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Molecular characteristics of dimethylnitrosamine induced fibrotic liver collagen

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Abstract

The molecular characteristics of purified pepsin solubilized collagen from rat liver was studied in control and dimethylnitrosamine administered animals. The α- and β-chains of purified pepsin solubilized liver collagen were separated by subjecting the denatured collagen to SDS-polyacrylamide gel electrophoresis. The $\alpha 1$(III) chains were resolved from the $\alpha 1$(I) chains by interrupted electrophoresis with delayed reduction of the disulfide bonds of type III collagen. The aldehyde content of the purified pepsin solubilized collagen was estimated in control and experimental samples in order to assess the extent of collagen cross-links. Fibril formation curves were studied with purified pepsin solubilized collagen to see the rate of formation of cross-links within the fibrillar mesh. The results of the unreduced electrophoretic studies revealed a significant increase in the β-subunit of type I collagen with a remarkable decrease of α/β ratio in DMN treated animals. Reduction with β-mercaptoethanol indicated the presence of type III collagen in the electrophoretic field with a proportionate increase on the 21st day. A significant increase in the aldehyde content and an increased rate of fibril formation were noticed in DMN induced fibrotic liver collagen. The data of the present investigation revealed that the DMN induced fibrotic liver collagen is more cross-linked than normal liver collagen and the deposition of type III collagen is more prominent than type I collagen in early fibrosis.

Keywords: Dimethylnitrosamine; Hepatic fibrosis; Liver collagen; Fibril formation

1. Introduction

The biochemical properties of the connective tissue depend essentially upon the structural and the three-dimensional mesh work of the collagen bundles embedded in a hydrated ground substance. During biosynthesis, collagen is modified by a series of post-translational modifications which are necessary to carry the nascent polypeptide chains to the extracellular matrix and to ensure proper fibril formation [1]. Disorders affecting any of these steps have profound effects on the connective tissue structure. A variety of human conditions, normal and pathological, involve the ability of tissues to repair and regenerate the collagenous framework. Some of these conditions are characterized by excessive formation and deposition of collagen in various tissues and are generally classified under the term 'fibrosis' [2].

To ensure the formation of functional extracellular matrix, it is necessary to control the production of collagen in vivo. In addition, the mechanism responsible for the control of this normally stable protein must be capable of adjusting the requirements of altered synthesis and degradation, as in periods of rapid growth or during the tissue remodelling that follows injury. Dimethylnitrosamine administration is found to cause excessive deposition of extracellular matrix proteins, especially of collagen in the rat liver [3–5]. This animal model appears appropriate for the study of the early events associated with the development of hepatic fibrosis [6]. Quantitation of various collagen types in normal and cirrhotic human liver has been carried out by Rojkind [7]. A detailed study on the role of intracellular enzymes in collagen biosynthesis in rat liver during hepatic injury induced by dimethylnitrosamine has been reported [8,9]. However no data is available on the influence of dimethylnitrosamine on the biochemical properties of connective tissue collagens during hepatic fibrosis. Similarly, no work has been carried out on the effect of dimethylnitrosamine on the cross-linking of liver colla-

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gen. Therefore, a systematic investigation has been undertaken to study the molecular characteristics of purified liver collagen in experimentally induced hepatic fibrosis.

2. Materials and methods

2.1. Chemicals

Dimethylnitrosamine, phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), pepstatin, pepsin, L-hydroxyproline, Chloramine-T, acrylamide, *N,N'*-methylene-bis-acrylamide, sodium dodecyl sulfate, ammonium persulfate, *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED), β-mercaptoethanol and Coomassie brilliant blue R-250 were purchased from Sigma (St. Louis, MO, USA). Formaldehyde and methyl cellosolve were procured from Fluka AG, Switzerland and *p*-dimethylaminobenzaldehyde from E. Merck, Darmstadt, West Germany. Ethylenediaminetetraacetic acid (EDTA) and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were obtained from Loba Chemie, Bombay, India. All other chemicals used were of analytical grade.

2.2. Animals

Adult male albino rats of Wistar strain at the age group of three months and weighing between 180–200 g were used for the induction of liver injury. The animals were bred and maintained under 12 h light/12 h dark cycles in the air-conditioned animal house with commercial rat feed (Hindustan Lever, Bombay, India) and water available ad libitum. They were housed in polypropylene cages with a wire mesh top and a hygienic bed of husk.

2.3. Induction of hepatic fibrosis

Hepatic fibrosis was induced by intraperitoneal injections of dimethylnitrosamine (DMN) in doses of 1 μl (diluted to 1:100 with 0.15 M sterile NaCl)/100 g body weight. The injections were given on the first three consecutive days of each week over a period of 21 days. Control animals also received an equal volume of 0.15 M NaCl without DMN. The animals were injected without anaesthesia. Treated animals were sacrificed on days 7, 14 and 21 of the experiment. Some of the control animals were sacrificed at the beginning of the experiment and some together with the treated animals on days 7, 14 and 21. All the animals were anaesthetized with diethyl ether before sacrifice. The livers were rapidly removed, rinsed in cold saline and stored at −70°C until analyzed.

2.4. Assessment of the degree of hepatic fibrosis

The degree of hepatic fibrosis was evaluated both histopathologically and biochemically. The histopathological examination of DMN treated liver tissue showed Mallory's hyaline within cytoplasm, mitosis, apoptosis, bridging necrosis and centrilobular fibrosis. The biochemical evaluation revealed a 4-fold increase in the total collagen content in the liver by the 21st day of DMN treatment.

2.5. Extraction of liver collagen

The liver collagen was extracted and fractionated according to the method described by Rojkind et al. [10] with certain modifications. About 50 g liver tissue was homogenized in a Polytron homogenizer (Kinematica GmbH, CH-6010 Kriens, Switzerland) in 10 volumes of double distilled water containing 20 mM EDTA for inhibiting metalloproteinases, 1 mM phenylmethylsulfonyl fluoride for inhibiting serine proteinases, 2 mM *N*-ethylmaleimide for inhibiting sulfhydryl proteinases and 1 μg/ml pepstatin for inhibiting carboxyl proteinases [11]. The entire operation was carried out at 4°C in a cold room in order to minimize bacterial contamination, enhance the solubility of native collagens and ensure the retention of native conformation on the part of the solubilized collagen. The homogenized suspension was stirred for 24 h using a magnetic stirrer and centrifuged at 15 000 × *g* for 30 min at 4°C in a Hitachi, model himac SCR 20B refrigerated centrifuge (Hitachi, Tokyo, Japan). The supernatant containing blood-borne contaminants was discarded and the residue was collected.

2.5.1. Neutral salt soluble collagen

The above residue was homogenized and resuspended in 10 volumes of 1 M NaCl with 0.05 M Tris (pH 7.5) containing all the proteinase inhibitors. It was stirred for 24 hours and centrifuged at 18 000 × *g* for 30 min in a refrigerated centrifuge. The supernatant was collected and the remaining residue was further extracted with 1 M NaCl containing 0.05 M Tris with inhibitors under identical conditions. It was centrifuged and the supernatant obtained was mixed with the first one.

2.5.2. Acid soluble collagen

The residue obtained after neutral salt soluble collagen extraction was homogenized and dissolved in 10 volumes of 0.5 M acetic acid with proteinase inhibitors. It was stirred for 24 h, centrifuged at 18 000 × *g* for 30 min and the supernatant was collected. The extraction was repeated and the supernatant obtained was combined with the above acid extract.

2.5.3. Pepsin solubilized collagen

The above residue was resuspended in 10 volumes of 0.5 M acetic acid and mixed with 500 mg pepsin per 10 g solid (5%). It was digested for 24 h at 4°C by stirring gently using a magnetic stirrer. The digested suspension was centrifuged at 18 000 × *g* for 1 h and the supernatant was collected. The digestion was repeated with further

addition of pepsin (5%) and the supernatant was combined with the above pepsin extract.

2.5.4. Insoluble collagen

The residue left behind after pepsin digestion was dissolved in 10 volumes of 0.5 M acetic acid and analyzed for collagen by hydroxyproline assay. The amount obtained was treated as insoluble collagen.

2.6. Purification of pepsin solubilized liver collagen

The pepsin solubilized liver collagen was purified according to the method described by Rojkind et al. [11] with modifications. To the collagen extract, crystalline NaCl was added slowly with constant stirring to a maximum of 3 M. The precipitation of collagen was completed by stirring for 2 hours in the cold. The precipitated collagen was allowed to settle down without stirring overnight at 4°C. It was centrifuged at 27 000 × *g* for 1 h in a Hitachi ultracentrifuge, model himac SCP 70G and the residue was redissolved in 0.5 M acetic acid and reprecipitated by adding 3 M NaCl. The precipitated collagen was again separated by centrifuging at 27 000 × *g* for 1 h. It was redissolved in 0.5 M acetic acid and dialyzed against 0.02 M disodium hydrogen phosphate until the precipitation was complete. The dissolution of collagen in 0.5 M acetic acid and precipitation by dialysis against 0.02 M disodium hydrogen phosphate was repeated for another two times. Finally the precipitated collagen was dissolved in a small volume of 0.5 M acetic acid, dialyzed exhaustively against 0.15 M acetic acid and lyophilized.

2.7. Estimation of total collagen in liver extracts

The total collagen in the liver extracts was determined by the estimation of hydroxyproline, a characteristic imino acid present in the collagen. For the determination of hydroxyproline, all the samples were hydrolyzed in 6 N HCl in sealed tubes at 110°C for 16 h. The hydrolyzed samples were evaporated to dryness in a boiling water bath to remove acid and the residue was redissolved in distilled water and made up to a known volume. It was treated with activated charcoal to remove the color and filtered through Whatman filter paper. The clear filtrate was used for the determination of hydroxyproline according to the method of Woessner [12]. The collagen content in the liver and collagen fractions were calculated by multiplying the hydroxyproline content by the factor 7.46 as postulated by Neuman and Logan [13] and the results were expressed as mg/g liver tissue wet weight.

2.8. Characterization of α and β-chains of collagen by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The α- and β-chains of purified pepsin solubilized liver collagen were separated by subjecting the denatured collagen to SDS-polyacrylamide gel electrophoresis according to the method of Chan and Cole [14]. The reagents were prepared as described by Blackshear [15]. The purified collagen molecule was dissolved (1 mg/ml) in sample buffer (pH 6.8) containing 2% SDS and denatured by heating at 50°C for 30 min. Polyacrylamide slab gel of 180 mm length, 150 mm width and 1.5 mm thickness was used. Collagen chains were separated on vertical slab gels consisted of 3.5% stacking gel and 5% resolving gel. The α1(III) chains were resolved from the α1(I) chains by interrupted electrophoresis with delayed reduction of the disulfide bonds of type III collagen using 5% β-mercapto-

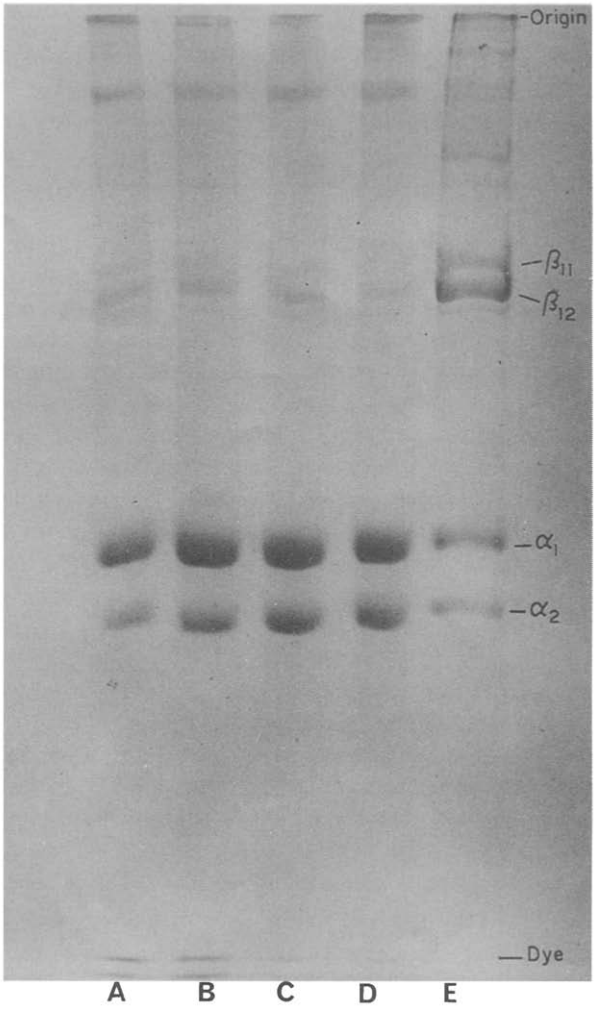

Fig. 1. Unreduced SDS-polyacrylamide gel electrophoretic pattern of purified pepsin solubilized liver collagen. The purified collagen chains were separated on vertical slab gel consisting of 3.5% stacking gel and 5% resolving gel. The collagen molecule was dissolved (1 mg/ml) in sample buffer (0.76 g Tris, 2 g SDS, 10 ml glycerol and water to 100 ml, pH 6.8) and denatured by heating at 50°C for 30 min. About 20 μl of this preparation was loaded into the well. Electrophoresis was carried out at a constant current of 150 V at 20°C until the tracking dye (Bromophenol blue) reached the bottom. Staining was performed with 0.25% Coomassie brilliant blue R-250. Purified rat tail tendon collagen was used as standard. lane A, control; lane B, day 7; lane C, day 14; lane D, day 21; lane E, standard type I collagen from rat tail tendon.

Table 1
Relative distribution of total liver collagen fractions in DMN induced hepatic fibrosis in rats

Collagen fractions	Control	Day 7	Day 14	Day 21
Total collagen	104.51 ± 4.30	184.60 ± 5.41 *	283.22 ± 6.14 *	381.64 ± 8.38 *
Neutral salt soluble collagen	9.46 ± 0.74	4.08 ± 0.39 *	3.82 ± 0.57 *	2.56 ± 0.45 *
Acid soluble collagen	4.46 ± 0.45	5.18 ± 0.47	5.20 ± 0.44	7.98 ± 0.50 *
Pepsin solubilized collagen	81.67 ± 3.10	162.12 ± 4.40 *	253.16 ± 11.18 *	345.51 ± 17.68 *
Insoluble collagen	8.92 ± 1.12	13.22 ± 1.13 *	20.94 ± 1.15 *	25.60 ± 1.78 *

Values are mean ± standard error ($n = 6$).
Values are expressed as mg collagen/100 g liver wet weight.
* $P < 0.001$ (by ANOVA).

ethanol. The separated collagen bands were stained using 0.25% coomassie brilliant blue R-250 for exactly 1 h and destained. The bands were scanned on a Molecular Dynamics personal densitometer, model PD-130 (Molecular Dynamics, 880 East Arques Avenue, Sunnyvale, CA, USA) and the subunit compositions were calculated.

2.9. Aldehyde content in pepsin solubilized collagen

The aldehydes present in purified pepsin solubilized liver collagen were estimated according to the method of Paz et al. [16]. In brief, 0.2 ml of 1% freshly prepared MBTH was mixed with 1 ml of 0.1% collagen in 0.5 M acetic acid and kept in a boiling water bath for 3 min. The tubes were cooled and 2.5 ml of 0.2% ferric chloride was added and allowed to stand at room temperature for 5 min. It was mixed with 6.5 ml of acetone and the intensity of the color was measured in a Shimadzu UV-160 spectrophotometer at 670 nm. Formaldehyde was used for the preparation of standard curve.

2.10. Fibril formation

The fibril formation curves of pepsin solubilized liver collagen were studied by the method of Gelman et al. [17] in order to investigate the rate of cross-link formation in DMN induced collagen. The fibril formation is attributed to alignment of collagen molecules in a quarter stagger form. Two ml of the ice cold collagen solution (1 mg/ml) in a cuvette was mixed with 2 ml of 30 mM phosphate buffer, 0.15 M NaCl (pH 7.3). It was transferred to the temperature controlled water jacketed sample compartment of a spectrophotometer maintained at 37°C through a thermobath. The increase in opacity was monitored continuously at 313 nm till a constant reading was obtained which indicated the completion of fibril formation.

2.11. Statistical analysis

The data were expressed as mean ± standard error. The results were statistically evaluated using one-way analysis of variance (ANOVA). The control mean collagen values were compared with the treated mean collagen values on days 7, 14 and 21 of the experiment using the least significant difference method. The value of $P < 0.01$ was considered as statistically significant. The linear relationship between the increase in collagen and aldehyde content was studied by calculating the correlation coefficient.

3. Results

3.1. Relative distribution of collagen fractions

The relative distribution of different fractions of collagen extracted from the livers of control and DMN treated rats are presented in Table 1. The results indicated a significant increase in the total collagen level in the livers of all DMN treated animals. The maximum increase was noticed on the 21st day of DMN treatment which was around 4-fold when compared to control values. In contrast to total collagen, a significant decrease was noticed in the case of neutral salt soluble collagen on all days of DMN treatment. In the case of acid soluble collagen, a significant increase was observed only on the 21st day. With regard to the pepsin solubilized collagen the increase was

Table 2
Subunit composition of pepsin solubilized liver collagen during DMN induced hepatic fibrosis in rats (unreduced SDS - PAGE)

Collagen subunit %	Control	Day 7	Day 14	Day 21
$\alpha 1$	45.6 ± 1.47	46.8 ± 1.51	43.2 ± 1.42	37.5 ± 1.28 *
$\alpha 2$	24.5 ± 1.12	23.9 ± 1.04	21.4 ± 0.98	19.3 ± 0.94 *
β	29.9 ± 1.29	29.3 ± 1.23	35.4 ± 1.26	43.2 ± 1.48 **
α/β	2.3 ± 0.08	2.4 ± 0.09	1.9 ± 0.06 *	1.3 ± 0.05 **

Values are mean ± standard error ($n = 5$).
* $P < 0.01$ and ** $P < 0.001$ (by ANOVA).

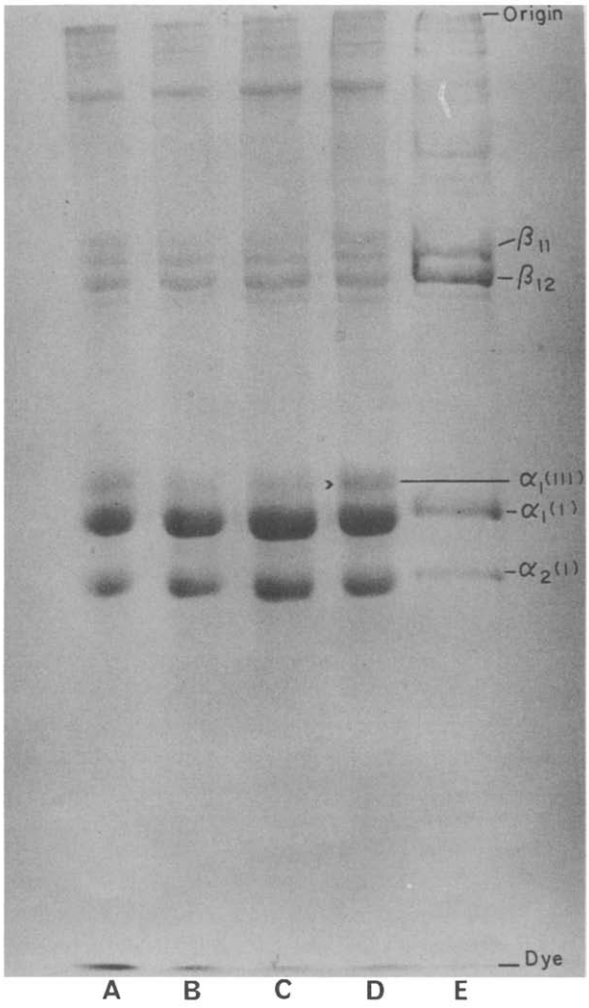

Fig. 2. Reduced SDS-polyacrylamide gel electrophoretic pattern of purified pepsin solubilized liver collagens indicating type III collagen with an increase on day 21 (arrow-head). The electrophoresis was carried out as described in Fig. 1. The α1(III) chains were resolved from the α1(I) chains by delayed reduction of the disulfide bonds of type III collagen molecule using 5% β-mercaptoethanol. lane A, control; lane B, day 7; lane C, day 14; lane D, day 21; lane E, standard type I collagen from rat tail tendon.

highly significant in all experimental animals with a maximum on the 21st day. Among all the fractions analyzed, the highest percentage yield was pepsin solubilized collagen with a minimum of 78% of total collagen in control

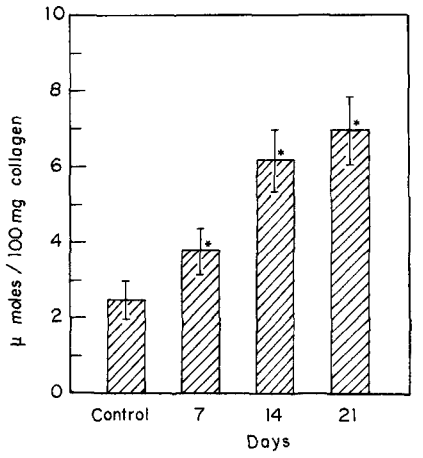

Fig. 3. Aldehyde content of pepsin solubilized liver collagen in DMN induced hepatic fibrosis in rats (* $P < 0.001$ by ANOVA). The aldehyde content was determined spectrophotometrically using *N*-methylbenzothiazolinone hydrazone (MBTH) [16] as colouring reagent and formaldehyde as standard. The values are mean and standard errors.

animals and a maximum of 91% on the 21st day of DMN treated animals. The analysis of the residue left behind after the pepsin digestion revealed a considerable amount of collagen by hydroxyproline analysis which was treated as insoluble collagen. The amount of insoluble collagen also increased significantly during DMN administration (Table 1).

3.2. SDS-polyacrylamide gel electrophoretic analysis of DMN induced liver collagen

The results of the unreduced SDS-polyacrylamide gel electrophoretic studies of DMN induced liver collagen (Fig. 1) revealed a significant increase in the β-subunit of type I collagen with a remarkable decrease of α/β ratio after DMN treatment (Table 2). Reduction with β-mercaptoethanol indicated the presence of α1 chain of type III collagen in the electrophoretic field. A proportionate increase was noticed in the α1 chain of type III collagen in DMN treated animals which was significant on the 21st day (Fig. 2 and Table 3). Laser densitometric scanning revealed a significant decrease in the α1(I)/α1(III) ratio of liver collagen on the 21st day of DMN treatment. The difference was not significant on other days studied (Table 3).

Table 3
Percentage distribution of α-chains of type I and type III liver collagens in DMN induced hepatic fibrosis in rats

α-Chain	Control	Day 7	Day 14	Day 21
α1 (Type III)	11.4 ± 0.68	9.2 ± 0.47	12.2 ± 0.59	23.7 ± 0.93 *
α1 (Type I)	60.3 ± 1.30	59.6 ± 1.07	57.0 ± 1.21	51.9 ± 1.16
α2 (Type I)	28.3 ± 0.86	31.2 ± 0.92	30.8 ± 0.82	24.4 ± 0.71
α1(I)/α1(III)	5.3 ± 0.23	6.3 ± 0.29	4.7 ± 0.18	2.2 ± 0.09 *

Values are mean ± standard error ($n = 5$).
* $P < 0.0001$.

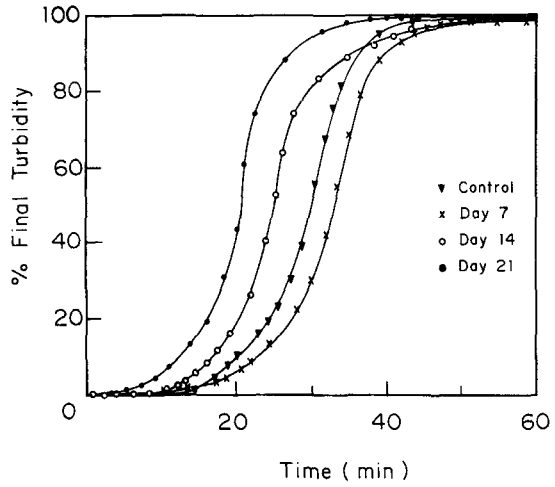

Fig. 4. Normalized fibril formation curves of pepsin solubilized liver collagen presented as percentage final turbidity versus time during varying periods of DMN treatment in rats. The collagen solution was prepared by dissolving purified pepsin solubilized liver collagen in ice cold 0.5 M acetic acid at a concentration of 1 mg/ml with gentle stirring. Two ml of this solution was mixed with 2 ml of 30 mM phosphate buffer (pH 7.3) containing 0.15 M NaCl. The cuvettes, one with blank were transferred to the water jacketed sample compartment of a double beam spectrophotometer maintained at 37°C. The increase in opacity was continuously monitored at 313 nm in order to obtain the fibril formation curve.

3.3. Aldehyde contents

The aldehyde content of the pepsin solubilized liver collagen was significantly increased on all days of DMN treatment (Fig. 3). The maximum increase was noticed on the 21st day of DMN administration which was about 3 times. The pattern of elevation coincided with the increase in total collagen. This was confirmed by the correlation coefficient studies which revealed a significant positive correlation ($r = 0.983$) between the two.

3.4. Fibril formation curves

The fibril formation curves of pepsin solubilized liver collagen of control rats and rats treated with DMN on different days were presented in Fig. 4. It was observed that under identical conditions, the fibril formation of DMN treated collagen started rapidly in comparison with control. This was very prominent in the case of the 21st day of DMN treated collagen (Fig. 4). A significant shift was observed in the fibril formation curves of the 14th and 21st days of DMN treated collagen due to quick and early formation of the gel. But regarding collagen on the 7th day, no such significant difference was observed in the curve, even though the fibril formation started much early. In all the cases, the fibril formation was completed at around 45 min with a maximum absorption on the 21st day of DMN treated collagen. An increase was noticed in the rate of fibril formation on the 14th and 21st days of DMN treated liver collagen.

4. Discussion

It is now well established that hepatic fibrosis is accompanied by deterioration of liver functions and accumulation of collagens in the liver [18–21]. An increase of total collagen content upto 10-fold in humans [22] and upto 8-fold in rats [23] were reported in various types of hepatic fibrosis. The approximate 4-fold increase of total liver collagen observed in the present study coincides with the previous investigations on DMN induced hepatic fibrosis [5,24].

Quantitative data regarding the relative distribution of total liver collagen fractions in DMN induced hepatic fibrosis is not available. An increase in type I and type III collagens were reported in human alcoholic cirrhosis [25,26]. The significant decrease noticed in the level of neutral salt soluble collagen on all days of DMN treatment with a maximum decrease on the 21st day clearly indicates the rapid and extensive formation cross-links in DMN induced liver collagen. This is further supported by the observation of increased aldehyde content and a rapid rate of fibril formation in the present investigation. It was reported that the increase in collagen content in hepatic fibrosis is associated with a higher proportion of insoluble collagen [27,28]. Kucharz [29] observed a reduction of collagen solubility during the induction of fibrosis of the liver which also substantiates the present findings. The absence of a significant increase in the case of acid soluble collagen and an increase of total and pepsin solubilized collagen except on the 21st day of DMN treatment indicates the rapid formation of cross-links which limits the solubility of collagen in dilute acid. The percentage increase noticed in the amount of pepsin solubilized collagen was analogous with an earlier report [7].

It was reported that the peptides derived by cyanogen bromide cleavage of type I collagen of normal and cirrhotic livers have the same electrophoretic mobility on polyacrylamide gels [25]. In the present investigation, both reduced and unreduced SDS-polyacrylamide gel electrophoretic studies provided evidence that the subunit composition of the α- and β-chains of type I collagen have the same electrophoretic characteristics in control and DMN treated animals. The ratio of the proα1(I) mRNA to the proα2(I) mRNAs was about 2:1 in the normal rat liver and remained unchanged during DMN treatment [5]. In the present study, laser densitometric scanning of polyacrylamide gels revealed almost 2:1 ratio for α1(I) and α2(I) chains of control and all the three groups of DMN treated animals (Table 3).

It was observed that fibrosis in the human liver is due to an increase in both type I and type III collagens and that the increase in type III was proportionately larger [25]. A relatively greater accumulation of type III collagen than that of type I collagen was reported in rats with carbon tetrachloride induced hepatic fibrosis [30]. Besides accumulation of other collagen types, an excess deposition of

type III collagen has been noticed in fibrotic human livers also [31]. In the present study, a proportionate increase in type III collagen was noticed in DMN treated animals which was significant on the 21st day (Table 3). This caused a significant decrease in the $\alpha 1(I)/\alpha 1(III)$ collagen ratio on the 21st day of DMN treatment. In this connection, it is interesting to note that slot-blot hybridization studies by using specific cDNA probes revealed that the procollagen mRNA of $\alpha 1(I)$ collagen chain increased 354% while the procollagen mRNA of $\alpha 1(III)$ collagen chain increased upto 486% on the 21st day of DMN induced hepatic fibrosis [5,24]. At the same time the procollagen mRNA of $\alpha 1(IV)$ collagen chain increased upto 655% [5] and upto 866% [24] on the 21st day of DMN treatment. The marked increase in the concentration of the mRNA for type IV collagen suggests that enhanced production of basement membrane collagen may be an early event in the development of hepatic fibrosis [5].

The native preparation of type III collagen molecules are trimers joined by disulfide bonded γ-component which can be reduced to α chains. Under reducing conditions, these bonds are cleaved and the released $\alpha 1(III)$ monomers migrate slightly slowly than $\alpha 1(I)$ chains [32]. In the present investigation, delayed reduction of disulfide linkages with β-mercaptoethanol permitted well separation of $\alpha 1(III)$ collagen chain from $\alpha 1(I)$ chain (Fig. 2).

One important post-synthetic alteration of collagen molecule is the formation of cross-links between peptide chains [33]. The cross-linkage is achieved in many ways. Siegel [34] observed that any increase in collagen synthesis leading to increase in newly formed collagen is associated with an increase in aldehyde content. It was also indicated that an increase in aldehyde content shows a greater potential for cross-link formation [35].

An increase in the level of insoluble collagen content, consistent with an enhanced activity of lysyl oxidase was reported in experimentally induced hepatic fibrosis in the rat [36,37]. An elevated level of serum lysyl oxidase activity was also observed in patients with various liver diseases [38]. Lysyl oxidase is an extracellular copper containing enzyme which oxidatively deaminates the ϵ-amino group of certain lysine and hydroxylysine residues of collagen yielding reactive aldehydes which form stable intra and intermolecular covalent cross-links by further reaction [39,40]. The significant increase observed in the aldehyde content of pepsin solubilized liver collagen obtained from DMN treated animals clearly indicates the higher degree of cross-linkage. In addition, the significant decrease in the neutral salt soluble collagen and the increase in the pepsin solubilized collagen levels noticed in this study further substantiate the above observation.

In the present investigation, it was also observed that the rate of fibril formation was more rapid in DMN induced liver collagen when compared to control. This is substantiated by the observation of reduction of collagen solubility [29] and the increase in the level of insoluble collagen [36,37] during the induction of hepatic fibrosis in rats. Fibril formation is a critical step in the cross-linking and self-assembly of collagen which purely depends upon the parent molecule. The results of the present study and the previous observations clearly indicate that the fibrotic liver collagen is more cross-linked than control liver collagen.

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