IN VITRO FORMATION OF INTRAMOLECULAR CROSSLINKS IN TROPOCOLLAGEN

Arvind D. Deshmukh and Marcel E. Nimni

Department of Medicine, School of Medicine, University of Southern California, Los Angeles, California 90033

Received May 8, 1969

Administration of penicillamine (β,β, dimethyl cysteine) to rats causes a disturbance of collagen metabolism that resembles lathyrism (Nimni and Bavetta, 1965). The neutral salt soluble collagen which accumulates in rats treated with penicillamine consists almost exclusively of α components (Nimni, 1965), but once extracted from tissues and purified, it forms stable fibers at 37°C (Deshmukh and Nimni, 1969a). This contrasts with lathyritic collagen which forms unstable fibers that redissolve in cold neutral salt solutions (Gross, 1963). This fundamental difference in behavior suggested that the soluble collagen extracted from the penicillamine treated animals should be capable of generating stable intramolecular as well as intermolecular crosslinks.

The present communication describes the formation of intramolecular crosslinks in vitro, which in turn, suggests a suitable model for the further study of the biosynthesis and characterization of this type of crosslink.

Materials and Methods. Preparation of acid soluble collagen: Dorsal skin of male Holtzman rats weighing 100-150 grams was used as a source of collagen. D-penicillamine and BAPN (β amino propionitrile) were administered to animals as previously

described (Nimni, 1968). Samples of finely cut skin, free of subcutaneous fat and muscle tissue were washed overnight with 0.15 M NaCl, extracted with 0.5 M acetic acid for 72 hours and the collagen purified by the procedure described by Deshmukh and Nimni (1968) for neutral salt soluble collagen. The final precipitate was dissolved in 0.05 M acetic acid, dialysed against the same solvent overnight and then centrifuged at 105,000 x g for two hours. This supernatant was used in all subsequent studies. All these operations were carried out at 4°C. Aliquots of the final preparation were hydrolysed and the concentration of collagen estimated by determining the hydroxyproline content (Stegemann, 1958; Grant, 1964).

Incubation: Purified acid soluble collagen was dialysed against excess 0.45 M NaCl, pH 7.0 at 4°C and then incubated at 37°C for various time intervals. Aliquots of the original samples were dialysed against 0.45 M NaCl, pH 7.0, containing 0.01 M D-penicillamine and were also incubated as above. At the end of each incubation, the samples were centrifuged at room temperature and the supernatants discarded. The pellets (containing the collagen fibers) were resuspended in 0.5 M acetic acid, shaken overnight at 4°C, and dialysed extensively against 0.5 M acetic acid. The suspensions were finally centrifuged at 105,000 x g for two hours and the supernatants used for further studies. Very little or no insoluble material resulted up to 72 hours of incubation, whereas, after 96 hours at 37°C in absence of D-penicillamine, a small amount of the initial pellet material remained insoluble in cold 0.5 M acetic acid.

Acrylamide disc electrophoresis: Aliquots of each of the collagen specimens were dialysed against cold 0.01 M acetic acid.

After thermal denaturation at 40°C, separation into α and β components was accomplished using the procedure of Nagai et al (1964) modified by Fessler and Bailey (1966). The relative concentration of these components were determined with a Canalco microdensitometer, a set of representative densitometric tracings are shown in Figure 2. Concentrations of collagen, between 10 to 60 mg, plotted against integration of areas corresponding to α and β subunits show a linear relationship using the procedure (Deshmukh and Nimni, 1969b).

CM Cellulose chromatography: 50 mg samples of each were chromatographed in the denatured form on a 2.0 x 18 cm. CM cellulose column (Whatman microgranular CM 32, capacity 1.0 meq/gm) and the fractionation of the material applied to the column was achieved essentially by the procedure of Piez et al (1963). A flow rate of 150 ml/hr was maintained using an LKB ReCyChrom peristaltic pump. The effluent was monitored at 230 mμ in a microflow cell in a Beckman DB-G spectrophotometer and the absorbancy was recorded. At the end of the salt gradient the modification of Veis and Anesey (1965) was introduced. Elution was continued with a 200 ml of 0.44 M NaCl in the starting buffer followed by 1.0 M NaCl containing 6.0 M urea. Collection of the fractions and monitoring of the effluent was continued throughout these elutions.

Results: It is evident from the electrophoretic patterns of the denatured material (Figure 1) that incubation of collagen obtained from penicillamine treated animals results in a decreased α/β ratio. With progressive incubation at 37°C, high molecular weight aggregates appear which were absent in the non-heated material. In contrast to the changes observed with collagen from penicillamine treated animals, collagen from

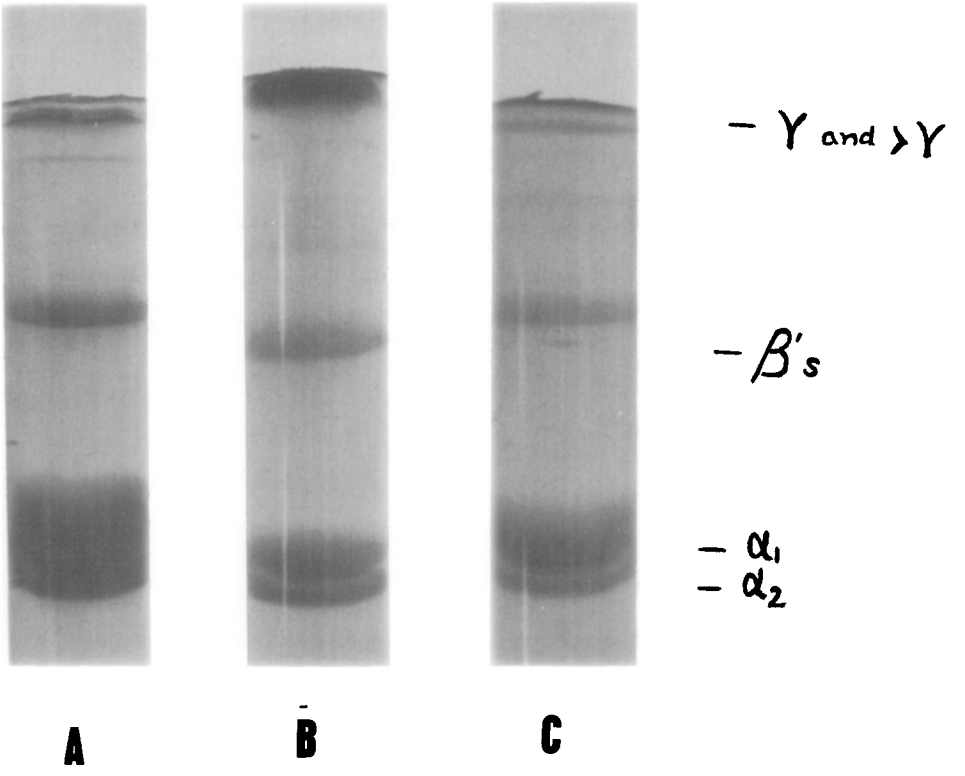

Fig. 1. Electrophoresis in polyacrylamide gels of denatured collagen samples originating from penicillamine treated animals. 25 µl of collagen solution (2.0 mg/ml in 0.01 acetic acid) were applied to the gel. Electrophoresis was conducted at pH 4.0 (Glycine buffer); current, 5 ma/sample; time, 90 min. A = Non-incubated (control), B = incubated for 96 hours at 37°C, C = incubated for 96 hours in the presence of 0.01 M D-penicillamine.

---

lathyritic animals when incubated at 37°C showed no significant change in the α/β ratio. Nevertheless, a small amount of high molecular weight aggregates was formed with a slight decrease in the concentration of β subunits. These observations can be seen more clearly when densitometric tracings corresponding to the denatured collagen subunits separated on acrylamide gel, before and after incubation at 37°C, are compared (Figure 2). In addition to the changes in relative concentration of the α and β components there is a net decrease of both types of

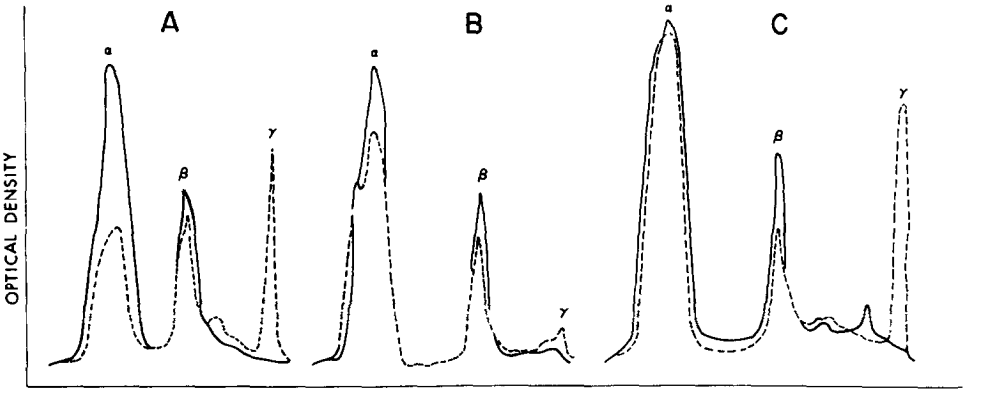

Fig. 2. Densitometric tracings of collagen subunits separated by acrylamide gel disc electrophoresis before (solid line) and after incubation at 37°C for 72 hours (dotted line). A = Acid soluble collagen from skin of D-penicillamine rats. B = Same collagen incubated in the presence of 10^{-2} M D-penicillamine. C = Acid soluble collagen from βAPN treated rats.

subunits which can be partially accounted for by the appearance of γ components or higher polymeric materials.

Table I summarizes the changes in the α/β ratio of acid soluble collagen from penicillamine and BAPN treated rats, incubated at neutral pH in 0.45 M NaCl at 37°C, for various time intervals. The time dependence for the formation of new β components can be clearly appreciated. Addition of 0.01 M penicillamine to the medium prevented the synthesis of β components. Collagen originating from BAPN treated rats was also unable to form new intramolecular crosslinks.

The profiles obtained for similar samples chromatographed on CM cellulose (Figures 3 and 4) support the above observations. Not only do the patterns show considerable changes with respect to α and β components, but they also show the presence of some unidentified peaks which are absent in the original sample.

TABLE I

Subunits present in acid soluble collagen originating from penicillamine and BAPN treated rats incubated at 37°C in 0.45 M NaCl pH 7.0 for various time intervals. Collagen solutions were denatured at 40°C in 0.01M acetic acid prior to disc electrophoresis. (average of 6 to 7 determinations)

Incubation time at 37°C, hours	Conc. of D-penicillamine in the medium	α/β ratio
Penicillamine treated		
0	0	6.0
1	0	6.0
2	0	6.0
4	0	5.0
8	0	5.0
16	0	4.0
24	0	4.0
48	0	2.6
72	0	2.1
96	0	2.0
48	0.01 M	6.0
72	0.01 M	6.0
96	0.01 M	5.7
BAPN treated		
0	0	4.0
96	0	3.8

DISCUSSION

It becomes clear from the data presented, that the soluble collagen which accumulates in large amounts in the soft tissue of animals treated with penicillamine, is normal and that the defect observed *in vivo* is dependent on the continuous presence of penicillamine in the environment.

The formation of a thiazolidine complex between α-amino thiols and aldehyde residues present on the collagen molecule

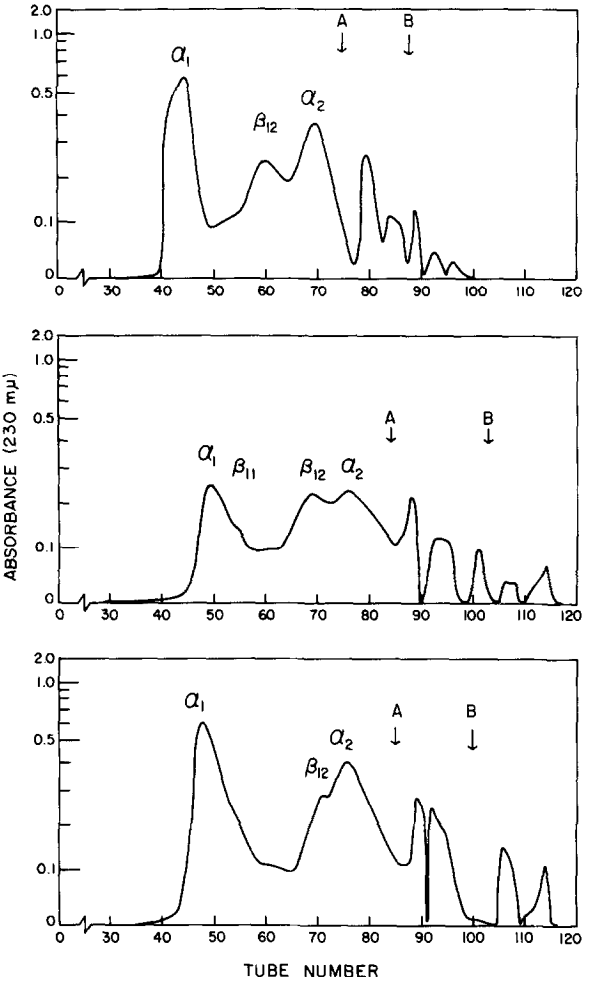

Fig. 3. Carboxymethyl-cellulose chromatograms of denatured skin collagen extracted from penicillamine treated rats with 0.5 M acetic acid. Conditions were: 50 mg collagen, 40°C, column size - 2 x 18 cm, flow rate - 150 ml/hr, buffer - sodium acetate pH 4.8 τ/2 - 0.06, linear gradient to 0.1 M NaCl. Arrow A and B indicate elution with starting buffer containing 0.44 M NaCl and 6.0 M urea plus 1.0 M NaCl, respectively. (Untreated sample -top; sample incubated for 96 hours - middle, and sample incubated in presence of 0.01 M penicillamine - bottom.)

---

has recently been postulated as the mechanism by which penicillamine prevents the formation of covalent crosslinks between adjacent tropocollagen molecules (Deshmukh and Nimni, 1969a). Dissociation of this complex (by prolonged dialysis or by a

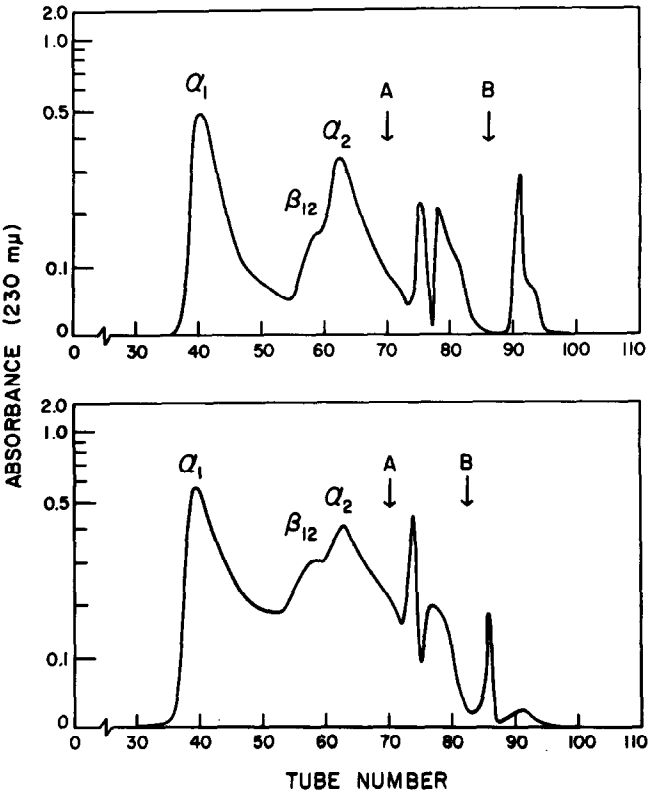

Fig. 4. Carboxymethyl-cellulose chromatogram of denatured skin collagen from BAPN treated rats. Conditions as described in Fig. 3. Untreated sample - top; sample incubated at 37°C. for 96 hours - bottom.

slower in vivo metabolic process) renders this collagen, which consists mostly of uncrosslinked α components capable of forming new intramolecular bonds. In addition, this soluble collagen is able to form macromolecular aggregates which barely penetrate the 7.5% acrylamide gel. Most of these aggregates, when placed on CM cellulose, remain on the column even after elution with urea indicating that they are probably held together by covalent crosslinks and are too large to be eluted.

The soluble collagen from BAPN treated rats, which is known to be defective in aldehydes (Bornstein et al, 1966, Rojkind and

Juarez, 1966), does not show a change in the α/β ratio when incubated at 37°C. Some of the existing subunits which may be normal, are nevertheless able to proceed to form small amounts of high molecular weight aggregates, which are more clearly detectable by acrylamide disc electrophoresis.

Neutral salt soluble collagen from normal rats, which has a high concentration of α components, is also capable of generating new intramolecularly crosslinked β components when incubated at 37°C, although with much lesser efficiency (Deshmukh and Nimni, 1969b).

From the present experiments it can be seen that β components of intramolecular origin can be synthesized in vitro if the precursor aldehydes are present on the molecule and if their reactivity is not hindered by reagents that react with carbonyl groups.

Acknowledgement: This study was supported by N.I.H. research grant #AM-10358.

REFERENCES

Bornstein, P., Kang, A.H., and Piez, K.A., Proc. Nat. Acad. Sci., 55, 417 (1966).
Deshmukh, K., and Nimni, M.E., Biochim. Biophys. Acta, 154 258 (1968).
Deshmukh, K., and Nimni, M.E., J. Biol. Chem., 244, 1787, (1969a).
Deshmukh, K., and Nimni, M.E., Biochem. J., in press (1969b).
Fessler, J.H., and Bailey, A.J., Biochim. Biophys. Acta, 117, 368 (1966).
Grant, R.A., J.Clin. Path., 17, 685 (1964).
Gross, J., Biochim. Biophys. Acta, 71, 250 (1963).
Nagai, Y., Gross, J., and Piez, K.A., Ann. N.Y. Acad. Sci., 121, 494 (1964).
Nimni, M.E., and Bavetta, L.A., Science, 150, 3698 (1965).
Nimni, M.E., Biochim. Biophys. Acta, 111, 576 (1965).
Nimni, M.E., J. Biol. Chem., 243, 1457 (1968).
Piez, K.A., Eigner, E.A., and Lewis, M.S., Biochemistry, 2, 58 (1963).
Rojkind, M., and Juarez, H., Biochem. Biophys. Res. Commun., 25, 481 (1966).
Stegemann, H., Hoppe Seylers Z. Physiol. Chem., 311, 41 (1958).
Veis, A., and Anesey, J., J. Biol. Chem., 240, 3899 (1965).