fitting onto the active site of the enzyme to allow the formation of the erythro isomers of methylephedrine (DMP IV). The results of Beckett and Mihailova [3] showed that by reduction of the size of the amino group of diethylephedrine (formed by keto reduction of diethylpropion) from *N*-diethyl to $NH_2$ the erythro isomer can be formed.

With DMP II, the bulk of the *N*-methyl groups in the molecule (of DMP I) is decreased, i.e. $N(CH_3)_2$ to $NHCH_3$, thus causing less steric hindrance, which seems to allow the molecule to be orientated either as shown in Fig. 2 or as shown in Fig. 3 (for DMP I). As a result of this both (±)-erythro and (±)-threo DMP V were produced by reduction of DMP II. Therefore, the erythro isomer of DMP V found in the urine after administration of DMP I (see Table 1) is not formed by reduction of DMP I to DMP IV followed by *N*-demethylation, but its demethylation first to DMP II and then reduction to form both isomers of DMP V.

Using the percentage stereoisomeric composition of the amino alcohols excreted in man after the oral doses of DMP I and DMP II (see Table 1), the contribution of each route of metabolism of DMP I to the formation of DMP V is calculated to be 45.6% from the route DMP I → DMP II → DMP V and 54.4% from the route DMP I → DMP IV → DMP V (see Fig. 1).

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# Inhibition of type II procollagen to collagen conversion by lysine derivatives and related compounds
## Mapping of the inhibitory structural features

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Collagens are a family of genetically distinct proteins which are initially synthesized and secreted as precursor forms, procollagens (for review on collagens, see Refs. 1–5). Type II procollagen, the hyaline cartilage-specific member of the family, contains non-collagenous extensions at both the amino- and the carboxy-terminal ends of the molecule. During the extracellular processing, these extensions are removed by specific proteinases, procollagen N-proteinase and C-proteinase respectively [6–8]. The removal of the extensions is necessary for the proper alignment of the collagen molecules to form fibril structures and for subsequent stabilization of the fibrils by intermolecular covalent cross-links [1–5, 7].

Previous studies have demonstrated that several naturally occurring amino acids, polyamines and structurally related compounds effectively inhibit the conversion of procollagen to collagen [9, 10]. Specifically, compounds such as lysine interfere with the removal of the carboxy-terminal extension, and consequently, pC-collagen accumulates in tissues incubated in the presence of the effective compounds. In this study, we have mapped the inhibitory structural features by testing several lysine derivatives and related compounds for their effectiveness in inhibiting the conversion of type II procollagen to collagen.

### *Materials and methods*

The test compounds used in this study (see Table 1) were purchased from the Sigma Chemical Co. (St. Louis, MO), except for Tris and $NH_3$ which were from the Fisher Scientific Co. (Fair Lawn, NJ). The purity of the acetylated lysine derivatives was verified by amino acid analyses. To test the effects of these compounds on the conversion of type II procollagen to collagen, pulse-chase experiments were performed by incubating 17-day-old chick embryo sterna, as indicated previously [10, 11]. All test compounds were soluble in concentrations used. The sterna were labeled for 30 min with [$^{14}$C]proline (pulse) and, after inhibition of the protein synthesis by the addition of 500 μg/ml of [$^{12}$C]proline and 200 μg/ml of cyclohexamide, the test compounds were added; the incubations were continued for 120 min (chase). Radioactive proteins were extracted from the sterna with 3% sodium dodecyl sulfate (SDS) at 100° in the presence of protease inhibitors either at the end of the pulse or following the chase period. The collagenous polypeptides were separated by SDS-polyacrylamide slab gel electrophoresis, and the radioactive peptides were visualized by fluorography, as indicated previously [12, 13]. The bands were quantitated by scanning at 700 nm using an automatic computing densitometer (ACD-18, Gelman Instrument Co.). The conversion of procollagen to collagen was calculated, as indicated previously [10, 11] and shown in Table 1.

### *Results*

When chick embryo sterna were pulse-labeled with radioactive proline for 30 min and then examined at the end of the pulse (t = 0), the major collagenous polypeptides could be identified migrating in the positions of pro$\alpha$ and pC$\alpha$ chains of type II procollagen (Fig. 1, lanes A and B). When the further incorporation of radioactive proline was inhibited and the incubation continued for an additional 60 min (lane C) or 120 min (t = 120), most of the collagenous polypeptides were converted to $\alpha$-chains (Fig. 1, lanes D–F). As demonstrated previously [10], addition of 50 mM L-lysine into the incubation medium at the beginning of the chase period inhibited the conversion of precursor polypeptides to $\alpha$-chains (Fig. 1, lanes G–I).

Utilizing similar pulse-chase experiments, several lysine derivatives were tested for their inhibitory effects on the procollagen to collagen conversion (Table 1). The results indicated that *N*-$\alpha$-acetyl-lysine inhibited the conversion by about 50% while *N*-$\varepsilon$-acetyl-lysine had little, if any, effect. In addition to L-lysine, 50 mM D-lysine was an effective inhibitor, and the conversion was abolished almost com-

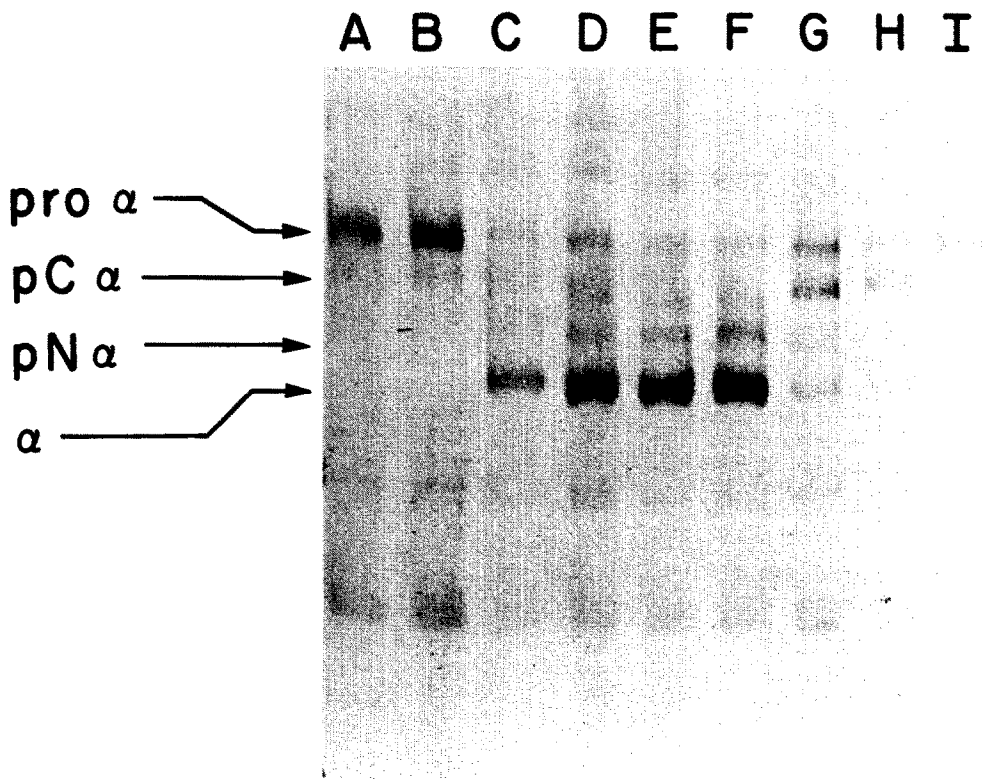


Fig. 1. Conversion of type II procollagen to collagen *in vitro*, and its inhibition by L-lysine. Sterna from 17-day-old chick embryos were pulse-labeled with [$^{14}$C]proline (pulse), and the incorporation of the radioactivity was inhibited by the addition of [$^{12}$C]proline and cyclohexamide, as indicated in Materials and Methods. The test substances were then added, and the incubations were continued for either 60 or 120 min (chase). The samples were examined by SDS-polyacrylamide gel electrophoresis, followed by fluorography. Key: Lanes A + B: samples at the end of the pulse; Lane C: sample after 60-min chase; Lanes D–F: samples after 120-min chase; and Lanes G–I: 50 mM L-lysine added at the beginning of a 120-min chase. The migration positions of pro$\alpha$, pC$\alpha$, pN$\alpha$ and $\alpha$-chains of type II procollagen and collagen are indicated in the figure.

pletely by D-lysine in 100 mM concentration. In addition to free lysines, several dipeptides containing an amino terminal lysine, such as Lys-Leu, Lys-Asp, and Lys-Val, effectively inhibited the conversion (Table 1). However, Lys-Phe was not effective. Also, a poly-L-lysine preparation in the molecular weight range of 1–4 kilodaltons (kD) effectively inhibited the conversion, while poly-L-lysine preparation with the molecular weight range of 30–70 kD was not inhibitory (Table 1).

In further experiments, additional amines were tested. $\alpha$-Aminobutyrate and $\beta$-aminobutyrate had little, if any, effect, while $\gamma$-aminobutyrate in 50 mM concentration caused about 50% inhibition of the conversion. Two small-molecular-weight amines, Tris and $NH_3$, both in 50 mM concentration, had little effect on the procollagen to collagen conversion.

*Discussion*

In this study, we have demonstrated that several amines and lysine-containing dipeptides effectively inhibit the conversion of type II procollagen to collagen. These studies extend our previous demonstrations [10] that several naturally occurring amino acids and polyamines are effective inhibitors of such conversion. The effective compounds were shown to preferentially inhibit the removal of the C-terminal extension from type II procollagen, while little or no effect was seen on the removal of the N-terminal extension [10]. For the purpose of mapping the structural features responsible for the inhibitory capacity of lysine, several lysine derivatives were tested in the conversion system. The *N*-$\alpha$-acetylated but not the *N*-$\varepsilon$-acetylated lysine derivative was an effective inhibitor of the conversion, suggesting that an unsubstituted $\varepsilon$-amino group is a required inhibitory feature on lysine. Similarly, $\alpha$- and $\beta$-aminobutyrates were not effective, while the $\gamma$-aminobutyrate was an effective inhibitor of the conversion. In addition, D-lysine was as effective an inhibitor as the L-isomer. These observations suggest that an inhibitory molecule requires a primary amino group at the end of an aliphatic carbon chain. Previous studies have shown that $\beta$-alanine is a less effective inhibitor than L-lysine [10], and our results here demonstrate that L-lysine is also a better inhibitor than $\gamma$-aminobutyric acid. Thus, the inhibitory capacity of these compounds increases with an increase in the chain length.

In addition to free lysines, two dipeptides, Lys-Leu and Lys-Val, which contain an amino terminal lysine and a nonpolar amino acid on the carboxyl end of the peptide, were potent inhibitors of the conversion. We have shown previously that free leucine and valine do not interfere with the conversion of procollagen to collagen [10] and, therefore, the inhibitory action of the dipeptides is probably attributable to the amino-terminal lysine. It was of interest, that Lys-Asp, which bears a charged amino acid on the carboxy-terminus, was less effective than either Lys-Leu

Table 1. Conversion of type II procollagen to collagen in the presence of amine-containing compounds

| Test compound | Concn. (mM) | Proportion of α-chains* (%) | Relative conversion† (%) |
|---|---|---|---|
| Experiment I | | | |
| Control t = 0 | | 3.9 | 0 |
| Control t = 120 | | 68.6 | 100.0 |
| L-Lysine | 50 | 18.7 | 22.9 |
| L-Lysine | 50 | 25.8 | 33.8 |
| *N*-α-Acetyl-lysine | 50 | 38.3 | 53.2 |
| *N*-α-Acetyl-lysine | 50 | 37.3 | 51.6 |
| *N*-ε-Acetyl-lysine | 50 | 57.6 | 83.0 |
| *N*-ε-Acetyl-lysine | 50 | 64.0 | 92.9 |
| α-Aminobutyrate | 50 | 55.4 | 79.6 |
| α-Aminobutyrate | 50 | 56.7 | 81.6 |
| β-Aminobutyrate | 50 | 66.7 | 97.1 |
| β-Aminobutyrate | 50 | 64.5 | 93.7 |
| γ-Aminobutyrate | 50 | 37.4 | 51.8 |
| γ-Aminobutyrate | 50 | 43.1 | 60.6 |
| Experiment II | | | |
| Control t = 0 | | 21.4 | 0 |
| Control t = 120 | | 90.8 | 100.0 |
| Lysyl-leucine | 50 | 43.2 | 31.4 |
| Lysyl-leucine | 50 | 47.1 | 37.0 |
| Lysyl-valine | 50 | 47.1 | 37.0 |
| Lysyl-valine | 50 | 41.3 | 28.7 |
| Lysyl-aspartate | 50 | 52.2 | 44.4 |
| Lysyl-aspartate | 50 | 57.3 | 51.7 |
| Experiment III | | | |
| Control t = 0 | | 17.0 | 0 |
| Control t = 120 | | 72.3 | 100.0 |
| D-Lysine | 50 | 50.1 | 41.3 |
| D-Lysine | 100 | 28.3 | 9.9 |
| Poly-L-lysine 1–4 kD | 50‡ | 36.2 | 34.7 |
| Poly-L-lysine, 1–4 kD | 50‡ | 34.7 | 32.0 |
| Poly-L-lysine, 30–70 kD | 50‡ | 66.4 | 89.3 |
| Poly-L-lysine, 30–70 kD | 50‡ | 65.9 | 88.4 |
| Lysyl-phenyl alanine | 50 | 71.2 | 98.0 |
| Lysyl-phenyl alanine | 50 | 64.1 | 85.2 |
| Tris | 50 | 65.2 | 87.2 |
| Tris | 50 | 66.0 | 88.6 |
| $NH_3$ | 50 | 66.0 | 88.6 |
| $NH_3$ | 50 | 62.4 | 82.1 |

Chick-embryo sterna were pulse-labeled with [$^{14}$C]proline for 30 min, and protein synthesis was inhibited by the addition of cycloheximide and unlabeled proline. The test compounds were added in the final concentrations indicated, and the incubations were continued for an additional 120 min (chase). The $^{14}$C-labeled collagenous polypeptides were extracted and examined by SDS-polyacrylamide slab gel electrophoresis either at the beginning (t = 0) or at the end (t = 120) of the chase.

* Calculated from the densitometric analyses of the fluorograms depicting the $^{14}$C-labeled collagenous polypeptides (see Fig. 1), as

$$100 \times \frac{\alpha}{\text{pro}\alpha + \text{pC}\alpha + \text{pN}\alpha + \alpha}\,(\%)$$

† Calculated from the proportion of α-chains as

$$100 \times \frac{(\%\ \alpha\,\text{experimental}) - (\%\ \alpha\,\text{control, t} = 0)}{(\%\ \alpha\,\text{control, t} = 120) - (\%\ \alpha\,\text{control, t} = 0)}$$

‡ Molar concentrations of lysyl residues.

and Lys-Val. Lys-Phe, which contains a bulky aromatic amino acid on the carboxy-terminus, was not an effective inhibitor. These observations suggest that the inhibitory capacity of the test compound can be modulated by changing the polarity and the size of the molecule. The differences in the inhibitory capacity of these compounds may also reflect the accommodation of the molecule at the active site of the enzyme, although possible variations in the tissue penetration in this system can not be excluded. Finally, poly-L-lysine in the molecular weight range of 1–4 kD was effective while a similar preparation with a molecular weight range of 30–70 kD was not effective. This difference could also reflect tissue penetration of the compounds.

The exact mechanism of the inhibition of procollagen to collagen conversion by the amines tested here is not clear at present. Previously, Leung *et al.* [9] suggested that arginine might prevent the removal of the C-terminal extension from type I procollagen by interfering with the aggregation of procollagen molecules. We have suggested that the inhibition of removal of the C-terminal extensions from type II procollagen might result from direct inhibition of the procollagen C-proteinase [10]. In support of the latter suggestion was our previous demonstration that the removal of the C-terminal extensions was also inhibited by ε-aminocaproic acid, as well-known proteinase inhibitor with a structural similarity to lysine. Helseth and Veis [14] suggested recently that amines might inhibit the procollagen to collagen conversion through a mechanism which involves elevation of the pH of the lysosomes, thus leading to the inhibition of acidic proteinases which were speculated to participate in the procollagen to collagen conversion. In support of their suggestion was the demonstration that 50 mM Tris was an effective inhibitor of the removal of the carboxy-terminal extension in their system [14]. Under our test conditions, little if any inhibition was noted with 50 mM Tris and ammonia. Thus, our observations further support the hypothesis that critical structural features are necessary for a molecule to be an effective inhibitor of the procollagen to collagen conversion.

Accumulation of collagen is a major pathological feature of various fibrotic diseases affecting the lungs, liver, skin and other animal tissues [5]. Previously, several pharmacologic agents were tested for their potential for limiting collagen deposition in fibrotic conditions [15, 16]. Unfortunately, most of these compounds are not specific for collagen, and their clinical efficacy is frequently compromised by toxicity and long-term side effects. The observations presented in this study suggest that compounds with structural features, which include the presence of a free amino group at the end of an aliphatic carbon chain, appear to inhibit procollagen to collagen conversion in a relatively specific manner. Thus, further development of pharmacologic preparations containing the effective compounds and targeted for tissues exhibiting fibrosis might be helpful in controlling the fibrotic processes.

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## A hydroxymethyl sulphate ester as an active metabolite of the carcinogen, 5-hydroxymethylchrysene

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5-Methylchrysene (5-MCR), a well known environmental carcinogen found in tobacco smoke [1] and having as high carcinogenicity to mouse skin as benzo[*a*]pyrene [2], is oxidized in rat liver microsomes to 5-hydroxymethylchrysene (5-HCR) as a major metabolite and to dihydrodiols and phenols [3]. The carcinogenicity of 5-HCR has been reported to be as high as 5-MCR [4], suggesting it to be one of proximate metabolites, while unsubstituted CR is known to have little or only a weak carcinogenic activity [2]. A similar relationship between the introduction of a methyl or hydroxymethyl group(s) to the polynuclear aromatic hydrocarbon and the remarkable increase in its