EXTRACTION OF α AND β COMPONENTS FROM INSOLUBLE COLLAGEN BY THIOL COMPOUNDS

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Received October 17, 1966

Penicillamine (β,β dimethyl cysteine) was reported to induce an accumulation of soluble collagen in the skin of rats (Nimni and Bavetta, 1965). The soluble material was shown to consist almost exclusively of α chains (Nimni, 1965). Subsequently these findings have been confirmed in individuals treated with penicillamine (Harris, Jaffe, and Sjoerdsma, 1966). During the course of studies to determine the "in vitro" effects of penicillamine on collagen metabolism it was observed that this compound, as well as other thiol reagents tested were able to solubilize significant amounts of collagenous material. The conditions for extraction as well as some characteristics of the subunits isolated are discussed in the present communication.

Methods. Dorsal skin from male Holtzman rats weighing 200-220 grams served as a source of collagen. The skin was clipped, cleaned from adhering adipose and muscle tissue and cut into small pieces. Insoluble collagen was obtained by washing the skin three times with 0.5M NaCl for 24 hours and with 0.5M citrate (pH 3.6) for 48 and 24 hours. After each extraction the suspensions were centrifuged at 40,000 x g for one hour and the supernatants analyzed for hydroxyproline. All operations were performed in the cold (4°C). In this way between 7.5 and 9% of the total skin collagen was removed. The residual material was lyophilized and served as a source of insoluble collagen. It was used immediately upon preparation inasmuch as solubility of these

preparations in thiol solutions decreased progressively with storage.

Extractions of the insoluble collagen were carried out in the cold (4°C) over a 72 hour period. After each extraction the preparations were centrifuged and the supernatants were dialyzed against distilled water. The resultant precipitate was hydrolyzed and analyzed for hydroxyproline as previously described (Nimni and Bavetta, 1964). The collagen fraction extracted with 0.1M cysteamine was precipitated by dialysis against water and redissolved in cysteamine solution, reprecipitated by dialyzing against water, and finally dissolved in 0.01M acetic acid and lyophilized.

For ultracentrifugal analysis the lyophilized product was shaken overnight in 0.15M citrate buffer (pH 3.6). For disc electrophoresis it was dissolved in 0.05% acetic acid and the collagen subunits separated following the procedure of Fessler and Bailey (1966). The samples were denatured prior to analysis by heating at 40°C.

Results. Figure 1 shows the percent of collagen that can be solubilized by the thiol compounds from a preparation of skin (not previously extracted

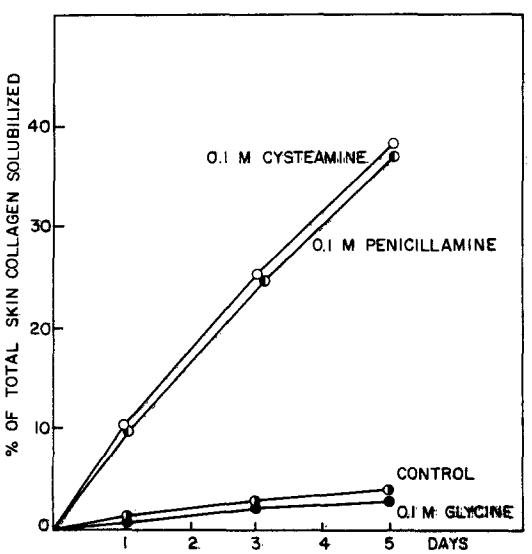

Figure 1. Rate of extraction of collagen from fresh skin by Krebs-Ringer-Phosphate (+ 0.4M NaCl) at 4°C (Control), and by a similar solution containing 0.1M cysteamine, D-penicillamine or glycine (pH = 7.0).

with 0.5M NaCl and 0.5M citrate). The fresh tissue was extracted with Krebs-Ringer-Phosphate (Umbreit et al, 1964) with an additional amount of salt (0.4M NaCl). To this solution (control), either 0.1M D-penicillamine, cysteamine or glycine were added and the pH adjusted to 7.0. Under these conditions the thiol compounds were able to extract significantly more collagenous materials than the controls. The large percent of non-dialyzable hydroxyproline coming into solution indicated that part of this must be derived from the insoluble collagen fraction. To further investigate this possibility all the subsequent work was done with the material remaining in skin after exhaustive extraction with 0.5M NaCl and 0.5M citrate (pH 3.6).

Figure 2A shows the effect of extracting insoluble collagen with varying concentrations of cysteamine. Approximately 80% of the insoluble collagen was dissolved at a concentration of 0.5M. Higher concentrations produced almost complete dissolution. Since it was noticed that the ionic strength seemed to play an important role in the solubilizing effect exhibited by cysteamine this variable was investigated. Maximum extractability in the presence of 0.1M cysteamine occurred when the solution contained 0.8M NaCl (Figure 2B).

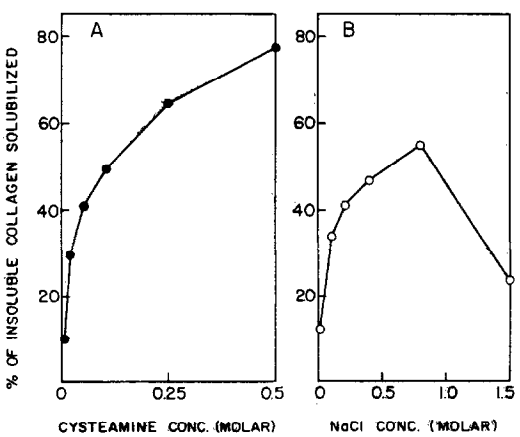

Figure 2. Extractability of insoluble collagen with cysteamine from insoluble collagen.
A. Effects of increasing cysteamine concentration in a constant salt environment (0.4M NaCl, 0.02M phosphate pH = 7.0).
B. Effects of increasing ionic strength in the presence of constant (0.1M) cysteamine buffered to pH 7.0 with 0.02M phosphate).

The effect of pH (with or without 0.1M cysteamine in the media) is shown in Figure 3. Below pH 5 the extractability of collagen was negligible except at the very low pH's were the hydrogen ion concentration begins to degrade the molecule as judged by the appearance of large amounts of dialyzable hydroxyproline peptides. Above pH 5, although in the absence of cysteamine there is some solubilization this was greatly enhanced by the presence of cysteamine. The extraction of further collagenous material by neutral salt solution may be due to the swelling of the insoluble collagen caused by its exposure to acid during the final stage of its preparation since at pH 7.0 and above, the amount of collagen extracted from fresh skin by 0.4M NaCl was negligible.

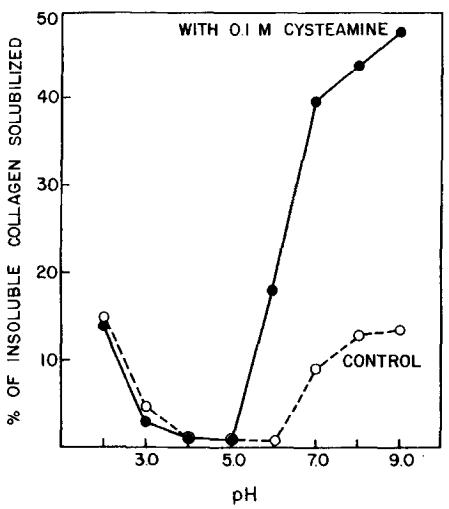

Figure 3. Extractability of insoluble collagen at different pH's by a solution containing 0.4M NaCl (buffered with 0.02M phosphate) with or without 0.1M cysteamine.

The nature of the collagenous material extracted by cysteamine was investigated. The ultracentrifuge pattern as well as the densitometric tracing of the acrylamide gel separations are shown in Figure 4. In all instances the acrylamide gels showed 2 clear bands and no traces of degradation products. The sedimentation coefficients after correcting for concentration and Johnston-Ogston effect (Speakman, 1963) as well as the

electrophoretic movement, served to characterize the presence of α and β components. Densitometry of the acrylamide gel using a Chromoscan and calculations of the areas under the peaks of the Schlieren patterns indicate a mixture consisting of 33% β and 66% α components.

Discussion. It has recently been reported that aggregated collagenous material from the cuticle of the worm Ascaris Lumbricoides, could be solubilized by β-mercaptoethanol (McBride and Harrington, 1965). This appeared to be due to the splitting of disulfide bridges between half-cysteine residues. Native collagen from this species is relatively rich in cysteine (27 residues/1000) whereas in mammalian collagen the presence of cysteine has not been confirmed. Therefore such an explanation cannot at this time be attempted in connection with our findings. Cysteamine, thioglycolic acid

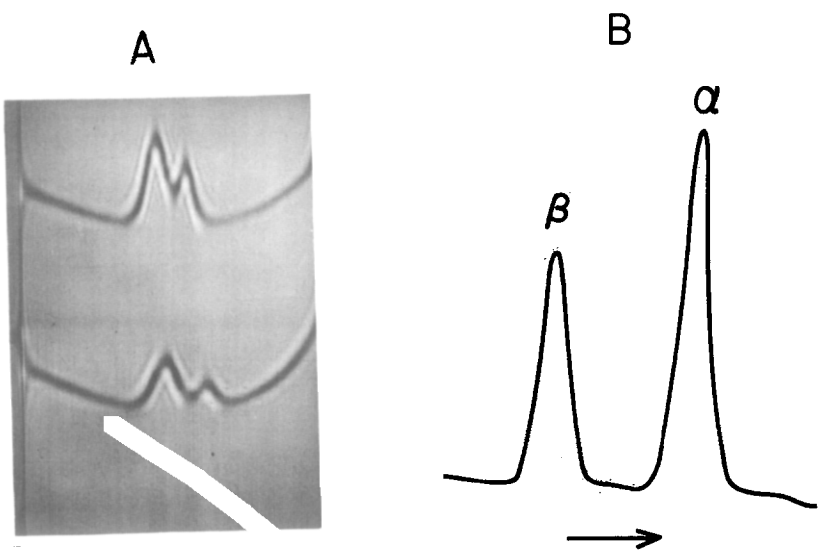

Figure 4. Separation of α and β components from cysteamine extractable collagen.
A. Schlieren pattern (Spinco, Model E) taken 96 minutes after reaching the speed of 59,780 rev/min (2.5 mg collagen in 0.15M citrate pH = 3.6). Upper tracing represents purified fraction extracted by cysteamine from insoluble collagen; lower tracing is a fraction extracted from fresh, untreated skin, by 0.1M cysteamine pH = 7.0.
B. Densitometer tracing of an acylamide gel electrophoretic separation of components present in a collagen fraction extracted by 0.1M cysteamine from insoluble collagen.

and other sulfhydro compounds have been shown to drastically reduce the tensile strength of skin. The effects were evident within a few hours of incubation in the cold, and could be reversed by iodine (10^{-2M}) (Harkness and Harkness, 1965, 1966). Labelled cysteine is rapidly incorporated into protein of granulation tissue (Williamson, 1966) and it was proposed that this amino acid may be required either for the formation of collagen precursors, their polymerization or their deposition in tissue. The proportion of sulfur amino acids in the diet was shown previously to correlate with the rate of wound healing. Epidermal cells are held together by intercellular bridges or desmosomes which seem to be keratin like in nature. Disintegration of these fibrillary elements and subsequent loss of organized structure can be caused by thioglycollate (pH 8.5) (Stoughton, 1959). In addition to epidermis, the endothelial lining of trachea, bronchi and intestine behave in the same way when exposed to disulfide and H-bond splitting agents.

Our findings on the solubilization of collagen by SH containing compounds in conjunction with observations made by other investigators seem to imply that disulfide bridges, or other bonds sensitive to thiol cleavage may play an important role in stabilizing the collagenous framework of connective tissue.

Acknowledgments. This study was supported by N.I.H. research grant #AM 10358-01. I am grateful to Drs. L.A. Bavetta, J. Fessler and R.D. Harkness for their helpful discussion.

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