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### Research Article

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The data indicate that in the absence of immuno-competent cells or their secretory products, tissue cultures from livers showing biopsy evidence of active fibrosis in vivo may demonstrate increased synthesis of collagen and GAG in vitro. Increased (soluble) procollagen synthesis in cultures from alcoholic hepatitis was not associated with histologically demonstrable [...]

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# The Rate of Synthesis of Glycosaminoglycans and Collagen by Fibroblasts Cultured from Adult Human Liver Biopsies

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**ABSTRACT** Adult human liver biopsies were cultured from normal, alcoholic hepatitis, chronic active hepatitis, fibrosis plus alcoholic hepatitis (active cirrhosis), inactive cirrhosis, and drug hepatitis. The synthesis of collagen was estimated in cultures from 58 livers by measuring the conversion of [ $^{14}\text{C}$ ]proline to the [ $^{14}\text{C}$ ]hydroxyproline of collagen; that of glycosaminoglycans in cultures from 57 livers by the incorporation of [ $^3\text{H}$ ]acetate and  $^{35}\text{SO}_4$  into glycosaminoglycans (GAG). The synthesis of procollagen was increased only in cultures from alcoholic hepatitis, both in the pulse medium ( $P < 0.05$ ) and in the chase medium ( $P < 0.02$ ). The synthesis of insoluble collagen was increased in cultures from chronic (active) hepatitis ( $P < 0.01$ ), fibrosis plus alcoholic hepatitis (active cirrhosis) ( $P < 0.001$ ), and inactive cirrhosis ( $P < 0.05$ ). Essentially all radioactive GAG was soluble in culture media. The predominant GAG were chondroitin-4 or -6- $\text{SO}_4$ . The synthesis of GAG was increased only in cultures from fibrosis plus alcoholic hepatitis (active cirrhosis) both in the pulse medium ( $P < 0.01$ ) and chase medium ( $P < 0.001$ ).

The data indicate that in the absence of immunocompetent cells or their secretory products, tissue cultures from livers showing biopsy evidence of active fibrosis in vivo may demonstrate increased synthesis of collagen and GAG in vitro. Increased (soluble) procollagen synthesis in cultures from alcoholic hepatitis was not associated with histologically demonstrable overt hepatic fibrosis in vivo, nor was it associated with increased GAG synthesis in vitro. No significant difference was demonstrable in collagen or GAG synthesis in paired cultures which contained either 300 mg/dl ethanol or 3.75 mg/dl methylprednisolone compared to their respective controls.

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## INTRODUCTION

Hepatic fibrosis and cirrhosis usually follow parenchymal necrosis and inflammation. It is not known, however, whether hepatic fibrosis is induced by hepatocellular necrosis, by the inflammatory exudate, or by an immune mechanism. Because cells from adult human livers can be maintained in tissue culture, it became possible to estimate the synthesis of collagen and glycosaminoglycans (GAG)<sup>1</sup> by cells when these are no longer exposed to the direct effect of their in vivo environment (1).

## MATERIALS

A random sample of the unselected liver biopsies was removed for culture. Those who obtained the liver biopsy did not participate in the laboratory work and those who performed the culture and biochemical work were not aware of the diagnosis.

### *Diagnostic categories*

**Normal.** The livers were classified as normal when there was neither clinical evidence of primary liver disease nor significant histopathology. GAG studies were performed in 9 and collagen studies in 13 cases. 4 of the 9 used for GAG and 2 of the 13 for collagen studies had intra-abdominal cancer with suspected hepatic metastasis at the time of biopsy. However, no evidence of tumor was seen on gross and microscopic examination of the liver removed by needle aspiration.

**Chronic (active) hepatitis.** The diagnosis was made by clinical data and by the biopsy demonstration of portal and periportal hepatitis, piecemeal necrosis, and scattered areas of lobular hepatitis. Cirrhosis was not seen in any of these livers. Fibrosis was predominantly in the portal and

<sup>1</sup> *Abbreviations used in this paper:* BSS, balanced salt solution; CM, chase medium; FCS, fetal calf serum; GAG, glycosaminoglycans; MEM, Eagle's minimal essential medium; PM, pulse medium; PPM, prepulse medium.

periportal areas. Collagen studies were performed in eight and GAG studies in seven cases.

**Alcoholic hepatitis.** The diagnosis was made by clinical data and biopsy demonstration of its characteristic histologic features (2). The biopsies in this group showed either no fibrosis or only minimal subsinusoidal or portal fibrosis. Each had confluent necrosis in zone 3 of the hepatic acinus (3). Collagen studies were performed in nine and GAG studies in seven cases.

**Fibrosis and alcoholic hepatitis (active cirrhosis).** These livers showed significant distortion of the lobular architecture by fibrosis in addition to active alcoholic hepatitis. 8 of the 12 used for collagen and 9 of the 16 livers used for GAG studies had established cirrhosis. The remaining biopsies showed well-developed subsinusoidal, intralobular, and periportal fibrosis but no definite septa were recognizable. In these biopsies several of the hepatic venules were compressed by fibrosis ("central hyalin sclerosis").

**Inactive cirrhosis.** Wedge biopsies were obtained from cirrhotic patients during elective shunt surgery. These contained broad and usually cellular septa. There was no significant active parenchymal necrosis and inflammation. These explants contained a much larger proportion of septum than those from other categories. Collagen studies were performed in 9 and GAG studies in 11 cases.

**Drug hepatitis.** These seven livers were from patients who had the clinical diagnosis of drug hepatitis (contraceptive pill [two], methylodopa [two], indomethacin [one], chlorpromazine [one], and isoniazid [one]). The biopsy findings confirmed this diagnosis. Each was negative for Hbs Ag.

## METHODS

**Tissue culture procedures.** Explant cultures were established from portions of liver biopsies (1). To avoid uncontrolled variations due to senescence of cultured cells, the studies were performed on subcultures between their 5th and 11th passages. Epithelial cells were no longer present in these subcultures. Studies were performed only on those cultures where monolayers were confluent and showed marked decrease of proliferative activity (4). There were no recognizable differences in cultures from various diagnostic categories.

To prepare for pulse-chase studies equal aliquots of a cell suspension were inoculated into 75-cm<sup>2</sup> Falcon flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) to establish replicate cultures. The monolayers were treated with prepulse medium (PPM)<sup>2</sup> for 72 h. The PPM for GAG studies contained no SO<sub>4</sub> other than streptomycin-SO<sub>4</sub>. The PPM for collagen studies contained no proline. The pulse medium (PM) was the same as the PPM and for collagen experiments it contained 20  $\mu$ Ci [<sup>14</sup>C]proline/dl and for GAG experiments it contained a 4.7 mCi [<sup>3</sup>H]acetate and 1 mCi of <sup>35</sup>SO<sub>4</sub>/dl. The specific activity of [<sup>14</sup>C]proline was 2.6 Ci/mol<sup>3</sup> and of <sup>35</sup>SO<sub>4</sub> was 58.8 Ci/mol, of [<sup>3</sup>H]acetate was 1.38 Ci/mmol at the beginning of the incubations. The amount of proline or acetate synthesized during incubation was not measured. After the 24-h pulse the PM was decanted, and all radioactivity was removed by rinses with 5-ml aliquots

of chase medium (CM).<sup>4</sup> The cells were incubated for 72 h in 15 ml CM; then the CM was removed and the monolayer was rinsed twice with 5 ml isotonic balanced salt solution (BSS). The respective PM, CM, and corresponding rinses from sets of subcultures were pooled and were stored frozen at -20°C. The monolayers with adherent insoluble material were then harvested by brief trypsinization followed by scraping; then they were centrifuged at 5,000 g at 4°C for 10 min and frozen (insoluble fraction).

When sufficient number of generation-specific subcultures were obtained, then both collagen and GAG were studied in sets of sister cultures; otherwise, only collagen or only GAG studies were performed. Furthermore, when the growth rate permitted, replicate sets of generation-specific cultures were used to evaluate the effect of compounds added to the PM and CM on collagen or GAG synthesis.

Ascorbic acid was added to give 3 mg/dl (a) only once at the start of the incubation or (b) at 6-h intervals during incubation; (c) controls.

Graded concentrations of ethanol in culture media, up to 600 mg/dl, produced no detectable cytologic changes under phase microscopy during experiments ranging from 7 to 14 days. Only ethanol concentrations of 1,000 mg/dl or greater produced pathologic alterations. Collagen or GAG synthesis in control cultures were compared with those which contained 300 mg/dl ethanol and with those which contained 3.75 mg/dl (0.1 mM) 6-methyl-prednisolone.

**Radioactivity.** All counts were the averages of replicates. The <sup>3</sup>H and <sup>35</sup>S were counted simultaneously in a Packard model 3321 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The three channels were set for <sup>3</sup>H, <sup>35</sup>S, and <sup>14</sup>C, respectively. The statistical counting error was less than 1.5%. The disintegrations per minute for each set were calculated by a computer program which corrected for quenching, counting efficiency, and, in the case of GAG experiments, decay of <sup>35</sup>S and spill of <sup>35</sup>S into <sup>3</sup>H channel. The counts were done in 10 ml toluene for <sup>14</sup>C counts and in Bray's scintillation solution for <sup>3</sup>H and <sup>35</sup>S.

**Cell protein.** The cell pellets were disrupted by freeze-thaw cycles and were homogenized. A 2-ml suspension was prepared and 0.1-ml duplicates were removed for measurement of total cell protein by the Lowry procedure (5) modified by increasing the NaOH incubation for 2 h at 18°C.

**[<sup>14</sup>C]Hydroxyproline containing soluble protein.** This protein could have been either C1q of complement, procollagen, or both. To detect any [<sup>14</sup>C]C1q, carrier C1q was added to 10-fold concentrates of pooled culture fluids and C1q was isolated by immunoprecipitation on aggregated IgG (courtesy of Dr. Charles Reimer and Mr. Don Phillips of Center for Disease Control, Atlanta, Ga.).

**Isolation of soluble procollagen.** The procollagen was precipitated from pooled culture fluids in ice-cold 10% TCA, washed in TCA, and hydrolyzed in 6.0 N HCl for 18 h at 115°C and 15 pounds per square inch. The [<sup>14</sup>C]hydroxyproline was estimated as described previously (6-8). Toluene extracts of [<sup>14</sup>C]pyrrole were eluted from 1  $\times$  7-cm silicic acid columns and replicate aliquots were used for <sup>14</sup>C counts and for colorimetric analysis. The recovery loss of hydroxyproline was calculated from differences between absorbencies of the samples and that of pyrrole standards. The recovery rates were 52 $\pm$ 4% (mean $\pm$ SD). The counts were appropriately corrected for the losses.

In addition, [<sup>14</sup>C]procollagen and [<sup>14</sup>C]collagen were demonstrated in 32 liver cultures; furthermore, procollagen and col-

<sup>2</sup> Phenol red-free Eagle's minimal essential medium (MEM; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS), 100 U penicillin, and 50  $\mu$ g streptomycin/ml.

<sup>3</sup> After 24 h of incubation, no significant <sup>14</sup>C was detectable by chromatography in amino acids other than proline.

<sup>4</sup> CM = phenol red-free MEM with 10% FCS, 0.08 M Na<sub>2</sub>SO<sub>3</sub>, and 0.001 M proline.

lagen were isolated from a pool of 87 liver cultures. Procollagen was isolated by a minor modification of the method described by Church et al. (9). In summary, procollagen was assumed to be soluble in PM, CM, and BSS. The crude procollagen was precipitated in 20% ammonium sulfate, dissolved in and dialyzed against potassium phosphate buffer (pH 7.6), precipitated in 18% ethanol, suspended in and dialyzed against 0.01 M Tris buffer (pH 7.4) in 2 M urea, and the precipitate from the dialysis bag was eluted from  $0.9 \times 20$ -cm column of DEAE-cellulose with the linear gradient containing 0.3 M NaCl in the limiting buffer. The fractions containing  $^{14}\text{C}$  were pooled and the hydroxyproline was isolated and counted (see above). Further analysis was performed on larger quantities which were available from pooled cultures. The procollagen peaks from the DEAE-cellulose columns were precipitated in 18% ethanol. The precipitate was dissolved in 0.046 M Tris, pH 8.1, containing calcium chloride and digested with trypsin-TPCK.<sup>5</sup> The hydroxyproline containing peptides were no longer detectable in the supernatant but were recovered in the precipitate. The precipitated collagen was then digested with collagenase<sup>6</sup> in a 0.05-M Tris (Tris buffer) containing calcium chloride at pH 7.5 at 37°C. All hydroxyproline was detected in the supernate of collagenase digest and no precipitable hydroxyproline remained.

**Isolation of insoluble collagen.** The insoluble residues of respective sister cultures were pooled and homogenized. Measured aliquots were digested with trypsin and then with collagenase. Collagenase solubilized [ $^{14}\text{C}$ ]hydroxyproline of insoluble collagen. The remainder was transferred to a glass ampule with 6 N HCl, sealed, and hydrolyzed. The [ $^{14}\text{C}$ ]hydroxyproline was estimated as described above.

**Isolation of GAG.** The pooled soluble fraction was loaded onto an ECTEOLA column ( $0.6 \times 3.0$ -cm with a 100-ml fluid reservoir). The unadsorbed GAG<sup>7</sup> in the effluent was digested with crystallized papain and was adsorbed on Dowex  $1 \times 2$  columns ( $1 \times 7$  cm, Dow Chemical Corp., Midland, Mich.). Both the ECTEOLA and Dowex columns were eluted in 2.5 N NaCl, precipitated with 0.5 mg carrier GAG in 66% (vol/vol) ethanol, and counted.

The insoluble fraction was digested with papain and analyzed as described above for soluble GAG.

**Electrophoresis of GAG.** Sufficient quantities of radioactive GAG were available only from one-third of the cultures. These were electrophoresed with carrier GAG (hyaluronic acid, chondroitin-4- $\text{SO}_4$ , and dermatan- $\text{SO}_4$ ) on cellulose acetate in 0.1 M formate-acetate buffer at pH 3.2. Hyaluronidase<sup>8</sup> incubation for 18 h at 37°C in pH 5.3 phosphate buffer

consistently eliminated any detectable amounts of hyaluronic acid and chondroitin-4- $\text{SO}_4$ , but left dermatan- $\text{SO}_4$  unaffected. The unstained bands of radioactive GAG (identified by adjacent stained bands) were counted in Bray's scintillation solution.

## RESULTS

In seven replicate experiments under our experimental conditions no radioactive precursor was incorporated into GAG or into the soluble or insoluble proteins in the absence of cells. No hydrolysis was detectable (i.e., no collagenase or hyaluronidase activity) when procollagen and collagen or chondroitin- $\text{SO}_4$  and hyaluronic acid were incubated with the monolayers for 72 h. All the added compounds were quantitatively recovered after the incubation.

In the culture fluids, no  $^{14}\text{C}$  was detectable in C1q by immunoprecipitation. Conversely, protein-bound hydroxyproline was quantitatively recovered in procollagen. No hydroxyproline was left unaccounted which could be attributed to more than trace amounts of C1q. These experiments demonstrated that sufficiently large amounts of C1q were not synthesized by human liver fibroblast strains in our cultures to affect the recovery of [ $^{14}\text{C}$ ]hydroxyproline of procollagen. Newly synthesized [ $^{14}\text{C}$ ]hydroxyproline in procollagen was identified (a) by its solubility characteristics in isotonic solutions at room temperature, in 20% ammonium sulfate, in 18% ethanol at 4°C, and in Tris-2 urea buffer; (b) by chromatography in DEAE-cellulose; (c) by conversion to collagen by tryptic digestion; and (d) by susceptibility of this collagen to collagenase.

**Procollagen (Fig. 1).** [ $^{14}\text{C}$ ]Procollagen increased both in the PM ( $P < 0.05$ ) and in the CM ( $P < 0.02$ ) only in cultures from livers with alcoholic hepatitis.

**Insoluble collagen (Fig. 1).** Collagen was unaffected by trypsin but was hydrolyzed by collagenase. [ $^{14}\text{C}$ ]Collagen was significantly increased in three diagnostic categories: (a) chronic (active) hepatitis ( $P < 0.01$ ), (b) fibrosis and alcoholic hepatitis (active cirrhosis) ( $P < 0.001$ ), and (c) cirrhosis ( $P < 0.05$ ). The ratios of [ $^{14}\text{C}$ ]hydroxyproline in procollagen:collagen were approximately 4:1 in cultures from alcoholic hepatitis and 2:1 in those from normal or drug hepatitis livers but it was 1:2 in cultures from fibrosis and alcoholic hepatitis (active cirrhosis) and from inactive cirrhosis.

Collagen synthesis was greater in cultures from active cirrhosis:  $39.8 \pm 7$  than in those from inactive cirrhosis:  $14.9 \pm 4.1$  (mean  $\pm$  SD,  $P < 0.01$ ).

Fig. 1 shows a distinct clustering of elevated insoluble collagen values of cultures either from chronic (active) hepatitis or from active cirrhosis. These clusters are well beyond 3 SD of the normal mean and

<sup>5</sup> Trypsin treated with L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone to inhibit contaminant chymotryptic activity.

<sup>6</sup> Purified collagenase A (from *Clostridium histolyticum*) EC 3.4.24.3, Worthington Biochemical Corp., Freehold, N.J. contained no peptidase activity.

<sup>7</sup> The reason for the adsorption of soluble GAG on ECTEOLA, followed by digestion of unadsorbed GAG and adsorption on Dowex  $1 \times 2$  was the suggestion that the ECTEOLA adsorbs the "free" GAG from untreated plasma and, after digestion, Dowex  $1 \times 2$  adsorbs the protein "bound" fraction (10, 11). Unfortunately, a substantial batch-to-batch variation of the binding of GAG by ECTEOLA introduced an uncontrollable variable. Consequently, only the sum of recoveries on both types of resins is reported.

<sup>8</sup> Highly purified testicular hyaluronidase (from Wyeth Laboratories, Marietta, Pa.). Lyophilized powder was dissolved in 0.1 M  $\text{PO}_4$  in 0.15 N NaCl, pH 5.3, buffer to give 150 National Formulary U/ml.

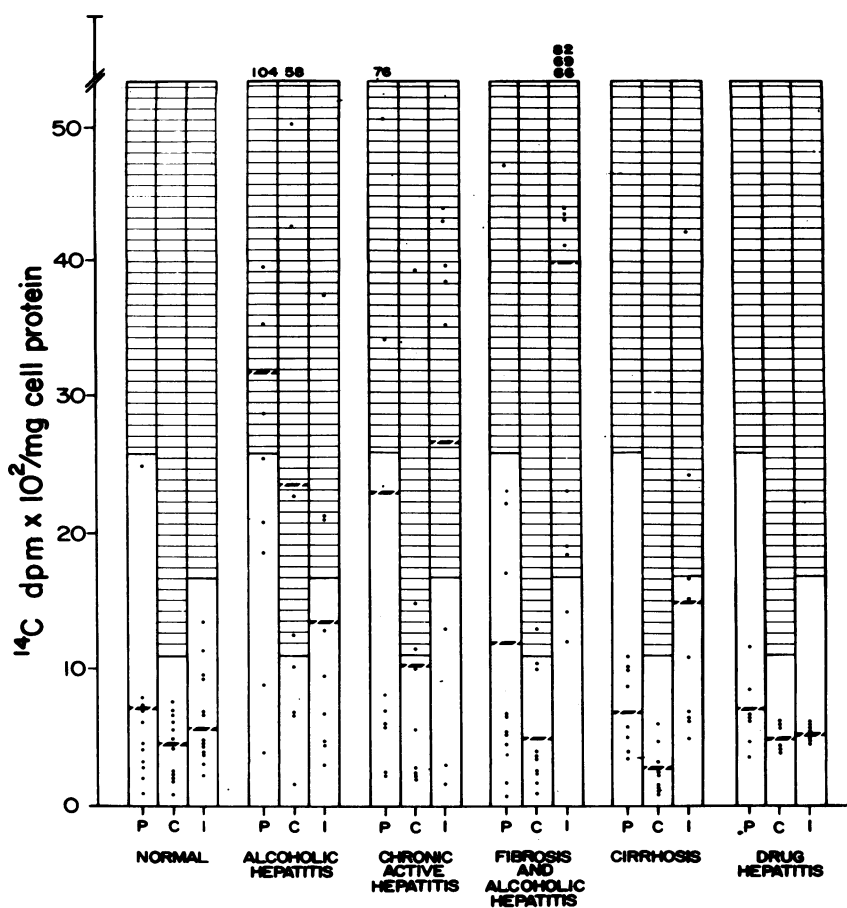


FIGURE 1 P, procollagen in PM: proline-free culture fluid (MEM in 10% FCS) which contained 20  $\mu$ Ci of [ $^{14}$ C]proline/dl (incubation, 24 h); C, procollagen in CM: culture fluid (MEM in 10% FCS) made 0.001 M proline (incubation, 72 h); I, insoluble collagen. Both P and C represent soluble procollagen. Heavy cross-hatched lines across the columns indicate the respective means. The clear area in the lower portion of each bar represents the values which are within 3 SD of the respective normal mean and the cross-hatched area above this represents values which are over the 99.7% confidence limit of normal.

include 5 of the 9 values from chronic (active) hepatitis and 7 of the 12 values from active cirrhosis.

**GAG (Fig. 2)** Essentially all the labeled GAG was soluble in the culture media. The insoluble fraction contained less than 1% of radioactivity and was not considered further. Significantly elevated GAG synthesis, in both the PM and CM, was observed only in cultures grown from livers with active cirrhosis. The increase was associated with an increase of  $^{35}\text{S}/^3\text{H}$  ratios. This ratio was over 1 in both PM and CM of over one-half of the cell strains from livers with alcoholic or chronic (active) hepatitis (Table I). GAG synthesis by cell strains from the other diagnostic categories did not differ from that of normal cultures. The only possible exceptions were the cultures grown from livers with drug hepatitis which appeared to have an increased synthesis of [ $^3\text{H}$ ]acetate but not of  $^{35}\text{SO}_4$ -labeled GAG in the PM only. This difference

was significant at the  $P < 0.05$  level. This could be a type I error.<sup>9</sup> Sufficient amounts of the  $^3\text{H}$ -labeled fraction were not available to document whether it represented a specific GAG or if the [ $^3\text{H}$ ]acetate-labeled one or more glycoproteins.

On electrophoresis, the  $^{35}\text{S}$ -labeled GAG migrated like sulfated GAG. The majority of  $^3\text{H}$ -labeled GAG migrated either like hyaluronic acid or chondroitin- $\text{SO}_4$ . Both of these bands were hydrolyzable by testicular hyaluronidase. The GAG in all groups contained a small proportion of unidentified sulfated GAG which resisted hydrolysis with testicular hyaluronidase and had the electrophoretic mobility of dermatan- $\text{SO}_4$ .

<sup>9</sup> 20 comparisons were made in this Figure; by definition one can expect to reject the null hypothesis by chance alone once in 20 trials (i.e., the  $P = 0.05$  level) when it should be accepted (type I error).

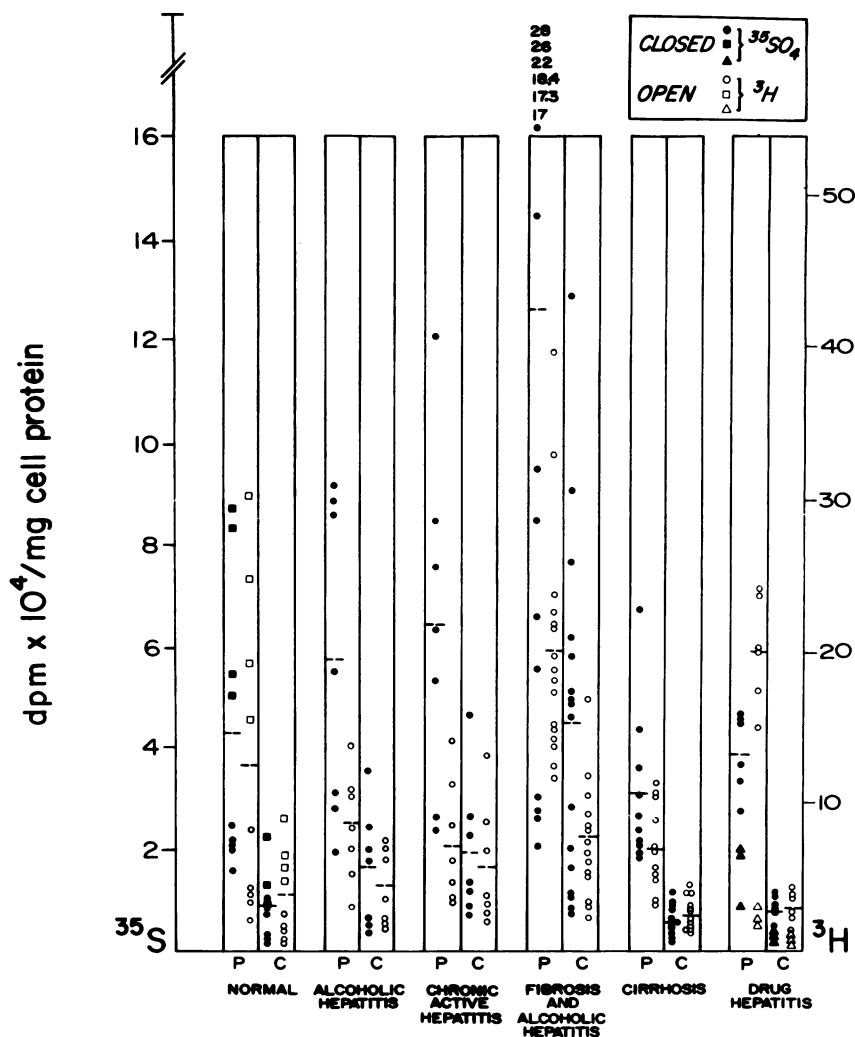


FIGURE 2 P, GAG in PM:  $\text{SO}_4$ -free culture fluid (MEM in 10% FCS) which contained 4.7 mCi  $^3\text{H}$ acetate and 1 mCi  $^{35}\text{SO}_4$ /dl (incubation, 24 h); C, GAG in CM: culture fluid (MEM in 10% FCS) made 0.008 M sodium sulfate (incubation, 72 h). The lines halfway across each column indicate the mean of the respective category. Solid symbols represent  $^{35}\text{S}$  counts and the open symbols represent  $^3\text{H}$  counts. Normal group: the circles represent normal biopsies where no liver disease was subsequently demonstrable. Squares represent biopsies which were obtained from livers which may have contained adenocarcinomas but the biopsy showed only normal appearing liver. Drug hepatitis: triangles represent three sets of replicate studies, the experiments were performed on the fifth, sixth, and seventh subcultures from a biopsy of methyldopa hepatitis.

The  $^3\text{H}$ GAG which did not contain  $^{35}\text{SO}_4$  behaved like hyaluronic acid.

The majority of newly synthesized GAG was probably chondroitin-4 or -6- $\text{SO}_4$  in cultures of hepatitis livers (chronic [active] or alcoholic with or without overt fibrosis). This was suggested by a high  $^{35}\text{S}/^3\text{H}$  ratio of radioactive GAG, by the electrophoretic mobility, and by the susceptibility to testicular hyaluronidase of the major radioactive bands. The composition of GAG synthesized in cultures from human liver biopsies, therefore, was similar to that found in human livers (12).

TABLE I  
 $^{35}\text{S}/^3\text{H}$  Ratios over 1 in PM or CM

	Normal	Alcoholic hepatitis	Chronic active hepatitis	Fibrosis + alcoholic hepatitis	Inactive cirrhosis	Drug hepatitis
n	9	7	7	16	11	7
PM	6	7	7	12	10	1
CM	2	5	6	14	2	4

n, number of cultures; PM, pulse medium; CM, chase medium.

**Experimental errors.** The reproducibility of the estimated rates of GAG synthesis is illustrated in the last two bars of Fig. 2. The experiments were performed during a period of 2 mo on three consecutive sets of subcultures from a liver with methyl-dopa hepatitis. These values are reasonably close to each other and illustrate the experimental error of the methods.

The effect of ascorbic acid was evaluated in replicate cultures from 11 liver strains and one skin strain. There was no demonstrable difference of collagen synthesis in cultures of liver strains to which ascorbic acid was added (*a*) once, (*b*) 6-hourly, or (*c*) not at all. However, in simultaneous studies, collagen synthesis in human foreskin cultures were significantly affected by the addition of ascorbic acid at 6-h intervals in accord with the observations made by Peterkofsky with L-929 fibroblasts (13).

The effect of ethanol was studied in replicate cultures on collagen synthesis from 10<sup>10</sup> liver strains, on GAG from 17<sup>11</sup> liver strains, and on that of methyl-prednisolone from 7<sup>12</sup> liver strains. There was no significant increase or decrease of collagen or GAG synthesis in the experimental cultures compared with their corresponding controls either in the ethanol or methyl-prednisolone experiments by the Wilcoxon signed ranks test (14).

## DISCUSSION

To the degree that the cultured cells from human livers represented *in vivo* biologic activity, the observations were compatible with the hypothesis that in active cirrhosis there was both an increase of collagen synthesis and a larger proportion of this collagen was insoluble at physiologic pH and ionic strength and was probably cross-linked. In the livers with alcoholic hepatitis (without overt fibrosis), there was also an increase of collagen synthesis; however, this was soluble procollagen which was not cross-linked. Procollagen would only lead to fibrosis if it were precipitated rapidly enough to become cross-linked collagen. Therefore, one may hypothesize that hepatic fibrosis depends not only on the rate of hydrolysis of procollagen to collagen but also on the composition of the ground substance which effects the rate and types of cross-linking of collagen.

Kupffer cells have been shown to contain collagenase activity (15) and hepatocytes a lysosomal enzyme with the substrate specificity similar to that of testicular

hyaluronidase (16). Our cultures, however, had no demonstrable collagenase or hyaluronidase activity. In the absence of cells, no incorporation of radioactivity was detectable. It is reasonable to assume, therefore, that the incorporation of radioactive precursors into procollagen and collagen or GAG was the function of their respective syntheses. It is also possible, however, that the observed changes in collagen [<sup>14</sup>C]hydroxyproline reflected increased prolyl hydroxylation in collagen, because our data did not clearly differentiate it from collagen synthesis. Increased prolyl hydroxylation without increased synthesis of collagen was an unlikely explanation because it had to postulate an over twofold increase of hydroxyproline in collagen or over fourfold increases of hydroxyproline/proline ratio per mole. Although the molar ratio of hydroxyproline varied some in collagens from different sources, this variation occurred within a narrow range.

The increased collagen and GAG synthesis by cell strains from human livers with biopsy evidence of overt and active fibrosis suggested that this technique could be used as a model for the development of rational therapy of fibrosis in chronic, precirrhotic liver disease. Preliminary observations suggested the suitability of these cultures for the evaluation of the effect of drugs, such as ethanol and methylprednisolone, in therapeutic concentrations.

If hepatic collagen or GAG synthesis was affected directly by ethanol in chronic alcoholics, then 300 mg/dl would be expected to exert an effect. The lack of a demonstrable effect in our cultures suggested that ethanol itself probably did not contribute to hepatic fibrosis. This was in accord with previous observations which suggested that, in alcoholic liver disease, fibrosis always follows alcoholic hepatitis (17), that there was a correlation between the progression of alcoholic hepatitis and of fibrosis (18), that the probability of cirrhosis was associated with the severity of alcoholic hepatitis (2, 19), and, most important in this regard, that fibrosis did not develop in one-half of the patients with alcoholic hepatitis who continued to drink but in others, fibrosis progressed and cirrhosis developed during abstinence (19).

If hepatic fibrosis was affected by corticosteroids, then 0.1 mM methylprednisolone would be expected to interfere with collagen and GAG synthesis. Although corticosteroid therapy had increased the survival rate and improved the clinical course of patients who had specific types of chronic hepatitis (20), there was no evidence that such therapy could prevent the development of cirrhosis (20–22). The lack of effect of methylprednisolone on collagen and GAG synthesis in seven sets of cultures was in accord with this clinical experience.

Peterkofsky demonstrated that in the absence of as-

<sup>10</sup> Normal (2), chronic active hepatitis (2), active cirrhosis (2), inactive cirrhosis (4).

<sup>11</sup> Normal (2), chronic active hepatitis (5), alcoholic hepatitis (3), active cirrhosis (3), inactive cirrhosis (4).

<sup>12</sup> Chronic active hepatitis (1), alcoholic hepatitis (1), active cirrhosis (2), inactive cirrhosis (3).

corbic acid, prolyl hydroxylation in collagen was 60% of maximum during the late stationary phase of L-929 fibroblast cultures (13). Fibroblast cultures of human foreskin behaved in a manner similar to that described for the L-929 cells (13). However, under the same conditions, ascorbic acid did not increase prolyl hydroxylation in collagen in cultures of human livers. This suggested that skin cultures would probably not be satisfactory models for the study of hepatic fibrosis.

The pathogenesis of fibrosis and cirrhosis has not been established. Recent studies have implicated immunologic mechanisms both for chronic hepatitis and for fibrosis and consequent cirrhosis (23-25). Not only chronic (active) hepatitis of viral or unknown etiology but also alcoholic hepatitis and its progression to cirrhosis has been attributed to immunologic mechanisms (24, 26). Our observations, however, do not support these suggestions that hepatic fibrosis required immunologic mechanisms.

If fibrosis was due to the direct and continuous effect of immunologic mechanisms, then increased collagen synthesis would not have been demonstrable in the absence of either immunocompetent cells or of their secretory products. Our subcultures of hepatic fibroblasts from various human liver diseases were not exposed to such cells or products. The most reasonable explanation for our observations, therefore, was that the cellular control of collagen and GAG synthesis was altered in livers with overt and active fibrosis or the explants from these livers contained a larger proportion of fibroblast. However, there was no evidence that variation of the numbers of fibroblasts in the explants affected the observed collagen or GAG synthesis in the cultures. If the higher proportion of fibroblasts in the explants were the explanation of the observed increases, then cultures from inactive cirrhosis should have shown the highest values because explants from inactive cirrhosis contained the largest amounts of fibrous septa and fibroblasts. Yet, this was not the case; collagen synthesis in cultures from fibrosis and alcoholic hepatitis (active cirrhosis) was significantly higher than that from inactive cirrhosis. Furthermore, GAG synthesis was not increased in cultures from inactive cirrhosis. Our findings did suggest the possibility that certain cells in human livers could have been induced in vivo either to synthesize collagen and GAG (hepatocytes) or to do so at an increased rate (fibroblasts). Our observations did not reflect on any possible effect that various lymphocytes or lymphokines could have on collagen or GAG synthesis in vivo (25).

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