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### Research Article

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# Collagen Polymorphism in Normal and Cirrhotic Human Liver

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**ABSTRACT** Collagens in normal human liver and in alcoholic cirrhotic liver were investigated. Collagens were solubilized by limited proteolysis with pepsin under nondenaturing conditions, and after purification, were fractionated into types I and III by selective precipitation with NaCl. After carboxymethyl cellulose and agarose chromatography, the resulting  $\alpha$ -chains from each of the collagen types were analyzed with respect to their amino acid and carbohydrate compositions. A comparison of the results obtained from normal liver with those from the diseased organ revealed no significant differences.

The isolated human liver  $\alpha 1(I)$  and  $\alpha 1(III)$  chains were digested with CNBr and the generated peptides were separated and purified by a combination of ion-exchange and molecular sieve chromatography. The molecular weight and the amino acid and the carbohydrate compositions of each of the peptides were identical to those of the corresponding human skin peptides except for the slightly higher content of hydroxylysine in some of the peptides.

The relative content of type III in relation to type I collagen in both normal and cirrhotic liver was determined by digesting washed liver homogenates directly with CNBr and quantitating the resultant  $\alpha 1(I)$  and  $\alpha 1(III)$  peptides after chromatographic separation. The relative quantities of these peptides indicated that normal human liver contained an average of 47% type III, with the remainder being type I. Cirrhotic liver, on the other hand, contained a significantly smaller proportion of type III, ranging from 18 to 34% in different samples, with a corresponding increase in type I. These findings indicate that, although the amino acid and carbohydrate compositions of collagens deposited in cirrhotic liver are normal, the fibrotic process of alcoholic liver disease

in humans is accompanied by an alteration in tissue collagen polymorphism, and suggest that the observed alterations may have pathogenetic implications.

## INTRODUCTION

Recent discovery of the existence of several different types of collagen molecules in various connective tissues has made more cogent the questions concerning possible physiological and functional significance of tissue collagen polymorphism and its alterations in disease states. So far, at least four genetically distinct types of collagen have been described. The most abundant and best-characterized collagen, referred to as type I, is present in nearly all connective tissues including skin, bone, tendon, and aorta. Its molecular structure consists of two  $\alpha 1(I)$  chains and a single  $\alpha 2$  chain, or  $[\alpha 1(I)]_2\alpha 2$  (1, 2). Type III collagen, which has been isolated from human skin, aorta, leiomyoma, placenta, periodontium, liver, and lung, contains three identical  $\alpha 1(III)$  chains,  $[\alpha 1(III)]_3$  (3-10). The remaining two genetically distinct collagens also consist of three identical but distinct  $\alpha$ -chains per molecule, i.e.,  $[\alpha 1(II)]_3$  and  $[\alpha 1(IV)]_3$ . However, their occurrence is more restricted; type II collagen occurring only in cartilage (11, 12) and type IV in basement membranes such as renal glomeruli and anterior lens capsule (13, 14).

Although there are no data directly pertaining to the biologic and functional significance of the observed collagen polymorphism, wide variations in the relative distribution of type I and type III collagens in various tissues and organs suggest that collagen polymorphism may play an important role in modulating the properties of the parent tissues in various physiologic and pathologic states (5, 7-10, 15). Metabolism of collagen in liver in normal and diseased states has been the subject of several investigations. Thus, it has been reported that normal human liver contains less than 2% collagen but that

Dr. Kang is a Medical Investigator of the Veterans Administration.

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TABLE I  
Relative Amounts of Collagen and Protein Extracted  
from Normal and Cirrhotic Human Liver\*

Extractants	Normal		Fibrotic	
	Collagen†	Protein§	Collagen†	Protein§
0.05 M Tris HCl, pH 7.4 <sup>  </sup>	0.3	18.2	0.6	28.0
First Pepsin Digestion	8.1	10.4	23.6	24.6
Second Pepsin Digestion	23.1	5.7	39.5	16.8
Third Pepsin Digestion	15.8	3.4	22.3	15.3
Total	47.0	37.7	85.4	56.7

\* The results are expressed as percent of collagen or protein solubilized by each extractant.

† Collagen content based on hydroxyproline analysis.

§ Total protein content was determined from amino acid analysis of a portion of each extract and converted to protein assuming a mean residue weight of 100.

<sup>||</sup> Includes distilled water washes. See Methods.

the collagen content may be increased to nearly 10% in hepatic fibrosis such as alcoholic cirrhosis (16–18). Increased activities of proyl- and lysylhydroxylases as well as glucosyl- and galactosyltransferases have also been demonstrated in experimental liver injury after injection of carbon tetrachloride to rats (19, 20). However, the important questions concerning the structure of collagens in liver and collagen heterogeneity in liver cirrhosis have not been clarified. Recent studies employing fluorescein-labeled antibodies to collagen had indicated the presence of relatively large amounts of type III collagen in human cirrhotic liver (8, 9). These investigators, however, were unable to identify the remaining collagen in the liver, as it was not stainable by antibody against type I collagen. In a separate study, Rojkind and Martinez-Palomo (10) reported that normal human liver contained as much as 80% type I collagen. Cirrhotic liver on the other hand was reported to contain increased quantities of both type I and type III collagen, but their relative distributions were abnormal in that type III collagen was present in nearly equal amounts with type I (10).

In the present studies we have utilized a quantitative approach to investigate both type I and type III collagens of normal as well as cirrhotic liver. Type I and type III collagens were prepared from both normal and diseased liver. The CNBr-derived peptides of the two collagens from cirrhotic liver were isolated and characterized. In addition the relative distribution of these two major types of collagen in normal liver and their alteration in alcoholic cirrhosis were determined by direct CNBr digestion of total liver collagen and quantitation of two sets of specific CNBr peptides of each collagen type. Our results

indicate that the content of type I collagen is markedly increased whereas the relative content of type III is markedly diminished in cirrhotic liver. Amino acid and carbohydrate analyses of the isolated  $\alpha$ 1(I),  $\alpha$ 2, and  $\alpha$ 1(III) chains and the CNBr peptides, however, revealed no significant differences in the diseased organ as compared with normal.

## METHODS

**Liver.** Human livers were obtained from adult males (40–60 yr of age) at autopsy. Normal organs were from individuals who died of unrelated causes and fibrotic livers were taken from individuals who died of liver failure due to alcoholic portal cirrhosis. All diagnoses were based on thorough examination of clinical data by one of the authors (A. H. Kang) as well as postmortem histopathologic findings by Dr. J. M. Young, Chief, Pathology Service, Veterans Administration Hospital, Memphis, Tenn. All autopsy materials were obtained as soon after death as possible but always within 12 h. The capsule and the portal vessels were surgically removed when possible. The remaining liver was cut into pieces, ground in a mechanical meat grinder with chips of ice, and homogenized for 60 s with a Waring Blender at 4°C (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.). The homogenate was extracted five times with cold 0.05 M Tris, pH 7.4, to remove large amounts of soluble, noncollagenous substances and finally washed exhaustively with cold distilled water. The insoluble liver residue, which contained over 99% of the original collagen, as determined by hydroxyproline analysis (21), was collected by centrifugation and used directly for pepsin solubilization of collagen or CNBr cleavage.

**Extraction of collagen by limited pepsin digestion.** The washed liver residue was suspended in cold 0.5 M acetic acid. The pH of the suspension was adjusted to 2.5 by the addition of formic acid, and digested with pepsin as previously described (7). The extracted collagen was purified by 0.01 M  $\text{Na}_2\text{HPO}_4$  precipitation, 1 M NaCl precipitation from 0.5 M acetic acid solutions, and selective precipitation of type III and type I collagen at 1.5 and 2.5 M NaCl, respectively (7).

**Cleavage with cyanogen bromide.** The washed liver homogenates were used directly for CNBr cleavage of total liver collagen. 0.5–1.0-g samples were suspended in 50–100 ml of 70% formic acid and digested with CNBr at 40°C. Purified collagen  $\alpha$ -chains were digested in an identical manner except that 50–100-mg samples were dissolved in 20 ml of 70% formic acid (7).

**Ion-exchange chromatography.** Separation of the  $\alpha$ -chains from pepsin-solubilized collagen was achieved by chromatography of heat-denatured collagen on a 2.5 × 10-cm column of carboxymethyl (CM)-cellulose (Whatman CM-32) at 43°C (1, 7). Samples were dissolved in starting buffer (0.02 M sodium acetate/1 M urea, pH 4.8), denatured by warming the solution at 43°C for 30 min, and applied to the column. Elution was performed using a linear gradient of NaCl from 0 to 0.1 M over a total volume of 1,200 ml at a flow rate of 200 ml per h.

The peptides obtained by CNBr digestion were chromatographed on a 0.9 × 15-cm column of CM-cellulose at 43°C (7, 22). Peptides from total liver digests (100 mg), or the isolated collagen chains (25–50 mg) were separated with a linear gradient formed between 250 ml each of starting buffer (0.02 N sodium citrate, pH 3.8) and limiting buffer (0.02 N sodium citrate/0.16 M NaCl, pH 3.8) (7). The peptides

obtained from the initial CM-cellulose chromatography at pH 3.8, were further purified by chromatography either on CM-cellulose at pH 4.8, phosphocellulose or gel filtration (7).

**Molecular sieve chromatography.** Separation and molecular weight determinations of denatured collagen chains and CNBr peptides were achieved on  $2 \times 120$ -cm calibrated columns of agarose beads, A-15 m, A-1.5 m (200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) or Sephadex G-50 SF (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Tritiated water was used to mark column volumes (23).

**Amino acid and carbohydrate analysis.** Samples for amino acid analyses were hydrolyzed in doubly distilled constant boiling HCl under an atmosphere of nitrogen for 24 h at 110°C. Analyses were performed with an automatic analyzer (model 121, Beckman Instruments, Fullerton, Calif.) using a four-buffer elution system previously described (24).

Hydroxylysine glycosides were determined after hydrolysis in 2 N NaOH in borosilicate-free tubes at 110°C for 24 h. Analyses were performed on a Beckman 121 analyzer employing the procedure of Askenasi and Kefalides (25). No correction factors were used to compensate for hydrolytic losses.

## RESULTS

Collagen from both normal and cirrhotic human liver was investigated. Only a small fraction of the total collagen present in normal liver was extractable by nondenaturing solvents, 1 M NaCl and 0.5 M acetic acid. However, limited pepsin digestion enabled solubilization of 47 and 85% of the total collagen from normal and cirrhotic liver, respectively (Table I). In general, much greater percentages of the total collagen were solubilized with pepsin from cirrhotic livers than from normal livers. This allowed isolation of sufficient quantities of liver collagen for chemical characterization.

Collagen extracted by limited proteolysis with pepsin was partially purified by dialysis against 0.01 M  $\text{Na}_2\text{HPO}_4$  and 1 M NaCl precipitation from dilute acetic acid solutions. Type III collagen was partially separated from type I by fractional precipitation between 1.0 and 1.5 M NaCl at pH 7.4 (0.05 M Tris). The more soluble type I collagen was separated as a 1.5–2.5 M NaCl precipitate at the same pH (3, 7).

Type III collagen in the 1.0–1.5 M NaCl precipitate was fractionated by CM-cellulose chromatography (Fig. 1). The elution patterns of normal and cirrhotic type III collagen were identical and similar to that observed for human skin or lung type III collagen (3, 7, 26). The peaks were identified by amino acid analysis after agarose A-15 m gel filtration (figure not shown). The molecular weight of  $\alpha 1(\text{III})$  was estimated to be 285,000 by the above procedures. The amino acid composition of the  $\alpha 1(\text{III})$  chain of human liver was indistinguishable from that of human skin  $\alpha 1(\text{III})$  reported previously except that the content of hydroxylysine was higher (8.4/1,000 residues as opposed to 5.0 for skin) (3, 26). The carbohydrate content was found to be 0.8 residue of *O*-glucosylgalactosylhydroxylysine

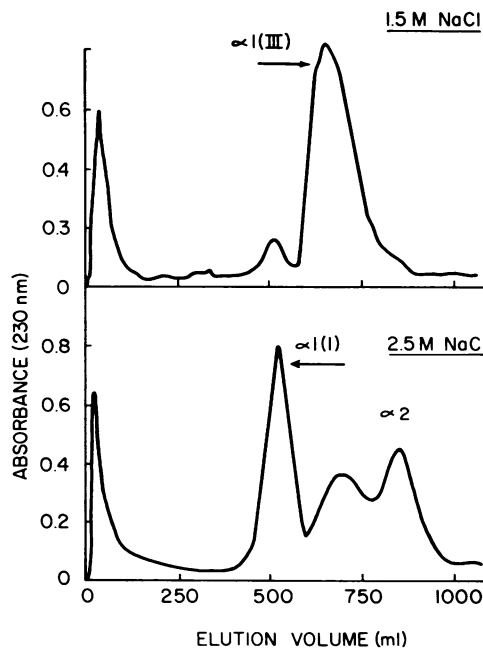


FIGURE 1 CM-cellulose chromatography of denatured collagens from cirrhotic human liver. The collagens were solubilized by limited pepsin digestion and separated by differential salt precipitation of 1.5 and 2.5 M NaCl, 0.05 M Tris yielding type III and type I collagens, respectively. Each fraction was further separated on CM-cellulose at pH 4.8 (0.02 M sodium acetate in 1 M urea), with a linear gradient of 0–0.1 M NaCl at 43°C. The upper panel represents the collagen fraction precipitating at 1.5 M NaCl at pH 7.4, and the lower panel indicates the fraction precipitating at 2.5 M NaCl at pH 7.4. The presence of  $[\alpha 1(\text{III})]_3$  chains eluting between  $\alpha 1(\text{I})$  and  $\alpha 2$  (lower panel) is due to the incomplete precipitation of type III at 1.5 M NaCl.

and 0.3 residue of *O*-galactosylhydroxylysine per  $\alpha 1$  chain. No significant differences were observed in either the amino acid or the carbohydrate composition between normal and fibrotic liver type III collagen.

Type I collagen precipitating at 2.5 M NaCl was separated into individual  $\alpha 1(\text{I})$  and  $\alpha 2$  chains by CM-cellulose chromatography (Fig. 1). Each of the  $\alpha$ -chains was further purified by gel filtration on agarose A-15 m (figure not shown), which allowed a clear-cut separation of  $\alpha 1(\text{I})$  or  $\alpha 2$  (mol wt 95,000 daltons each) from  $\alpha 1(\text{III})$  (mol wt 285,000 daltons). No peaks corresponding to  $\beta 11$  or  $\beta 12$  (of type I) were observed on gel filtration. The amino acid composition of the  $\alpha 1(\text{I})$  and the  $\alpha 2$  chains was identical, within experimental error, to that reported previously (27, 28) for the type I chains from human skin except for the higher hydroxylysine content of the liver  $\alpha 1(\text{I})$  chains (6.5 vs. 5.0). The content of hydroxylysine glycosides was similar to that of type III collagen. Again no significant

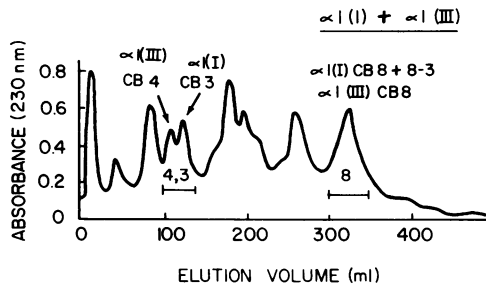


FIGURE 2 CM-cellulose chromatography of a CNBr digest of an artificial mixture containing 40%  $[\alpha 1(\text{III})]_3$  and 60%  $\alpha 1(\text{I})$  from cirrhotic human liver. The collagen chains were obtained as indicated in Fig. 1. The mixture containing 20 mg of  $[\alpha 1(\text{III})]_3$  and 30 mg  $\alpha 1(\text{I})$  was digested with CNBr and chromatographed on CM-cellulose at pH 3.8 (0.02 N sodium citrate), using a linear gradient of NaCl from 0.02 to 0.16 M at 43°C. The peaks labeled  $\alpha 1(\text{III})\text{-CB}4$  and  $\alpha 1(\text{I})\text{-CB}3$  were pooled together as indicated by the bar and chromatographed on agarose A-1.5 m yielding a single peak corresponding to 13,500 mol wt. From the amino acid composition of this mixture the  $\alpha 1(\text{I})$  content was found to be 58%. The other peak labeled  $\alpha 1(\text{I})\text{-CB}8$  and (8-3) and  $\alpha 1(\text{III})\text{-CB}8$  was pooled as indicated by the bar. Separation of these latter three peaks into  $\alpha 1(\text{I})\text{-CB}(8-3)$ ,  $\alpha 1(\text{I})\text{-CB}8$  and  $\alpha 1(\text{III})\text{-CB}8$  with agarose A-1.5 m allowed an alternative procedure for calculation of  $\alpha 1(\text{I})$  and  $\alpha 1(\text{III})$  ratio based on the total hydroxyproline in each peak. In this case, the  $\alpha 1(\text{I})$  content was determined to be 62% of the total (Table II).

differences were noted in the amino acid or carbohydrate content between collagens of normal and cirrhotic liver.

To further characterize the covalent structure of type I and type III collagens of liver, the  $\alpha 1(\text{I})$  and the  $\alpha 1(\text{III})$  chains obtained by CM-cellulose and agarose chromatography were digested with CNBr, and the resulting peptides were fractionated on CM-cellulose at pH 3.8 (7). Each of the CNBr peptides was then further purified by a combination of ion-exchange and molecular sieve chromatography (7, 22, 28), and analyzed for molecular weight and amino acid composition. All of the peptides were, within experimental error, identical with the corresponding peptides from human skin (22, 28).

Next, the question of collagen polymorphism in normal and cirrhotic liver was investigated. Because of the fact that pepsin solubilization yielded variable portions of the total collagen present in the liver, determination of the  $\alpha 1(\text{I})$ : $\alpha 1(\text{III})$  ratio in the extracted collagen would not necessarily have reflected their distribution in the tissue. Thus, the washed total liver homogenates were subjected to CNBr digestion directly. Exhaustive prior extraction of the homogenates with 0.05 M Tris, pH 7.4 and distilled water was essential to remove large quantities of noncollagenous proteins and other substances. Under the experimental conditions used in the present study, 90% or more of

the total hydroxyproline was solubilized by CNBr digestion. CNBr digests were then fractionated on CM-cellulose and gel filtration. The relative quantities of two separate sets of specific CNBr peptides unique to  $\alpha 1(\text{I})$  and  $\alpha 1(\text{III})$  were determined to calculate the ratio of the two types of collagen in the original tissue homogenates. These peptides are  $\alpha 1(\text{I})\text{-CB}8$  in relation to  $\alpha 1(\text{III})\text{-CB}8$  (5, 7, 26), and  $\alpha 1(\text{I})\text{-CB}3$  to  $\alpha 1(\text{III})\text{-CB}4$  (6).

To ascertain the reliability of this approach, artificial mixtures containing various proportions of isolated  $\alpha 1(\text{I})$  and  $\alpha 1(\text{III})$ , ranging from 20 to 40% type III, were initially analyzed. As an example, a CM-cellulose chromatogram of a CNBr digest of a sample containing 40%  $\alpha 1(\text{III})$  and 60%  $\alpha 1(\text{I})$  is shown in Fig. 2. The peptide  $\alpha 1(\text{III})\text{-CB}8$  coeluted with  $\alpha 1(\text{I})\text{-CB}8$  and the uncleaved  $\alpha 1(\text{I})\text{-CB}(8-3)$  (5, 7, 26). Incorporation of tracer amounts of purified, [ $^{14}\text{C}$ ]glycine-labeled  $\alpha 1(\text{I})\text{-CB}8$  prepared from chick skin collagen (29) into CNBr digests before chromatography enabled pooling of consistent fractions from the CM-cellulose effluents. The three peptides with molecular weights of 12,000, 24,000 and 37,000, respectively, were separated

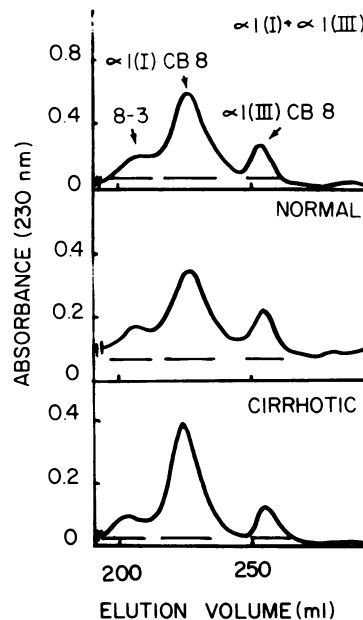


FIGURE 3 Agarose A-1.5 m gel filtration of the CNBr peptides eluting in the CB8 region from CM-cellulose. The samples were derived from the synthetic mixture, (upper panel), normal liver (middle panel), and cirrhotic liver (lower panel). The CB8 region was collected, lyophilized, and separated by gel filtration on agarose A-1.5 m using 0.05 M Tris/1 M  $\text{CaCl}_2$ , pH 7.4, as the eluting solvent. The collagen peptides,  $\alpha 1(\text{I})\text{-CB}8$ ,  $\alpha 1(\text{I})\text{-CB}(8-3)$  and  $\alpha 1(\text{III})\text{-CB}8$  were eluted from the column at positions corresponding to mol wt of 37,000, 24,000 and 12,000, respectively. Molar ratios based on hydroxyproline analysis of these three peptides were used to calculate molar ratios of type I and type III collagens (Table II).

TABLE II  
Quantitation of the Ratio of  $\alpha 1(I):\alpha 1(III)$  Collagen Chains in Artificial Mixtures \*

$\alpha 1(I):\alpha 1(III)$ Used	Hydroxyproline		Measured $\alpha 1(I):\alpha 1(III)^{\ddagger}$	$\alpha 1(I)\text{-CB3} + \alpha 1(III)\text{-CB4}^{\S}$		Measured $\alpha 1(I):\alpha 1(III)$
	$\alpha 1(I)\text{CB8} + \text{CB8} - 3\ddagger$	$\alpha 1(III)\text{-CB8}\ddagger$		Threonine	Valine	
	nm			nm		
80:20	111	26	81:19	32	87	78:22
70:30	98	39	72:28	26	45	68:32
60:40	98	60	62:38	33	38	58:42

\* Artificial mixtures were prepared from isolated purified  $\alpha 1(I)$  and  $\alpha 1(III)$  of human cirrhotic liver.

† The CB8 region was collected in from CM-cellulose chromatography as indicated in Fig. 2, with the aid of an incorporated authentic radiolabeled  $\alpha 1(I)\text{-CB8}$  and further chromatographed on agarose A-1.5 m as shown in Fig. 3. The fractions indicated by the bars were collected and analyzed for hydroxyproline.

§ The CB 4,3 region was collected from CM-cellulose chromatography as indicated in Fig. 2 and further fractionated on agarose A-1.5 m. The peak eluting at the position corresponding to a molecular weight of 13,500 was collected, and analyzed on an amino acid analyzer.

|| The calculations were based on 29 nmol hypro per  $\alpha 1(I)\text{-CB8}$ , 15 nmol hypro per  $\alpha 1(III)\text{-CB8}$ , and five threonine residues for  $\alpha 1(III)\text{-CB4}$ , 0 threonine for  $\alpha 1(I)\text{-CB3}$ , four valine residues for  $\alpha 1(III)\text{-CB4}$  and 0 valine for  $\alpha 1(I)\text{-CB3}$ .

from each other by agarose A-1.5 m filtration (Fig. 3), and quantitated by hydroxyproline analysis on aliquots of each peptide. The composition of such mixtures calculated from this type of analysis is presented in Table II.

The standard samples were also analyzed for the ratio of type I to type III using a second set of CNBr peptides,  $\alpha 1(I)\text{-CB3}$  and  $\alpha 1(III)\text{-CB4}$ . The two peptides are homologous and have an identical molecular weight of 13,500, but differ in amino acid composition. The former peptide contains four residues of valine but no threonine, while the latter contains five residues of threonine but no valine. The ratio of these two amino acids, therefore, could be used to calculate the relative proportions of type I and type III in the sample. Thus, the CM-cellulose fractions containing the two peptides (Fig. 2) were pooled, desalted, and subjected to gel filtration on agarose A-1.5 m. The peak eluting at the position corresponding to a molecular weight of 13,500 was collected, desalted, and analyzed in triplicate for amino acid composition. From the observed values of threonine and valine, the ratio of type I to type III was calculated. The results, given in Table II also, are in close agreement with the predicted values and with the results obtained by analyses of the CB8 peptides. The CNBr peptides derived from the  $\alpha 2$  chain of type I collagen have previously been shown not to coelute with the above  $\alpha 1(I)$  and  $\alpha 1(III)$  peptides and therefore were not a source of error (7).

A CM-cellulose chromatogram of a CNBr digest of a normal liver is presented in Fig. 4 (upper panel), along with that obtained from a cirrhotic liver (Fig. 4, lower panel). The peaks corresponding to  $\alpha 1(I)\text{-CB8}$ , CB(8-3), and  $\alpha 1(III)\text{-CB8}$  were pooled,

desalted on Sephadex G-25, lyophilized, and the peptides were further separated by gel filtration on agarose A-1.5 m (middle and lower panels), as shown in Fig. 3. Hydroxyproline analysis was used to deter-

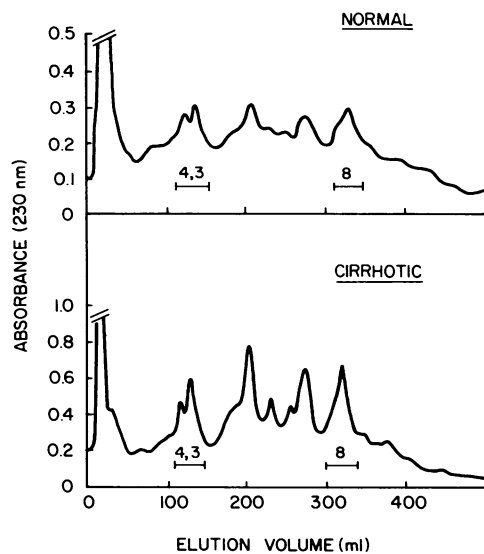


FIGURE 4 CM-cellulose chromatography of peptides solubilized by CNBr digestion of normal and cirrhotic human liver. The tissue was washed exhaustively with the 0.05 M Tris, pH 7.4, then distilled water, and digested with CNBr at 40°C for 4 h. CM-cellulose chromatography of a 150-mg sample was performed using identical conditions as indicated in Fig. 2. The upper panel represents a CNBr digest of normal liver and the lower panel contains the peptides from cirrhotic liver. The peaks designated 4, 3, and 8 were pooled as indicated by the bars and used for measurement of relative proportions of type I and type III collagens as indicated in Fig. 3.

TABLE III  
Percentage of Type III Collagen in Normal and  
Cirrhotic Human Liver\*

Normal liver	Cirrhotic liver
	% type III collagen
48	18, 16
45	26, 26, 24, 22, 28
49	34, 36, 34
	32
	30
	27

\* Each line represents an individual liver. Actual values in the case of multiple analysis of individual liver homogenates are included.

mine the quantities of these peptides, and from the known composition of the peptides, molar ratios of the two collagens in liver was calculated. In addition, the ratio of the two collagens was also determined by amino acid analysis in triplicate of the peptide fractions containing a mixture of  $\alpha 1(I)$ -CB3 and  $\alpha 1(III)$ -CB4 as described above for the standard mixtures. The results obtained by the two types of analyses were averaged and are shown in Table III. Normal human liver contained 45–49% type III collagen. In the case of alcoholic liver cirrhosis, the relative content of type I was increased with a corresponding decrease in type III collagen with values ranging from 18 to 34% type III.

## DISCUSSION

The process of liver fibrosis after injury has been well-characterized morphologically (30, 31). The large variety of chemical agents capable of inducing liver fibrosis in experimental animals has enabled us to obtain a relatively clear understanding of the morphologic progression of the disease process. The increased deposition of collagen is one of the major pathologic processes associated with chronic hepatic fibrosis. However, the chemical nature of collagen in liver from normal and cirrhotic states has not been fully characterized and represents the aim of this investigation.

Type I and type III collagens were isolated and purified from normal and cirrhotic liver, and the component  $\alpha$ -chains were obtained by CM-cellulose and molecular sieve chromatography. The molecular weight of each of the two  $\alpha$ -chains of type I collagen and the type III chains was identical to that found in human skin, aorta, leiomyoma, and lung. The amino acid composition of each of the polypeptide chains was also similar to that found for human skin chains with the exception of hydroxylysine. Higher values were found for the hydroxylysine content of the

$\alpha 1(I)$  and  $\alpha 1(III)$  chains of liver as compared to the skin chains, the increase being two to three residues per chain (3, 26–28). These results probably reflect a tissue-specific variation in the activity of lysyl-hydroxylase. Liver type III collagen contained identical quantities of *O*-glucosylgalactosylhydroxylysine and *O*-galactosylhydroxylysine as type III of lung (7). In all respects examined, i.e., molecular weight, amino acid composition, lysine and proline hydroxylation, and glycosylation, no significant differences were noted between normal and cirrhotic liver collagen. This is interesting because the activities of the post-translational enzymes, prolyl- and lysyl-hydroxylases, and collagen glucosyl- and galactosyl-transferases have all been shown to increase several-fold during experimentally induced hepatic fibrosis (19, 20). The fact that these increased enzymatic activities do not lead to increased collagen hydroxylation or glycosylation suggests that the increased enzymatic activities may reflect an overall increased collagen synthesis incident to the fibrotic process.

The CNBr peptides of cirrhotic liver  $\alpha 1(I)$  and  $\alpha 1(III)$  were isolated and characterized with respect to amino acid and carbohydrate composition and molecular weight. The amino acid compositions are in close agreement with those obtained from human skin  $\alpha 1(I)$  and  $\alpha 1(III)$  (3, 28).

The specific nature of the CNBr cleavage and of the resulting peptides from both collagen types allowed investigation of collagen polymorphism in normal and cirrhotic liver. Artificial mixtures of isolated  $\alpha 1(I)$  and  $\alpha 1(III)$  were used initially to establish elution positions of the peptides from each  $\alpha$ -chain. The peptide  $\alpha 1(III)$ -CB8 coelutes from CM-cellulose with  $\alpha 1(I)$ -CB8 and CB(8-3) (26). The molecular weights of the latter two peptides, 24,000 and 37,000, respectively are greater than that of  $\alpha 1(III)$ -CB8 (12,000) and therefore they could be separated by agarose A-1.5 m chromatography. Incorporation of tracer amounts of radiolabeled  $\alpha 1(I)$ -CB8, prepared from chick skin, into samples of tissue CNBr digests ensured collection of consistent fractions. Quantitation of the peptides eluting from agarose A-1.5 m was achieved using hydroxyproline analysis on aliquots to prevent possible errors from noncollagenous proteins. A second procedure of quantitating the relative amounts of  $\alpha 1(I)$ -CB3 and the homologous  $\alpha 1(III)$ -CB4 (6) provided substantiation of the above quantitation methods. After an initial separation on CM-cellulose, the above two peptides coeluted from agarose A-1.5 m as a single peak corresponding to 13,500 mol wt. From their known amino acid compositions, the proportion of each peptide was calculated from quantitative amino acid analysis of the mixture. The above two procedures were generally in close agreement, assuring the

measurements were reproducible. Close agreement obtained by five identical analyses of one liver sample and duplicate and triplicate analyses on two other samples (Table III) also supports the reliability of the determinations.

The normal liver contained an average of 47% type III collagen. This is much higher than that found in adult human skin (26). The content of type III collagen in cirrhotic liver was quite variable ranging from 18 to 34% depending on the patient. Close agreement however, was generally obtained on repeated analyses of the same tissue. No attempts were made in the present study to correlate the severity of liver involvement with the reduction in type III collagen. The consistent reduction in the type III content, however, indicates the fibrotic response of this tissue to alcoholic injury is to result in a net increase in type I collagen deposition. Whether this is due to a preferential increase in the degradation of type III collagen or solely to increased synthesis of type I collagen is currently under investigation.

Recently, Kuhn and his colleagues (8, 9) have reported that human liver contains at least two apparent populations of collagen; one stainable by fluorescein-labeled antitype III collagen antibody but not by antitype I antibody, and the other not stainable by either. It was suggested that human liver contains, in addition to type III, either a new type of collagen as yet undefined or type I collagen which differs from skin type I in its immunologic properties. The present investigation indicates that type I collagen is a major constituent of liver, and that biochemically it is indistinguishable from type I collagen from other tissues. The explanation for these discrepant observations is not clear at present.

Rojkind and Martinez-Palomo (10) on the other hand reported that normal liver contained 80% type I collagen whereas cirrhotic liver contained higher amounts of both type I and III. However, the proportion of type III was increased in cirrhotic as compared to normal liver (10). These conclusions, however, were based entirely on the selective precipitation of the two types of collagen after pepsin solubilization. Utilizing identical procedures, we found a relatively high degree of cross-contamination in the 1.5 and 2.5 M NaCl precipitates rendering accurate quantitation of the relative proportions difficult. To circumvent these uncertainties as well as the problem of variable pepsin solubilization (Table I) of normal vs. cirrhotic liver collagens, we instead utilized CNBr digestion of the total liver collagen which consistently yielded over 90% of the collagens as soluble CNBr peptides. These were then separated and quantitated by well-established procedures to yield peptides specific for both types of collagen.

Whether a diminution in the relative content of type III with an increase in type I is characteristic of fibrotic repair in general is not known. Similar changes have been reported for chronic pulmonary fibrosis (7) and dermal scar (32), suggesting that this may be the case. However, the possibility also exists that the collagen change in fibrosis may depend on the nature of injury which incites the fibrotic response. For example, rheumatoid synovium reportedly (33) contains elevated levels of type III collagen. This facet of the problem is currently under investigation in our laboratory.

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