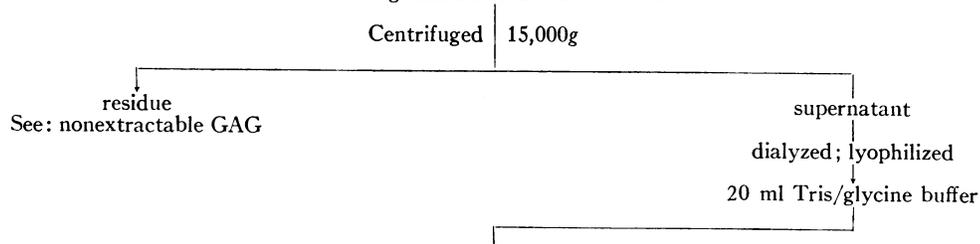
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gastrointestinal bleeding, three with hepatorenal syndrome, two with infection, one with coma, and one with trauma. Autopsies were performed within 1–5 h after death. There was no relationship between the delay of autopsy and the four diagnostic categories. Livers were fixed in 4% formaldehyde and histologic examinations were done on hematoxylin-eosin, Mallory's aniline blue, alcian blue-periodic acid-Schiff, and van Gieson-stained sections. The case was excluded from the study if postmortem autolysis was seen. The following diagnostic criteria were used: normal, either no histopathology or only passive congestion was seen; steatosis, $\geq 50\%$ of hepatocytes contained fatty vacuoles and only minimal or no necrosis or inflammation was seen; alcoholic hepatitis, the morphologic criteria for the diagnosis were previously described in detail (8). Briefly, the diagnosis of alcoholic hepatitis required portal edema and inflammation with invasion of the limiting plate, a zonal hepatocellular necrosis involving at least zone 3 of the hepatic acinus of Rappaport (9) and a predominantly polymorphonuclear (PMN)-leukocytic inflammatory exudate. Because this type of alcoholic injury characteristically affected zone 3 of the hepatic acinus, degenerating, ballooned hepatocytes, parenchymal necrosis, and edema were most prominent around the efferent ("central") vein. This process resulted in compression of this vein, and often it could no longer be identified. The ballooned hepatocytes contained weblike cytoplasmic remnants and eosinophilic clumps of various shapes. In some liver, groups of these enlarged hepatocytes contained alcoholic hyalin but the typical hyalin was not seen in every liver. PMN leukocytes were usually seen within or around these enlarged, degenerating hepatocytes or in areas of necrosis. Steatosis was seen in each of the livers included in this study. The livers with alcoholic hepatitis contained intralobular and periportal fibrosis, but fibrous septums and nodular regeneration were not seen. Cirrhosis: septa and nodules were well developed but only minimal parenchymal necrosis and inflammation were present. Those livers containing both cirrhosis and significant alcoholic hepatitis were excluded from this study.

The livers were cut in about $\frac{1}{2}$ -cm thin slices, sectioned to 2–5-cm strips and put in acetone. The containers were agitated to prevent adherence of fragments. The strips of fixed liver tissue were meticulously dissected free from capsular, vascular, and other visible *extraneous* connective tissue. Care was taken *not* to remove fibrous septa of cirrhotic livers, but all visible blood vessels were stripped off the tissues. (These structures were removed and discarded because their GAG content could have contaminated hepatic GAG). The fragments of liver were homogenized and were repeatedly extracted with acetone, with acetone-ether, and with ether. The dried defatted liver powder (DDL) was

Extraction.

0.05 M Tris/0.38 M Glycine pH 8.2 100 ml/5 g DDL² stirred in VirTis³ for 30 min at 0° with magnetic stirrer for 18 h at 4°C



² The fibrous fragments of cirrhotic liver were included in the DDL.

³ VirTis Co., Inc., Gardiner, N. Y.

TABLE I

Diagnosis	Number of livers	DDL g
Normal*	10	210
Fatty liver‡	5	85
Alcoholic hepatitis	7	270
Cirrhosis	10	220

* 6 of the 10 had passive congestion due to heart failure.

‡ Over 50% of hepatocytes of these chronic alcoholics were distended with fat.

The diagnosis of alcoholic hepatitis or cirrhosis was made according to the morphologic criteria described previously (8). Two of the livers with alcoholic hepatitis had early septum formation and five had intralobular fibrosis on histologic examination.

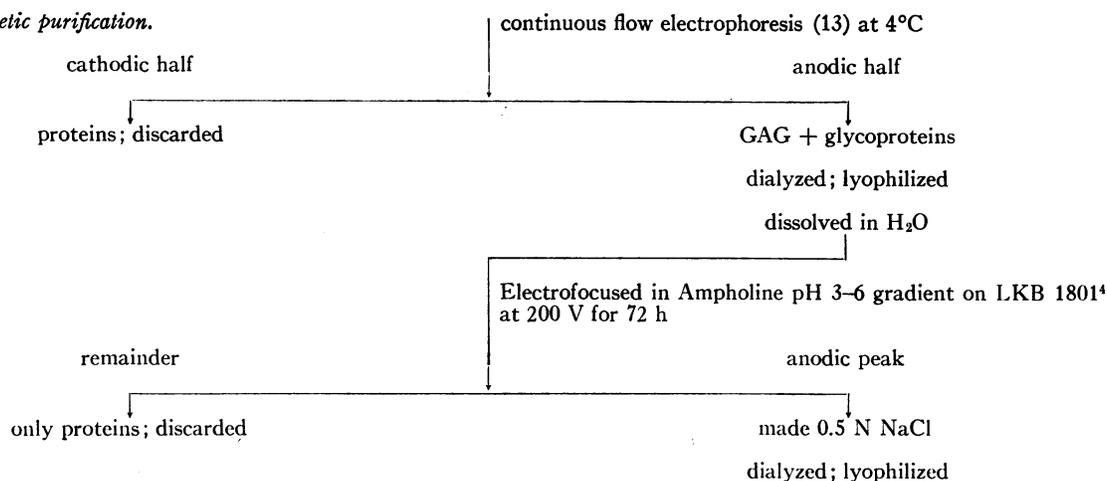
stored in sealed plastic bags in a desiccator at 4°C until it was used.

Hepatic collagen. 10 mg of the DDL was hydrolyzed in vacuum-sealed vials in 6 N HCl at 120°C for 18 h for determination of total hydroxyproline (HO-Pr); 100 mg of liver powder was used for the determination of 1 N NaCl-extractable HO-Pr (10–12).

GAG. The number of cases in each diagnostic category and the total amount of DDL used in each are listed in Table I. At least 20 g of DDL were used from each normal and cirrhotic liver, 15 or 20 g of DDL from each fatty liver, and 30 or 40 g of DDL from each alcoholic hepatitis liver.

Extractable GAG and proteoglycans. Because the native state of GAG in connective tissue is probably protein-polysaccharide macromolecules (proteoglycans), gentle extraction procedures were used to keep their degradation to a minimum. Proteoglycans are one of the largest negatively charged macromolecules in mammalian tissue; therefore, their isolation was based on these two properties: low isoelectric point (pI) and large size. In the liver the concentration of proteoglycans and GAG is low and these are extracted with much larger amounts of proteins and glycoproteins. Complex and prolonged procedures were required to separate the proteoglycans from contaminating glycoproteins. All procedures were done on replicates.

Electrophoretic purification.

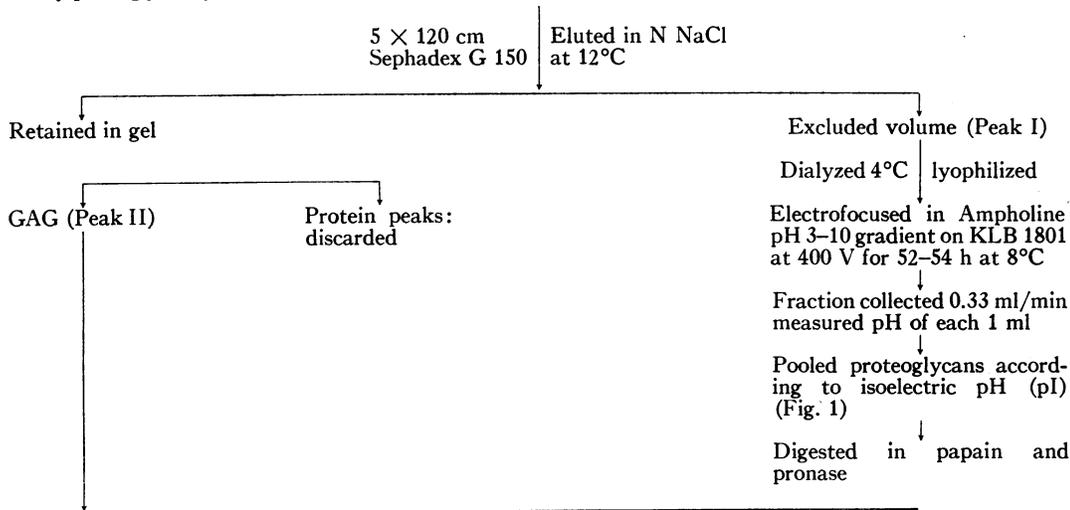


Estimation of total extractable GAG/5 or 10 g DDL from each liver.

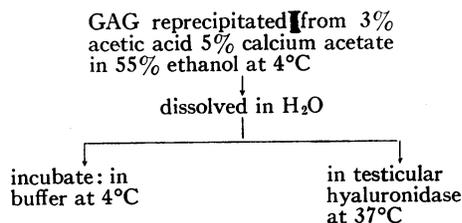
Dissolved in 5 ml H₂O and hexuronic acids were measured by the modified carbazole reaction (14)

$\frac{1}{3}$ of each were pooled according to the four diagnostic categories and were lyophilized.

Separation of proteoglycans from GAG.



Characterization of extractable GAG according to Calatromi and diFerrante (15).



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0.9 × 7.0 cm Dowex 1 × 8
(Fig. 2)

Eluted with increasing NaCl concentrations, each fraction was precipitated in 70% ethanol dissolved in water, and GAG were estimated by the carbazole reaction (14).

Recoveries. Because of contaminating proteins, the carbazole reaction (14) did not give reliable values on the original extracts but did so after the initial electrophoretic purifications. 80–105% of “total absorbency” of the carbazole color was recovered from Sephadex G-150 chromatography and 88–109% after electrofocusing. The recovery averaged 74% when purified proteoglycans (16, 17) from

bovine nasal cartilage were carried through the procedure. *The amino acid* composition of the proteoglycans was determined by the method of Malawista and Schubert (16), as modified by Heinegard and Gardell (17). Pooled representative samples were assayed from fractions excluded from Sephadex G-150 (Peak I) and had pI ≤ 3.1 from normal and from alcoholic hepatitis livers.

Nonextractable GAG

Preliminary purification. Residues of Tris/glycine extracts were digested by papain, were incubated at 4°C in 0.5 N NaOH in 0.05 N NaBH₄ (18), and were precipitated in 10% trichloroacetic acid

Precipitate, discarded

Supernate and washes were neutralized, dialyzed at 4°C, and eluted with H₂O from 2.5 × 20 cm Dowex 50-X8(H⁺), were dialyzed at 4°C, lyophilized.

Estimation of total nonextractable GAG/10 g DDL from each liver.

Dissolved in 10 ml H₂O, and hexuronic acids were measured by the modified carbazole reaction (14).

The remaining 9.5 ml was made 3% acetic acid, 5% calcium acetate; GAG were fractionated by precipitations in 40% and 55% ethanol at 4°C according to Meyer, Linker, Davidson, and Weissmann (19).

Soluble in 40% ethanol
ppt. in 55% ethanol—Fraction G (Fig. 3)

ppt. in 40% ethanol
dissolved in 0.035 N Na₂SO₄
ppt. with cetylpyridinium chloride (CPC)
1 × 22 cm column of acid-washed celite⁵
washed in 0.04 N NaCl in 0.1% CPC until washes did not absorb at 210 nm
Eluted in 0.35 N NaCl 0.1% CPC—Fraction A
Eluted in 0.6 N NaCl 0.1% CPC—Fraction B
Dissociate CPC-GAG with ethanol
Eluted in 430 ml logarithmic gradient of 58% ethanol and aqueous 3% acetic acid—5% calcium acetate at 20 ml/h at 12°C
Ethanol concentrations (vol/vol):
58%–38%—Fraction C
38%–28%—Fraction D
28%–20%—Fraction E
Eluted in aqueous 3% acetic acid 5% calcium acetate.—Fraction F

⁵ Mesh 120/140 (Gas-Chrom-P) Applied Science Labs., Inc., State College, Pa.

After Fraction F further elution of the columns with 2 N NaOH did not remove any additional carbozole-reacting material. Replicate analyses from the same liver were within $\pm 10\%$ of the average estimates of hexuronic acids.

Recovery of GAG in Fractions A-F

Hyaluronic acid. 91-96% was in the 0.35 N NaCl elution of the CPC precipitate (Fraction A).

Heparan-SO₄. 86-95% was in the 0.6 N NaCl elution of the CPC precipitate (Fraction B).

Chondroitin-6-SO₄ (shark). 81-90% was in the 58%-38% ethanol gradient (Fraction C), but none of chondroitin-4-SO₄ (bovine cartilage) was removed by this fraction.

Chondroitin-4-SO₄. 25-40% was in the 38%-28% ethanol gradient (Fraction D) and 60%-75% in 28%-20% ethanol gradient (Fraction E).

Dermatan-SO₄. 92-100% was in the aqueous 3% acetic acid 5% calcium acetate (Fraction F). None was removed in the ethanol gradient.

The replicates of each liver were within $\pm 10\%$ of the average.

Characterization of GAG in Fractions A-G

Each fraction was analyzed for hexuronic acid, hexosamines, SO₄, hexose, sialic acid, and fucose and for susceptibility to testicular hyaluronidase. The range of molar ratios of hexosamines and SO₄ varied $\pm 14\%$ among the four diagnostic groups. Duplicates were within $\pm 10\%$. Assays for SO₄ and hyaluronidase digestion were not performed on the following fractions because sufficient amount of material was not available for analysis: normal C and E; fatty liver A, B, F, and G.

The GAG fractions A through G were pooled according to the four diagnostic categories. Duplicate samples of each were incubated in buffer at 4°C or with testicular hyaluronidase (Wydase, Wyeth Laboratories, Inc., Div. of American Home Products, Inc., Philadelphia) at 37°C and were chromatographed on 0.9 × 7.0 Dowex 1 × 2 columns according to Calatroni and diFerrante (15).

The recovery of chondroitin-4 and -6-SO₄ and of a mixture of hyaluronic acid, heparin-SO₄, and dermatan-SO₄ on Dowex 1 × 2 columns ranged from 89% to 97%.

Electrophoresis. Samples were divided in two: one was incubated with hyaluronidase and the other in buffer. Both were precipitated in 71% ethanol. The precipitates were dissolved in 20 μ l water and applied to cellulose acetate membrane. Electrophoresis was performed in a Beckman microzonal apparatus (Beckman Instruments, Inc., Electronic Instruments Div., Schiller Park, Ill.) in 0.6 M acetate-formate pH 2.55 buffer at 5 mA for 30 min. The membranes were stained for 8 min with 1% alcian blue in 1:1 ethanol/acetate (pH 5.8, I = 0.05).

Analytical procedures

Dialysis was performed in cellophane bags prepared according to Korn (20) by being heated in a dry oven at 65°C for 3 days. Hexuronic acid was determined by Galambos modification (14) of the carbozole reaction. The presence of sulfamate in this modification (14) permitted the reliable estimation of hexuronic acid in proteoglycans that produced considerable yellow-brown discoloration after the addition of carbozole in the absence of sulfamate. Even in the presence of sulfamate, slight orange discoloration developed in addition to the typical carbozole color in proteoglycan fractions with $pI > 3.1$. In these fractions hex-

uronic acid was probably overestimated. Hexose was estimated by the anthrone procedure with galactose as standard (21), fucose according to Tsiganos and Muir (22), sialic acid by the resorcinol method (23), protein according to Lowry, Rosebrough, Farr, and Randall (24), and sulfate according to Muir (25). Hexosamines were determined after hydrolysis in 2.5 N HCl at 100°C in vacuum-sealed ampules for 20 h (these conditions gave maximum recoveries). The tubes were dried three times in vacuum and the glucosamine and galactosamine were determined on a Beckman 120C automatic amino acid analyzer.

The amino acids were determined after hydrolysis in 6 N HCl for 24 h at 100°C in sealed tubes from which oxygen was removed to reduce excessive destruction of amino acids in the presence of carbohydrates. The tubes were dried in a vacuum three times and the amino acids were measured on a Beckman-120C automatic amino acid analyzer. No correction was made for the presence of carbohydrates.

Criteria for identification

Hyaluronic acid. (a) CPC precipitate was soluble in 0.35 N NaCl, (b) glucosamine was the amino sugar, (c) fraction contained no SO₄, (d) elution pattern from Dowex 1 × 2 resin and electrophoretic mobility were similar to that of commercial hyaluronic acid, and (e) it was susceptible to hydrolysis with testicular hyaluronidase. (Hyaluronic acid standards were from Nutritional Biochemicals Co., Cleveland, Ohio.)

Heparan SO₄. (a) CPC precipitate was soluble in 0.6 N NaCl, (b) glucosamine was the amino sugar, (c) the fraction contained SO₄, (d) elution pattern from Dowex 1 × 2 resin and electrophoretic mobility were similar to those of purified heparan SO₄, (e) the fraction resisted hydrolysis with testicular hyaluronidase. (Heparan SO₄ standard was isolated from crude commercial product according to Linker and Hovingh [26]).

Chondroitin-6 and -4-SO₄. (a) were not removed from their CPC precipitate with 0.6 N NaCl, (b) galactosamine was their amino sugar, (c) they contained SO₄, (d) the elution pattern from Dowex 1 × 2 resin and electrophoretic mobility were the same as those of shark and bovine chondroitin-SO₄s, (e) they were susceptible to testicular hyaluronidase hydrolysis. It was assumed that the chondroitin-SO₄ soluble in high concentrations of ethanol is predominantly chondroitin-6-SO₄, and that having low ethanol solubility is predominantly chondroitin-4-SO₄.

Dermatan SO₄. (a) was not removed from its CPC precipitate by 0.6 N NaCl, (b) galactosamine was the amino sugar, (c) the precipitate contained SO₄, (d) the elution pattern from Dowex 1 × 2 resin and electrophoretic mobility were similar to those of dermatan SO₄, (e) the precipitate resisted hydrolysis by testicular hyaluronidase, and (f) was not soluble in 20% ethanol at 12°C. (Dermatan SO₄ standard was a gift from Roger W. Jeanloz.)

On histologic sections collagen and alcian blue-reactive GAG were graded on 0-4. The histologic gradings were correlated with biochemical estimates of collagen and GAG respectively.

RESULTS

Collagen

The total and 1 N NaCl-extractable collagen concentrations are described in Table II. Compared to normal

TABLE II
Collagen Content of Normal Liver and in
Alcoholic Liver Disease

	<i>n</i>	Total collagen‡	N NaCl-extractable collagen
		<i>mg HO-Pr/g POL</i>	
Normal	10	6.9*±0.37	0.13±0.06
Fatty liver	5	7.2±1.5	0.4±0.12
Alcoholic hepatitis	7	28.1±3.1	2.01±0.39
Cirrhosis‡	10	27.4±2.8	1.09±0.21

The data presented in this Table and in Table III are means ±SE of each diagnostic group. The average estimate of replicate analyses per liver was used to compute the mean per group. The number of livers in each group and not the number of samples analyzed per liver was used as “*n*” and *df* in statistical computations.

n, number of livers per diagnostic category.

* Mean±SE of livers per diagnostic category.

‡ All visible fibrous scar tissue was excluded.

liver, total or neutral salt-soluble collagen concentration in fatty livers was not different ($P > 0.1$); however, both were increased in alcoholic hepatitis and in cirrhosis ($P < 0.001$). Total collagen concentration was the same in alcoholic hepatitis and cirrhosis, but the concentration of 1 N NaCl-extractable collagen in alcoholic hepatitis was almost twice that found in cirrhosis ($P = 0.06$).

GAG and proteoglycans

The total, extractable, and nonextractable GAG are described in Table III.

Extractable GAG (proteoglycans)

Fig. 1 describes the distribution of proteoglycans according to their *pI*. Duplicates were within ±10% of the average. The distribution pattern (percent of total in each fraction) of proteoglycans was similar in normal liver, fatty liver, and cirrhosis. The two fractions with *pI*s between 2 and 3.1 accounted for most of the increase of proteoglycans in livers with alcoholic hepatitis (Fig. 1, Table IV, $P < 0.01$). The proteoglycan content of cirrhotic livers in fractions with $pI \leq 3.1$ appeared to be intermediate between that of alcoholic hepatitis and normal or fatty livers (Fig. 1). The data are insufficient to define precisely the probability of chance occurrence of the observations. As the *pI* increased above 3.1, in each fraction both the protein and the hexosamine-to-hexuronic acid ratios increased, and the hexosamines consisted exclusively of glucosamine (Table IV). These changes were due to increasing proportions of glycoproteins and decreasing glucosaminoglycuronans in fractions with increasing *pI*. Further analysis of these com-

TABLE III
Extractable (Proteoglycans) and Nonextractable
GAG Hexuronic Acid

	Normal (<i>n</i> -10)	Alcoholic			
		Fatty (<i>n</i> -5)	Hepatitis (<i>n</i> -7)	Cirrhosis (<i>n</i> -10)	
		<i>μg/10 g DDL</i>			
Extractable	243*±55	178±68	744§±151	310±89	
Nonextractable	325±61	273±83	492‡±47	713‡±160	
Sum of means	568	441	1236	1023	

The observed difference of extractable GAG between alcoholic hepatitis and cirrhosis was due to chance: $P < 0.05$, and that of nonextractable GAG: $P = 0.19$. *n*, number of livers per diagnostic category. See Note from Table II.

* Mean±SE of livers per diagnostic category.

‡ $P < 0.05$.

§ $P < 0.01$.

pounds has not been undertaken because sufficient material was not available for their identification. Furthermore, even the modified carbozole reaction (14) probably overestimated the hexuronic acid content in fractions with $pI > 3.1$. No hexuronic acid was detectable in fractions with $pI > 4.4$.

The proteoglycans with $pI \leq 3.1$ contained neither sialic acid nor fucose.

GAG in proteoglycans

The elution pattern of GAG from Dowex 1 × 2 columns and the proportion of hyaluronidase-digestible GAG in each diagnostic category are illustrated in Fig. 2. The affinity to the resin for GAG from normal liver was lower (all were removed with 1.5 N NaCl) than those from fatty liver, alcoholic hepatitis, and cirrhosis, where all GAG were not removed even with 2 N NaCl. Each diagnostic category contained various proportions of the GAG removed at 0.1 N NaCl. These were small, dialyzable fragments of GAG precipitable in 70% etha-

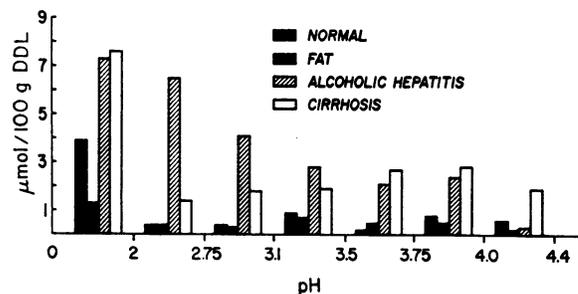


FIGURE 1 Extractable GAG (proteoglycans) (mean of replicates). The proteoglycans were electrophoresed in a continuous sucrose gradient in an electrofocusing column. After equilibrium was reached, the compounds were eluted according to their *pI*. The fractions with $pI > 3.1$ contained increasing proportions of glycoproteins.

TABLE IV
Distribution of Protein, Hexuronic Acid, Glucosamine in Extractable GAG (Proteoglycans)
Excluded from Sephadex G-150 (means of duplicates)

pI	Normal			Fatty			Alcoholic			Hepatitis			Cirrhosis		
	Protein	Hexu- ronic acid	Glucos- amine *	Protein	Hexu- ronic acid	Glucos- amine	Protein	Hexu- ronic acid	Glucos- amine	Protein	Hexu- ronic acid	Glucos- amine	Protein	Hexu- ronic acid	Glucos- amine
		$\mu\text{g}/\text{aliquot}$	%		$\mu\text{g}/\text{aliquot}$	%		$\mu\text{g}/\text{aliquot}$	%		$\mu\text{g}/\text{aliquot}$	%		$\mu\text{g}/\text{aliquot}$	%
<2	110	77	57‡	141	25	73‡	41	141	57‡	73	148	75‡	73	148	75‡
2-2.75	10	8	66‡	6	8	—	27	125	66‡	11	27	56‡	11	27	56‡
2.76-3.1	10	7	67	5	6	—	19	80	90	4	33	—	4	33	—
3.11-3.5	22	15	100	15	13	100	12	55	100	11	36	100	11	36	100
3.51-3.75	49	4	100	44	10	100	31	41	100	21	52	100	21	52	100
3.76-4.0	53	16	100	97	10	100	47	47	100	31	54	100	31	54	100
4.01-4.4	76	12	—	23	3	—	24	6	—	46	36	100	46	36	100

* Percent of total hexosamine as glucosamine.

‡ The hexuronic acid: hexosamine ratio was approximately unity in fractions $pI \leq 2.75$.

—, data are not available.

nol. These short GAG fragments represented in normal livers 55%, in fatty livers 15%, in alcoholic hepatitis 17%, and in cirrhosis 20% of total GAG. The composition of remaining GAG was in normal liver, 28% was heparan SO_4 and dermatan- SO_4 , and 17% was chondroitin-4 or -6- SO_4 ; in alcoholic fatty liver, 85% was chondroitin-4 or -6- SO_4 ; in alcoholic hepatitis, 27% was chondroitin-4 or -6- SO_4 , and 56% was heparan- SO_4 and dermatan- SO_4 ; and in cirrhosis, 80% was chondroitin-4 or -6- SO_4 .

The amino acid composition of pooled extractable proteoglycans with $pI \leq 3.1$ from normal livers was similar to that from alcoholic hepatitis. The amino acid composition of proteoglycans extracted from livers was similar to that of cartilage (27, 28). The most common amino acids were aspartic acid, glutamic acid, glycine, serine, and proline (Table V).

Peak II. A fraction of the extractable GAG was retained on Sephadex G-150 in 6 of 10 normal livers (average of 29%) and in 4 of 10 cirrhotic livers (average of 18%). The retention of extractable GAG on the Sephadex G-150 gel indicates smaller molecular size. The most likely explanation for Peak II is antemortem anoxia which probably reduced hepatic pH to levels where lysosomal enzymes hydrolyzed detectable amounts of GAG from proteoglycans (27, 28).

The GAG in Peak II from normal livers were dermatan- SO_4 and heparan- SO_4 , and from cirrhotic were chondroitin-4 or -6- SO_4 (52%), and dermatan- SO_4 (48%).

Nonextractable GAG

The isolated GAG contained up to 3% protein (assuming that hexuronic acid was 30% of molecular weight) but no trend was detectable in the GAG fractions or disease categories. Neither sialic acid nor fucose was detectable in any of the GAG fractions.

The total nonextractable GAG are described in Table III and Fig. 3. Both alcoholic hepatitis and cirrhosis contained more GAG than normal or fatty livers ($P < 0.05$). However, the GAG content in normal livers was similar to that in fatty livers, and in alcoholic hepatitis it was not different from that in cirrhosis ($P = 0.19$). The composition of each of the GAG fractions described

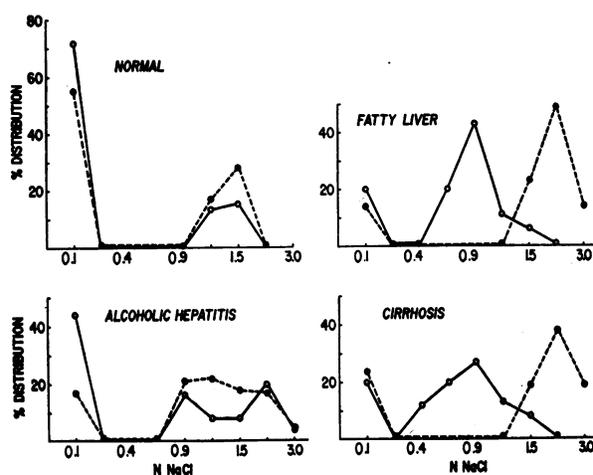


FIGURE 2 Extractable GAG, Mean of duplicates. The GAG were isolated from proteoglycans. Duplicates were eluted from 0.9×7 cm Dowex 1×2 columns with increasing NaCl concentrations (\bigcirc - - - \bigcirc). Duplicates were incubated with hyaluronidase and were chromatographed in the same manner. (\bigcirc - - \bigcirc) The hyaluronidase-susceptible GAG were hydrolyzed and the smaller fragments were eluted by lower NaCl concentrations. Therefore, a shift to the left indicates hydrolyzed GAG.

in Fig. 3 was not affected by diagnostic categories. (The variations between diagnostic categories of the same GAG fraction were not significantly greater than variations of duplicates of the fraction of the same diagnostic category.)

Fraction A contained both hyaluronidase-susceptible and -resistant GAG. Hexosamine composition was 95% glucosamine and 5% galactosamine. On electrophoresis the major band was indistinguishable from hyaluronic acid. Two smaller bands accounted for approximately half of this fraction and both were resistant to hyaluronidase: one migrated with hyaluronic acid, the other one moved faster and was indistinguishable from heparan-SO₄. One or both of these unknown fractions contained SO₄.

Fraction B consisted of two-thirds heparan-SO₄, and one-third was susceptible to hyaluronidase digestion. Approximately 88% of the latter was hyaluronic acid and approximately 12% of this fraction was galactosaminoglycuronan, such as chondroitin or chondroitin-SO₄.

Fractions C, D and E were chondroitin-6 and -4-SO₄.

Fraction F was mostly dermatan-SO₄; less than a fourth was hyaluronidase-susceptible chondroitin-4-SO₄.

Fraction G was chondroitin-6-SO₄ and probably contained keratan-SO₄, but the latter was not further identified.

Normal. The dominant GAG were chondroitin-SO₄, mostly chondroitin-6-SO₄. The dermatan-SO₄ and heparan-SO₄ contents of normal livers was not significantly different from those of livers with alcoholic liver damage.

Alcoholic fatty liver. Most of the GAG were chondroitin-SO₄s.

Alcoholic hepatitis liver. It contained more hyaluronic acid (Fraction A) than normal, fatty, or cirrhotic livers ($P < 0.01$). The dominant GAG were chondroitin-SO₄s.

Cirrhosis. Over 81% of GAG was in Fraction D and over 91% of GAG were chondroitin-SO₄s.

Histologic examinations showed many more fibers in groups of livers with high collagen content (alcoholic hepatitis and cirrhotic) than in normal or fatty livers, confirming previous observations (29). However, no correlation was demonstrable between chemically measured collagen concentration and histological estimates of fibrosis within the high collagen groups (alcoholic hepatitis or cirrhosis) or within the low collagen (fatty or normal) livers. Similarly, histologic estimates of GAG clearly separated the groups of livers with high GAG content (alcoholic hepatitis and cirrhosis) from normal and fatty livers. There was no definite correlation between alcian blue-reactive ground substance and chemically estimated GAG within the alcoholic hepatitis and cirrhosis groups. In normal or fatty livers alcian blue did not stain extracellular GAG outside blood vessel walls.

TABLE V
Amino Acid Composition of Proteoglycans

	Range*
	μmol/100 μmol
Aspartic	105-129
Threonine	52-65
Serine	127-147
Glutamic acid	155-179
Proline	71-79
Glycine	140-149
Alanine	52-61
Valine	54-66
Leucine	41-54

* The range of duplicate analyses of the nine most common amino acids from normal and from alcoholic hepatitis livers.

DISCUSSION

The acetone-ether treatment of each noncirrhotic liver yielded a homogeneous powdery material. Cirrhotic livers, however, had readily recognizable tough fibrous fragments, presumably scar tissue, mixed with the homogeneous but granular DDL. Although intralobular and periportal fibrosis were seen in alcoholic hepatitis on histologic examinations, even in these livers the DDL was homogeneous and powdery. It is reasonable to assume that the homogeneous DDL represented hepatic parenchyma with its connective tissue framework.

The HO-Pr concentrations of selected fibrous fragments from cirrhotic livers were two-to-threefold higher than that of the homogeneous DDL from which visible fibrous pieces were removed. The HO-Pr concentrations of these fibrous scar fragments were between 4%

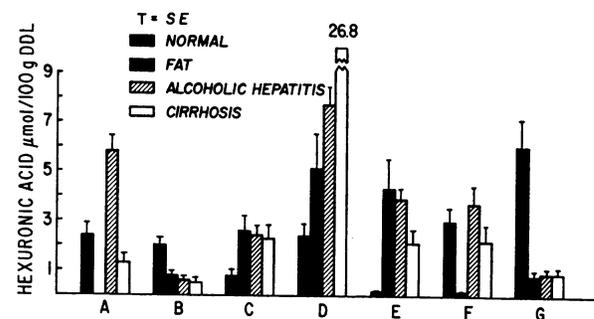


FIGURE 3 Nonextractable GAG, Mean \pm SE per diagnostic category. (Number of cases per category: see Table III.) The composition of each fraction is described in the text. The dominant GAG in each fraction were: Fraction A, hyaluronic acid; Fraction B, heparan-SO₄; Fraction C, chondroitin-6-SO₄; Fraction D, chondroitin-6 and -4-SO₄; Fraction E, chondroitin-4-SO₄; Fraction F, dermatan-SO₄ and chondroitin-4-SO₄; and Fraction G, chondroitin-6-SO₄ (contained also keratan-SO₄).

and 9%, as compared to 13.6% found in our collagen standard. Because inadvertent and arbitrary variations of the proportions of these fibrous fragments in DDL would have significantly affected the apparent collagen concentrations of cirrhotic livers, all visible fibrous fragments were removed from DDL before HO-Pr contents were estimated. Consequently, only the homogeneous, granular DDL was studied in cirrhotic livers. This probably represented the connective tissue of regenerative nodules and those portions of the septa easily fragmented during homogenization in acetone.

Our observations are in accord with those of Kent, Fels, Dubin, and Popper (29), who found that the total HO-Pr concentration in cirrhotic livers was four times that of normal livers and that the collagen content of livers with alcoholic hepatitis^a was similar to that of Laennec's cirrhosis.

The insoluble and soluble collagen contents of normal livers were not significantly different from that of alcoholic fatty livers; however, the content of both types of collagen was significantly greater in alcoholic hepatitis and in cirrhosis. Our data do not provide kinetic information about the turnover rate of hepatic collagen. Nevertheless, it is generally accepted that neutral salt-soluble collagen is composed of young, recently synthesized fibrils (30). The large increase of young collagen content in alcoholic hepatitis and alcoholic cirrhosis is compatible with active fibrillogenesis.

The large increase of extractable GAG macromolecules and the increase of soluble collagen content in alcoholic hepatitis but not in fatty livers support the suggestion of increased fibroblast activity in alcoholic hepatitis. These data provide important support to clinical observations that suggested that alcoholic hepatitis is, but fatty liver is not, a stage in the pathogenesis of alcoholic liver disease to cirrhosis (31).

For GAG analyses over 1,000-fold larger amounts of DDL were used than for the estimation of collagen content. In the study of hepatic GAG content, therefore, a significant error was avoided by not removing visible fibrous fragments from cirrhotic DDL, as either the entire specimen or a very large proportion of the liver powder was used. Therefore the original proportions of liver powder and scar tissue were maintained in each specimen.

However, all visible vascular tissue was removed from each liver because of its high content of chondroitin-SO₄s and heparan-SO₄ which could have significantly affected the apparent extravascular hepatic GAG.

^aThe term "alcoholic hepatitis" was introduced in 1961. In the 1950's a variety of terms were used to describe this lesion. These authors (29) called alcoholic hepatitis "early diffuse septal cirrhosis with fat."

The composition of increased GAG in the ground substance in alcoholic hepatitis shows similarities to that found in early scar formation. It has been shown that hyaluronic acid concentration increases rapidly in the ground substance during the early stage of wound healing (32). The increase of hyaluronic acid both in the extractable and the nonextractable GAG of alcoholic hepatitis is responsible in part for the increased GAG content in these livers. Hyaluronic acid accounted for about 75% of the increased proteoglycans with $pI > 2$, < 3.1 (Fig. 1, Table IV), and of 50% of the increased Fraction A in the nonextractable GAG (Fig. 3).

The most common GAG in the cirrhotic liver were the chondroitin-SO₄s, which accounted for over 90% of the nonextractable molecules. In the cirrhotic liver most of the GAG was bound firmly to insoluble collagen fibers, in contrast to that found in alcoholic hepatitis. This indicates a more mature fibrous organization in the cirrhotic liver.

The statistical significance of the increase of extractable GAG with $pI < 2$ in cirrhotic livers over that in fatty or normal livers could not be documented because of the unavailability of enough material to permit the estimation of these compounds in individual livers to provide adequate data for statistical analysis. Nevertheless, the extractable GAG in cirrhotic livers with $pI < 2$ were similar to that of alcoholic hepatitis (Fig. 1).

Proteoglycans are the native state of GAG in connective tissue. These compounds were shown to be essential for the formation of collagen fibrils from newly secreted tropocollagen and also for the organization of mature collagen fibers. Fiber formation seems to be accelerated by large aggregates of proteoglycans. These aggregates consist of complexes of proteoglycans and glycoproteins. There seems to be little doubt that the proteoglycans function as extracellular assembly and architecture of collagen during fibrogenesis in vivo (33, 6). On the basis of physicochemical and electron-microscopic observation it seems probable that GAG are firmly bound to collagen in fibrous connective tissues (34-36).

The amino acid composition of the protein moiety and the carbohydrate chains of proteoglycans in the liver were similar to that found in other tissues. It is reasonable to assume that the process of scar formation and fibrosis in the liver is not unique but is similar to that found elsewhere in the body. Therefore, it is likely that the GAG in the hepatic connective tissue also play an important role in the rate of formation, orientation, and cross-linking of collagen fibrils, and promote the formation of fibrous collagen bundles. In that case it is probable that the GAG of the ground substance could play a deciding role in the conversion

of the early and apparently reversible stage of fiber formation into the mature and apparently irreversible stage of hepatic fibrosis.

It has been shown that the hepatic lysosomes contain a hydrolytic enzyme that has substrate specificity similar to testicular hyaluronidase (7). It was suggested that the increase of GAG in cirrhotic livers is due to the accumulation of hepatic hyaluronidase-resistant GAG such as dermatan-SO₄ (37). However, in alcoholic hepatitis and in cirrhosis, the increased GAG were not due to hyaluronidase-resistant GAG. Furthermore, even normal livers had high dermatan-SO₄ and heparan-SO₄ content, as was also noted by Delbruck (38). We could not demonstrate an increase of these GAG in cirrhosis. It is probable that the accumulation of large amounts of GAG in alcoholic hepatitis and cirrhosis was due to increased rate of production rather than decreased rate of destruction of GAG. In order to confirm this suggestion, additional studies are required to directly measure the kinetics of GAG metabolism or estimate hyaluronidase-like activity in the immediate vicinity of GAG in hepatic connective tissue. These studies will require patients, because in experimental alcoholic or toxic liver injury, neither alcoholic hepatitis nor increased accumulation of GAG were demonstrable.

The finding of increased content of both young collagen and GAG suggests persistence of fibroblast activity in cirrhotic livers. Yet these livers showed only minimal parenchymal necrosis and no PMN-leukocytic inflammatory exudate on hematoxylin-eosin-stained sections. Continued active fibrosis is compatible with the observations that demonstrated the transformation of the micronodular (Laennec's) cirrhosis to a macronodular (postnecrotic-type) cirrhosis (39, 40). This type of transformation was observed in those cirrhotic patients who stopped drinking and in whom alcoholic hepatitis subsided. Indeed, serial biopsies in patients with alcoholic hepatitis showed the development of cirrhosis even in those who stopped drinking and in whom the acute alcoholic hepatitis subsided (31).

It seems reasonable to postulate that some unknown mechanism can "induce" hepatic fibroblasts during alcoholic hepatitis. These affected fibroblasts may continue to remain active even after the injurious agent, i.e. ethanol, has been withdrawn and active parenchymal necrosis and PMN-leukocytic inflammation have subsided. The persistence of hepatic fibroblast activity was associated with ductular proliferation and with the prominence of mononuclear cells (31). Further observations are required to document whether one or more of the mononuclear cell types is responsible for continued activity of hepatic fibroblasts after the acute parenchymal injury.

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