Collagen proline hydroxylase activity and $^{35}$S sulphate uptake in human liver biopsies

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SUMMARY In an effort to assess connective tissue biosynthetic activity in human liver disease, collagen proline hydroxylase (a key enzyme in collagen biosynthesis) and the uptake of $^{35}$S sulphate (a precursor of sulphated mucopolysaccharides) were measured in hepatic tissue obtained mainly by percutaneous biopsy.

A procedure is described for the quantitation of collagen proline hydroxylase in cryostat sections which allows for the simultaneous histopathological examination of the liver specimen. A three to eightfold increase in the activity of this enzyme was found in four cirrhotic livers compared with the mean value of four normal livers and two biopsies from patients with Gilbert's syndrome. Elevated hydroxylase levels were found also in five patients with hepatic dysfunction but without cirrhosis (four alcoholics and one patient with persistent hepatitis associated with serum smooth muscle antibody). It is suggested that the hepatic level of collagen proline hydroxylase may be a useful quantitative index of fibroblastic activity in human liver disease.

Autoradiographic studies of radioactive sulphate uptake in biopsy specimens from patients with chronic liver disease showed an exaggerated incorporation of isotope not only at sites of established fibrogenesis but also in the walls of sinusoids throughout the liver.

Active hepatic fibrogenesis compared with passive stromal condensation may be involved in the progression of some liver diseases to cirrhosis. This implies an increase in the biosynthesis within the liver of the connective tissue components, collagen and mucopolysaccharide (Galambs, 1966; Popper and Udenfriend, 1970). Morphological examination of liver can determine the presence or absence of hepatic fibrosis, but it is impossible by this means to assess the amount of collagen or mucopolysaccharide being produced at any given point in time. Similarly, it is not feasible to use the standard isotopic procedures for measuring collagen and mucopolysaccharide synthesis in percutaneous needle biopsies of liver because of the limited amount of tissue available.

It has been reported that the activity of collagen proline hydroxylase$^1$, the enzyme responsible for hydroxyproline biosynthesis in collagen, generally reflects the rate of collagen synthesis in a variety of tissues (see Grant and Prockop, 1972, for review). It has also been shown that the uptake of $^{35}$S sulphate determined autoradiographically reflects the rate of sulphated mucopolysaccharide synthesis in experimental liver disease (McGee and Patrick, 1969a and b). Since it seemed possible that this enzyme and $^{35}$S sulphate uptake could be measured in needle biopsies of liver taken for diagnostic purposes, the present investigation was undertaken with a small number of normal specimens and with other specimens from a variety of hepatic diseases. The techniques used allow for simultaneous histopathological examination of the biopsy material.

Materials and Methods

3,4 $^3$H-proline (specific activity 38Ci per mole) was purchased from New England Nuclear Enterprises, Frankfurt, Germany; $^{35}$S sulphate (specific activity 100 mCi per mole) from the Radiochemical Centre, Amersham, England; minimum essential medium from Biocult, Paisley, Scotland; Triton X-100 and

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$^1$Collagen proline hydroxylase has also been referred to as prolyl-hydroxylase, protocollagen proline hydroxylase, and peptidyl proline hydroxylase.

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ferrous ammonium sulphate from British Drug Houses, Liverpool, England; all other reagents

Most of the liver biopsies were carried out by the
percutaneous route using a Menghini needle and
a few by open surgical operation.

MEASUREMENT OF COLLAGEN PROLINE
HYDROXYLASE ACTIVITY

Entire needle biopsy specimens were frozen im-
mediately onto a metal cutting block on top of a
layer of partially frozen 0.15 M NaCl by immers-
ing the base of the chuck in a mixture of ethanol and
solid CO$_2$. Surgical wedge biopsy specimens were
trimmed to exclude the edges of excision and the
liver capsule before freezing in the same manner.
A total of seven or 12 sections were cut from the
needle and surgical specimens respectively in a crys-
stat maintained at $-15^\circ\text{C}$. The first and last sections
(8 $\mu$m thick) were processed for histological exami-
nation to determine the purity of the samples used
for assay; those contaminated with other tissues,
such as skeletal muscle or large pieces of blood clot,
were excluded from the study. The intervening un-
thawed sections (20 $\mu$m thick) were used for the
measurement of collagen proline hydroxylase activi-
ty. These were transferred by a cold needle to a
1 ml glass-teflon homogenizer which was stored in
the cryostat on ice. The sections were homogenized
at 4°C in 0.5 ml of 0.05 M Tris-HCl, pH 7.2, con-
taining 0.25 M sucrose, 10$^{-5}$ M ethylenediamine-
tetraacetic acid, 10$^{-3}$ M diethiothreitol, 0.1% Triton
X-100, and 50 $\mu$g/ml of phenylmethylsulphonyl
fluoride. Aliquots of the homogenate were assayed
in duplicate for enzyme activity by the tritium release
method using a 3,4$^3$H-proline-labelled substrate
(Hutton, Tappel, and Udenfriend, 1966); these
assays were performed immediately since the ac-
tivity of the enzyme rapidly deteriorates in crude liver
homogenates. The reaction mixture for the assay
contained the following reagents in a final volume of
1 ml: 50 $\mu$moles of Tris - HCl (pH 7.2); 5 $\mu$moles of
ascorbate; 1 $\mu$ mole of ferrous ammonium sulphate;
2 mg of heat-denatured bovine serum albumin;
0.4 mg of catalase; 1 $\mu$ mole of $\alpha$-ketoglutarate; 0.1
$\mu$ mole of diethiothreitol; 0.1 ml of $^3$H-proline-labelled
substrate; 0.05-0.20 ml of liver homogenate as
enzyme. The complete mixture was incubated at
30°C and the reaction terminated after 30 minutes
by the addition of 0.1 ml of 50% trichloroacetic acid.
The concentration of Fe$^{2+}$, ascorbate, and $\alpha$-ketog-
lutarate is 10 times higher than that usually em-
ployed (McGee, Langness, and Udenfriend, 1971) in
order to increase the sensitivity of the assay. It has
been shown that the tritium released into water in
this assay is proportional to the formation of hy-
droxyproline (Hutton et al., 1966). Separate aliquots
of the homogenate were taken for protein estimation
by the method of Lowry, Rosebrough, Farr, and
Randall (1951) using bovine serum albumin as a
standard. The collagen proline hydroxylase activity
of each biopsy specimen was expressed as counts per
minute (cpm) released from the tritium-labelled sub-
strate per milligram of tissue protein in the homo-
genate. Tissue protein was chosen as the reference
point for enzyme activity rather than tissue mass
since it is impracticable on a routine basis to weigh
cryostat sections. Variation in protein concentration
of the homogenates did not affect the linearity of the
enzyme assay (see results).

The tissue remaining after sections had been taken
for the hydroxylase assay was processed routinely
for histopathological examination. It was entirely
adequate in size and preservation for diagnostic
purposes.

$^{35}S$ SULPHATE UPTAKE

Immediately after the biopsy specimen was taken a
small piece (3 mm x 1 mm) was cut from each end
and placed in 5 ml of HEPES-buffered minimum
essential medium, pH 7.2, containing 10$^{-3}$M ascor-
bate, 10$^{-4}$M ferric nitrate, and 250 $\mu$Ci of $^{35}S$ sul-
phate in a sterile universal container. The tissue was
incubated at 37°C for 90 minutes in a 95% O$_2$, 5% CO$_2$
atmosphere. After incubation the medium was
decanted, the tissue washed three times with 10%
buffered formalin, pH 7.0, and fixed in the same
solution for 16 hours. Thereafter it was processed
routinely to paraffin and autoradiographs were pre-
pared using Ilford G5 nuclear track emulsion. The
autoradiographs were exposed for six weeks, de-
veloped by routine methods, and then stained with
neutral red.

Results

The procedure used for the measurement of collagen
proline hydroxylase activity in human liver was
worked out initially on a large sample of tissue
obtained from a renal transplant donor immediately
after death. After excision the liver was immersed
in liquid nitrogen and stored at $-80^\circ\text{C}$. Of the various
homogenization conditions used for the measure-
ment of hepatic collagen proline hydroxylase it was
found that 0.05 M Tris-HCl (pH 7.2) containing
0.1% Triton and 10$^{-3}$ dithiothreitol yielded optimum
enzyme activity (table I). This buffer was used
throughout the study. As shown in fig 1 the release of
tritium from 3,4 $^3$H-proline-labelled substrate is
directly proportional to the amount of liver homo-
genate. The linearity of the reaction was not affected
by changes in the protein concentration of the
Additions to Buffer

<table>
<thead>
<tr>
<th>Collagen Proline Hydroxylase (cpm/mg protein)</th>
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<tbody>
<tr>
<td>10⁻⁴M Dithiothreitol</td>
</tr>
<tr>
<td>10⁻⁴M Dithiothreitol + 0.1% Triton X-100</td>
</tr>
<tr>
<td>10⁻³M Dithiothreitol</td>
</tr>
<tr>
<td>10⁻²M Dithiothreitol + 0.1% Triton X-100</td>
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</tbody>
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Table I Hepatic collagen proline hydroxylase activity in different homogenization buffers

1In each experiment the buffer consisted of 0.05 M Tris-HCl (pH 7.2) containing 0.25 M sucrose, 10⁻⁴M EDTA, and phenylmethylsulphonic fluoride to a concentration of 50 μg/ml. Additions to this buffer were made as indicated. An approximately equal weight of human liver was homogenized in 40 volumes of each buffer and aliquots were assayed immediately for collagen proline hydroxylase activity.

Homogenates over the range 0.5-2.2 mg/ml. Tritium release is entirely dependent on α-ketoglutarate (fig 1), a cofactor not required by other hepatic hydroxylases. The variability of the assay in this laboratory was determined by measuring the activity of partially purified mouse skin collagen proline hydroxylase (Rhoads and Udenfriend, 1970). The results of assaying an aliquot of this enzyme preparation with the same substrate on five consecutive days were 951, 1081, 1120, 980, and 901 cpm.

Collagen proline hydroxylase activity in four normal livers, in two cases of Gilbert’s syndrome, and in four examples of hepatic cirrhosis are recorded in table II.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Proline Hydroxylase (cpm/mg protein)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>1158 (726, 893, 1459, 1553)</td>
</tr>
<tr>
<td>Gilbert’s disease</td>
<td>1310 (946, 1674)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td></td>
</tr>
<tr>
<td>Mildly active</td>
<td>3585</td>
</tr>
<tr>
<td>Moderately active</td>
<td>6000</td>
</tr>
<tr>
<td>Aggressive</td>
<td>9366 (9144, 9588)</td>
</tr>
</tbody>
</table>

Table II Collagen proline hydroxylase activity in histochemically normal liver and in cirrhosis

1The diagnosis in each instance was established by clinical and histopathological criteria.

2The mean value is shown for each group where more than one patient was studied. The individual values within each group are shown in brackets.

3See figs 2a-c.

The normal liver values were derived from patients with no biochemical or histological hepatic abnormality; two were undergoing operation for staging of Hodgkin’s disease (726 and 1459 cpm/mg), the third was a psychotic patient with minimal hepatomegaly (893 cpm/mg), and the fourth was the transplant donor referred to above (1553 cpm/mg). The mean value for those patients with Gilbert’s syndrome (serum bilirubin 3 mg/100 ml)—a hepatic disorder not associated with connective tissue formation—fell within the normal range. In contrast, the proline hydroxylase activity in four patients with cirrhosis was several-fold higher than normal. The case of cirrhosis, which was aggressive (fig 2a) by standard histological criteria (Popper, 1966), had an enzyme value six times higher than the highest control, while that of moderately active cirrhosis (fig 2b) and of mildly active cirrhosis (fig 2c) were four-and two-fold higher than the highest control.

Of particular interest was a small group of patients with raised hepatic collagen proline hydroxylase activity who did not have cirrhosis, and some of whom had little or no morphological evidence of collagen deposition at the time of biopsy. The enzyme levels together with the morphological diagnosis and the degree of fibrosis in these cases is shown in table III. Patients 1-4 had in common a history of chronic alcoholism and all had at least one frankly abnormal liver function test on admission; all had proline hydroxylase values several times normal. The main clinical finding in patients 1 and 2 was hepatomegaly. Patients 3 and 4 had marked portal and periportal fibrosis together with the other morphological and clinical stigmata of alcoholic liver disease.
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Fig 2  Light micrographs of (a) aggressive, (b) moderately active, and (c) mildly active cirrhosis. In (a) there is marked chronic inflammatory cell infiltration of fibrous septa, piecemeal necrosis and fine (immature) collagen fibre deposition (arrows) at the periphery of regenerative nodules. In (b) there is inflammatory cell infiltration of the septa with slight erosion of the edge of regenerative nodules. The collagen proline hydroxylase activity found in these biopsies expressed as cpm/mg protein in the liver homogenate was (a) 9144, (b) 6000, and (c) 3585. (a) Haematoxylin and Van Gieson’s stain, $\times$ 225; (b and c) haematoxylin and eosin, $\times$ 140.

Fig 2c  In (c) the fibrous septa consist mainly of thick (mature) collagen bundles and there is little inflammatory cell infiltrate.
Table III  Collagen proline hydroxylase activity in non-cirrhotic liver disease

1Fibrosis was assessed morphologically. Patients 3 and 4 had moderate portal and periportal fibrosis.

Patient 5 had a persistent hepatitis with intrahepatic cholestasis, but no evidence of hepatic fibrosis at the time of biopsy. She also had a serum smooth muscle antibody but no antimitochondrial antibody. This woman, possibly a developing case of active chronic hepatitis, had a hydroxylase level five times higher than the mean normal value.

The method adopted for the assessment of sulphated mucopolysaccharide biosynthesis was the uptake of $^{35}$S sulphate. Representative results from a study of nine cases of chronic liver disease are illustrated in figures 3-5. A case of congenital hepatic fibrosis, an example of non-progressive hepatic fibrosis, was used as a control in this series. As shown in fig 3b there is little uptake of $^{35}$SO$_4$ except in a few cells in the connective tissue bands and in isolated sinusoidal cells. This is comparable to the degree of cell labelling in normal liver. In comparison, the uptake in vitro of the isotope in aggressive liver disease typified by primary biliary cirrhosis and acute alcoholic hepatitis is much more marked (figs 4b and 5). It is pronounced at the sites of connective tissue formation and in the portal tracts. Rather surprisingly, however, the isotope is also avidly taken up by numerous sinusoidal cells throughout the liver. Mast cells, which are known to incorporate $^{35}$S sulphate, were inconspicuous in these biopsies as determined by toluidine blue staining, and could not account for the results obtained.

![Fig 3a](http://gut.bmj.com/content/15/4/260/F3.a)

**Fig 3a** Liver in congenital hepatic fibrosis. (a) The light micrograph shows numerous small bile ducts in mature fibrous septa. H and VG, $\times$ 159.

![Fig 3b](http://gut.bmj.com/content/15/4/260/F3.b)

**Fig 3b** The autoradiograph shows uptake of $^{35}$S sulphate confined to a few mesenchymal cells (arrows) in the fibrous bands and to mucinous material in bile ducts, $\times$ 159.
**Fig 4** Liver in primary biliary cirrhosis. (a) The light micrograph shows chronic portal and periportal inflammation with fibrosis and an abnormal dilated interlobular bile duct. H and E, × 105. (b) The autoradiograph shows considerable uptake of $^{35}$S sulphate at the sites of connective tissue deposition. There is also labelling of many hepatic sinusoidal cells, × 105.

**Fig 5** Liver in alcoholic hepatitis with fatty change. This autoradiograph illustrates a notable degree of $^{35}$S sulphate uptake by sinusoidal cells (arrows) in this form of chronic aggressive liver disease, × 560.
Discussion

Collagen proline hydroxylase, an enzyme specific to the collagen pathway, catalyses the biosynthesis of prolylhydroxylase, an amino acid found only in collagen and to a very limited extent in elastin, by hydroxylating certain prolyl residues in growing collagen α chains (Miller and Udenfriend, 1970). It does not hydroxylate free proline (Hutton and Udenfriend, 1966) but only peptidyl proline occurring in sequences of the general structure -X-Pro-Gly- where X can be one of several amino acids (McGee, Rhoads, and Udenfriend, 1971); the bulk of the collagen molecule is composed of repeating -X-Pro-Gly- triplets. Collagen proline hydroxylase requires as cofactors for its action O2, Fe++, α-ketoglutarate, and a reducing reagent such as ascorbate. It is the prototype of a new class of oxygenases which stoichiometrically decarboxylate α-ketoglutarate during the hydroxylation process (Udenfriend, 1970). In this respect it differs from the many other hepatic hydroxylases. The fact that the enzyme activity assessed in this study was totally dependent in the presence of α-ketoglutarate indicates that only collagen proline hydroxylase activity was measured. Several observations suggest that the tissue level of collagen proline hydroxylase is in general an indicator of fibroblastic biosynthetic activity. In physiological conditions where collagen production is elevated, as in the developing embryo (Mussini, Hutton, and Udenfriend, 1967) and in the pregnant uterus (Halme and Jääskeläinen, 1970), activity is increased. High enzyme levels have also been found in other collagen formative conditions such as healing wounds and granulomas (Mussini et al, 1967), developing experimental cirrhosis (Takeuchi and Prockop, 1969), alcoholic liver disease in animals, (Feinman and Lieber, 1972), and experimental pulmonary fibrosis (Halme, Uitto, Karhunen, and Linay, 1970). In acute liver disease induced in mice it has been shown that there is in fact a rough parallelism between hydroxylase activity and the rate of collagen synthesis in liver (McGee, O'Hare, and Patrick, 1973). On the basis of this evidence it is reasonable to assume that the high levels of collagen proline hydroxylase found in human cirrhosis in the present study are probably a manifestation of a general increase in fibroblastic biosynthetic activity. This interpretation of the results in the cirrhotic patients is in keeping with the finding that the hydroxylase level in individual cirrhotics showed a good positive correlation with the degree of aggressive activity in each case assessed morphologically; collagen formation at the periphery of regenerating nodules is a prominent feature of progressive liver disease (Popper, 1966). The measurement of the hepatic level of this enzyme may be, therefore, a useful quantitative index of progression of the cirrhotic process in individual patients. It may also prove to be helpful in assessing the effect of therapeutic agents on collagen metabolism in chronic liver disease. The increase in collagen proline hydroxylase activity in the alcoholic patients and in the case of persistent hepatitis without noteworthy fibrosis requires discussion. If, as argued above, this observation is a reflection of a general increase in collagen biosynthetic activity, it has to be postulated that collagen degradative mechanisms at the time of biopsy were capable of preventing a net morphological increase in collagen deposition. In experimental hepatic fibrosis collagen proline hydroxylase activity begins to increase before fibrosis (McGee and Patrick, 1972; McGee et al, 1973) or cirrhosis (Takeuchi and Prockop, 1969) is morphologically evident. If the latter experimental observations are applicable to human liver disease the measurement of hepatic collagen proline hydroxylase activity may be of value in predicting which types of liver damage may eventually progress to chronic liver disease and cirrhosis. The group of patients under discussion is being enlarged and followed up therefore to determine whether this is so.

Until recently it has been difficult to understand why collagen proline hydroxylase should rise in conditions characterized by increased collagen formation since it has not been shown that this enzyme is rate limiting in collagen biosynthesis. The recent finding that proline hydroxylation is required for the assembly of a thermally stable triple helical collagen molecule (Jimenez, Harsch, and Rosenbloom, 1973; Berg and Prockop, 1973), however, indicates that elevated hydroxylase levels in cirrhosis and other collagen formative conditions is biologically necessary for the production of collagen molecules which will not spontaneously denature at body temperature.

The results of the 35S sulphate investigation demonstrate that there is a very marked increase in sulphated mucopolysaccharide synthesis in active chronic liver diseases. Rather surprisingly it was found that this increase was not confined to the areas of connective tissue formation in the portal tracts but was also prominent within hepatic sinusoids. It would seem therefore that connective tissue biosynthesis is increased throughout the entire liver in chronic progressive liver disease. Although autoradiography is a useful procedure for determining the sites of intrahepatic fibrogenesis it suffers from the disadvantage that it is not easily made quantitative and the results are not available for a period of weeks. The results of collagen proline hydroxylase measurements, however, are quantitative and are
available about 2-5 hours after the biopsy has been taken, while the tissue remaining is entirely adequate for histopathological diagnosis.

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References


