# Synthesis of Aldehydes and Their Interactions during the *in Vitro* Aging of Collagen*

Arvind Deshmukh,† Kalindi Deshmukh, and Marcel E. Nimni‡

ABSTRACT: Incubation of neutral salt soluble collagen from rat skin at 37° is accompanied by a conversion of some lysine residues into peptide-bound α-aminoadipic δ-semialdehyde. This oxidative deamination is enhanced by a tissue extract with lysyl oxidase activity and is inhibited by β-aminopropionitrile, EDTA, isonicotinic acid hydrazide, and D-penicillamine. Cooling of these incubated specimens to 4° yielded two fractions, a residue and a supernatant, both of which were reduced with $NaBH_4$. The supernatant contained increasing amounts of ε-hydroxynorleucine and traces of an aldol condensation product. The insoluble residue, in addition to significant amounts of the above mentioned constituents, showed the presence of Schiff base components which steadily increase during the first 2-weeks incubation. After this time, the concentration of all reducible components begins to decline, reaching very low values at the eighth week. This disappearance of reducible cross-linking entities is accompanied by the inability of this collagen to become soluble in 0.5 M acetic acid or 0.2 M cysteamine pH 7.0. These experiments suggest that the Schiff-base components identified in collagen fibers are the labile intermolecular cross-links cleaved by weak organic acids. The heat and acid stable intermolecular cross-links which characterize insoluble collagen appear to be nonreducible by $NaBH_4$.

The conversion of peptide-bound lysine located near the N-terminal region of collagen to α-aminoadipic δ-semialdehyde by oxidative deamination and its subsequent involvement in an aldol condensation reaction with a similar aldehyde on a neighboring chain seem to be well established (Bornstein *et al.*, 1966; Bornstein and Piez, 1966). Impairment of this biosynthetic process by β-aminopropionitrile (βAPN)[1] or by induction of a copper deficiency results in a collagen molecule deficient in aldehydes as well as in its capacity to form stable intra- and intermolecular cross-links (Levene and Gross, 1959; Chou *et al.*, 1969; Weissman *et al.*, 1963; Bornstein and Piez, 1966; Rojkind and Juarez, 1966; Page and Benditt, 1967a,b).

A lysyl oxidase obtained from embryonic chick cartilage has been shown to catalyze the formation of the lysine-derived aldehyde (allysine) (Pinnell and Martin, 1968). This enzyme which has been partially purified requires copper for activity and is irreversibly inhibited by βAPN (Siegel *et al.*, 1970). Earlier it had been shown that an amine oxidase present in pig plasma could also be inhibited by βAPN, in a competitive and reversible fashion (Page and Benditt, 1967c). Besides the aldehydes which participate in the intramolecular cross-linking process, additional aldehyde groups are synthesized during this metabolic pathway (Deshmukh and Nimni, 1968, 1969). Analysis of the cyanogen bromide peptides of the fraction extracted by cysteamine from rat skin has allowed us to locate these aldehydes and identify them as being derived from lysine (Deshmukh and Nimni, 1971).

The present paper reports the changes in aldehyde content and their nature which accompany the aging of collagen *in vitro*, as well as their time-dependent involvement in the intramolecular and intermolecular cross-linking process.

## Materials and Methods

Dorsal skins of male rats (weighing 80–100 g) were cleaned, cut into small pieces, defatted, and washed with 0.15 M NaCl for 24 hr. They were then extracted with 0.45 M NaCl in 0.02 M sodium phosphate buffer (pH 7.0) for 72 hr. The extract was centrifuged at 15,000 rpm for 30 min and the supernatant was purified as described previously (Deshmukh and Nimni, 1968). The final preparation of neutral salt soluble (NSS) collagen was centrifuged at 105,000*g* for 2 hr prior to use. All the operations were carried out at 4°.

*Synthesis of Aldehydes during Incubation at 37°.* NSS collagen was incubated at 37° for various time periods ranging from 0 to 8 days. A small amount of toluene was added to each tube to prevent bacterial growth. At the end of each incubation period, the gels were cooled at 4° for 24 hr and centrifuged at 15,000 rpm for 30 min. After removing the supernatant the residue was dissolved by shaking with 0.5 M acetic acid overnight and centrifuged. The supernatant obtained by cooling the gels and the acetic acid extract were dialyzed against 0.1 M glycine-HCl buffer (pH 4.0). The residue which remained insoluble in 0.5 M acetic acid was suspended in water and gelatinized by heating at 60°. In some experiments the supernatant was treated with $NaBT_4$ (specific activity 9.4 mCi/mmole) at pH 7.0–7.5 and dialyzed, and the radioactivity of the retentate counted using a Beckman liquid scintillation counter. Aldehyde content of these solutions was determined by the spectrophotometric method of Paz *et al.* (1965), using *N*-methylbenzothiazolone hydrazone hydrochloride (MBTH).

*Action of Copper Chelators and Enzyme Inhibitors on Aldehyde Synthesis.* To solutions of NSS collagen were added different concentrations ($10^{-2}$ and $10^{-4}$ M) of (i) D-penicillamine, (ii) βAPN, (iii) ethylenediaminetetraacetic acid (EDTA), or (iv) isonicotinic acid hydrazide (INH). These mixtures were incubated at 37° for various time periods. The gels were then

* From the School of Medicine, Department of Medicine, Rheumatic Disease Section, University of Southern California, Los Angeles, California. *Received December 21, 1970.* This research was supported by grants from the National Institutes of Health (AM-10358 and DE-02471) and from the Arthritis Foundation.

† Present address: Max Planck Institute, Munich, West Germany.

‡ To whom to address correspondence.

[1] Abbreviations used are: βAPN, β-aminopropionitrile; INH, isonicotinic acid hydrazide; NSS, neutral salt soluble; MBTH, *N*-methylbenzothiazolone hydrazone hydrochloride.

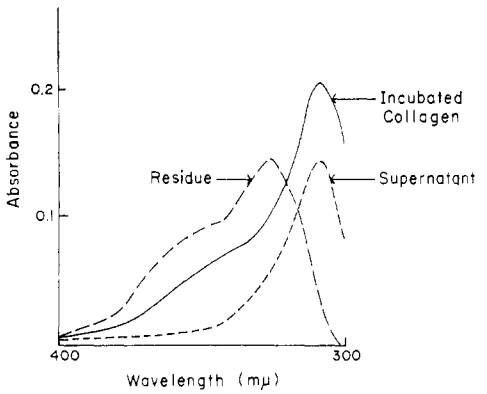


FIGURE 1: Absorption spectra of NSS collagen, incubated at 37° for 8 days, and reacted with MBTH (pH 4.0). (----) Supernatant obtained after cooling to 4° and centrifuging. (— — —) Residue dissolved in 0.5 M acetic acid at 4°. (———) Incubated collagen: total specimen heated at 60° for 30 min at pH 4.0.

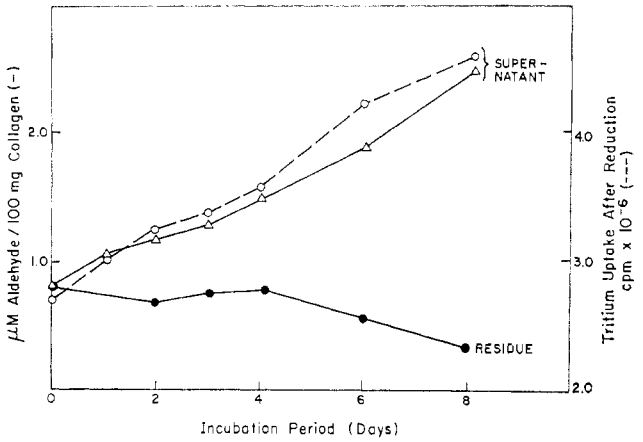


FIGURE 2: Changes in aldehyde content and tritium uptake of NSS collagen incubated at 37° from 0 to 8 days. (Δ—Δ) Aldehyde content (as estimated by reaction with MBTH) of supernatant obtained after incubation at 37° and cooling at 4° overnight followed by centrifugation. (●—●) Aldehyde content of the residue dissolved in cold 0.5 M acetic acid. (○---○) Tritium uptake of supernatant after reduction with $NaBT_4$.

cooled at 4° overnight and centrifuged. Except for control NSS collagen, all other gels dissolved almost immediately upon cooling. The supernatants were dialyzed against water and then against 0.1 M glycine-HCl buffer (pH 4.0) and the aldehyde content was determined using MBTH.

*Measurement of Lysyl Oxidase Activity Using Biosynthetically Labeled Collagen.* A group of rats weighing 60–70 g was fed a normal diet while another group received a diet containing 0.4% βAPN for 1 week. At this time all the rats were injected with 25 μCi of [U-$^{14}$C]lysine (specific activity 260 mCi/mmole) and 50 μCi of DL-[6-$^{3}$H]lysine (specific activity 8.3 Ci/mmole) each day for 2 days. The rats were maintained on the same diets for 4 more days and sacrificed.

Normal and lathyritic NSS collagen was extracted from the dorsal skins of these rats, purified extensively as reported earlier (Deshmukh and Nimni, 1968), and centrifuged at 105,000*g* for 2 hr. The clear supernatants served as labeled substrates.

Extracts with lysyl oxidase activity were obtained from weanling rats. Cleaned dorsal skins were extracted for 4 hr with 0.15 M NaCl buffered to pH 7.5 with 0.05 M sodium phosphate, followed by centrifugation at 105,000*g* for 2 hr. The supernatant contained 5–6 mg/ml of protein, as estimated by the Lowry method (Lowry *et al.*, 1951), and less than 0.4 mg/ml of collagen.

Two milliliters of labeled normal and lathyritic NSS collagen (2.5 mg/ml) was incubated at 37° with 0.5 ml of the following solutions: (a) enzyme extract, (b) enzyme extract heated previously at 100° for 15 min, (c) enzyme extract + 0.1 M βAPN, or (d) 0.15 M NaCl (pH 7.5). At the end of different incubation periods, the gels were centrifuged at room temperature at 3000 rpm for 30 min. The residue was washed twice with distilled water and centrifuged. The supernatant and the washings for each gel were pooled and the aliquots were used to count $^{14}$C and $^{3}$H activity using a Beckman liquid scintillation counter.

*Formation of Intra- and Intermolecular Cross-Links during in Vitro Aging.* NSS collagen from normal rat skin was incubated at 37° for periods of up to 8 weeks. At the end of the incubation period the gels were cooled for 24 hr at 4° and centrifuged at 15,000 rpm for 30 min. The residue and the supernatant from each gel were treated with 500-fold molar excess of $NaBT_4$ (specific activity 9.4 mCi/mmole) for 2 hr, maintaining the pH of the reaction mixture between 7.0 and 7.5. At the end of the reaction, the pH was lowered to 4.0 by addition of 0.5 N HCl. The reduced samples were divided in two aliquots. One batch was hydrolyzed with 3 N HCl and the other with 2 N KOH in sealed ampules at 110° for 24 hr. The acid hydrolysates were evaporated to dryness to remove excess HCl. The alkali hydrolysates were adjusted to pH 4.0 with perchloric acid, chilled, and centrifuged. The residue was washed with water. The supernatant and the washings were chilled together and centrifuged, and the final clear supernatant was dried. The acid and alkaline hydrolysates were taken in 0.01 N HCl and the amino acid analysis was carried out using a Jeol amino acid analyzer. The fractions were collected using a split-stream device and aliquots were counted for tritium activity using a Beckman liquid scintillation counter.

## Results

The absorption spectrum of NSS collagen aged *in vitro* at 37° for 8 days and reacted with MBTH is shown in Figure 1. When this collagen was fractionated by cooling the supernatant gave rise to a spectrum which resembles that of simple aldehydes with a peak at 308 mμ. The residue, soluble in 0.5 M acetic acid, shows a shift in the peak to 320 mμ and the presence of a prominent shoulder at 350 mμ, a characteristic of α,β-unsaturated aldehydes (Paz *et al.*, 1965; Miller and Fullmer, 1966). The supernatant obtained after cooling showed a progressive increase in aldehydes synthesized during the incubation period. The tritium uptake of this supernatant following reduction with $NaBT_4$ parallels the increase in aldehydes measured spectrophotometrically (Figure 2). The residue (soluble in 0.5 M acetic acid) showed a gradual decline in spectrophotometrically measured aldehydes. The amount of residue increased with longer incubation periods. Except for those samples incubated for 8 days, all precipitates dissolved completely in 0.5 M acetic acid. After 8 days a small percentage of the collagen residue (10–15%) remained insoluble in acetic acid. This insoluble collagen showed very little or no aldehyde content after gelatinization and reaction with MBTH.

The effect of various compounds on the rate of formation of aldehydes during *in vitro* aging is described in Figure 3.

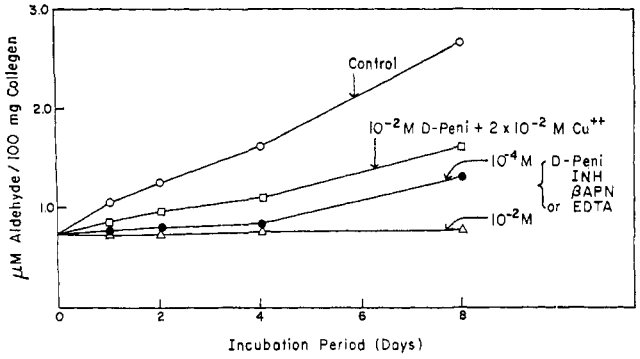


FIGURE 3: Effect of D-penicillamine, βAPN, INH, and EDTA on the synthesis of aldehydes during incubation of NSS collagen at 37° for 8 days.

D-Penicillamine and EDTA are copper chelators, INH inhibits enzymes requiring pyridoxal phosphate as a cofactor, and βAPN is a powerful osteolathyrogen. The presence of these compounds at a concentration of $10^{-2}$ M completely inhibited aldehyde formation. At lower concentrations ($10^{-4}$ M) no aldehyde formation occurred during the first 4-days incubation but the aldehyde content increased thereafter, though at much slower rate than the controls. The effect of D-penicillamine was partially overcome by copper sulfate.

The action of the tissue extract with lysyl oxidase activity on the double-labeled collagen substrates is summarized in Figure 4A,B. Within 8-days incubation, 10% of the tritium at position 6 on lysine was released in the media from normal NSS collagen (Figure 4A). This reflects the formation of aldehydes by a process of oxidative deamination of the ε-amino group of lysine. This leads to the formation of peptide-bound α-aminoadipic δ-semialdehyde with the release of tritium attached to this terminal carbon (Pinnell and Martin, 1968). This tritium release was inhibited by the presence of βAPN. However, it is important to note that if the enzyme was inactivated by heating or if 0.15 M NaCl replaced the enzyme in the mixture, 5–6% of $^{3}H$ was still released into the media. This observation suggests either a close association of enzyme with the highly purified collagen we have used as a substrate, or that the process of oxidative deamination of lysine is partially nonenzymatic. Lathyritic NSS collagen showed a similar trend, but the rate of release of 6-$^{3}H$ was in all instances slower than from normal NSS collagen. With neither of the two

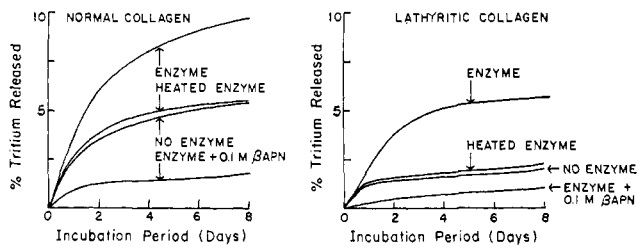


FIGURE 4: Rate of release of tritium from NSS collagen (labeled with [U-$^{14}C$]- and [6-$^{3}H$]lysine) after addition of enzyme extract and incubation at 37° for periods of up to 8 days. The specific activity for the normal collagen was 3000 dpm/mg for the $^{14}C$ label and 5080 dpm/mg for $H^{3}$. For the lathyritic collagen from the βAPN-treated animals the values were 3150 and 5100 dpm per mg of collagen, respectively. (A) Normal collagen and (B) lathyritic collagen.

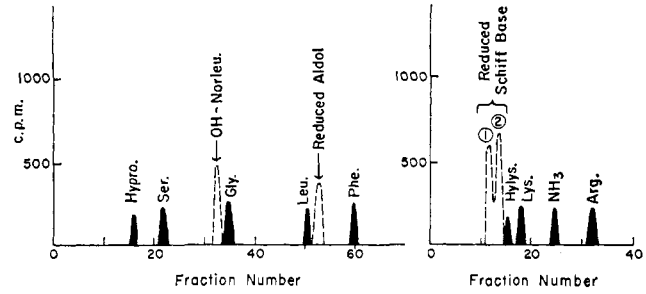


FIGURE 5: Elution pattern of amino acids from the amino acid analyzer and localization of tritium activity after incubation of NSS collagen at 37°, reduction with $NaBT_4$, and hydrolysis. This figure is a composite of separate acid and base hydrolysis.

labeled substrates was the presence of $^{14}C$ observed in the media.

To obtain an insight into the overall process of aldehyde formation and to monitor their interactions during the formation of intra- and intermolecular cross-links, NSS collagen was aged *in vitro* from 0 to 8 weeks and reduced with $NaBT_4$ at various time intervals. The reduced collagen was hydrolyzed and analyzed on an amino acid analyzer simultaneously with the measurement of the tritium activity incorporated into the various components (Figure 5). After 2 weeks at 37° the gels did not redissolve by cooling, indicating that the formation of intermolecular cross-links stable at neutral pH and at 4° was completed. Figure 6 shows the results from the supernatant obtained after cooling the gels to 4°. The unincubated NSS collagen (zero time) showed most of its tritium activity associated with reduced α-aminoadipic semialdehyde (ε-hydroxynorleucine). A small amount of radioactivity was also seen where the reduced aldol product appears, *i.e.*, near leucine, reflecting the small proportion of intramolecular cross-links in this collagen (8–10% β chains). There was a threefold increase in ε-hydroxynorleucine activity within 2 weeks of incubation, and only a slight increase in the reduced aldol product.

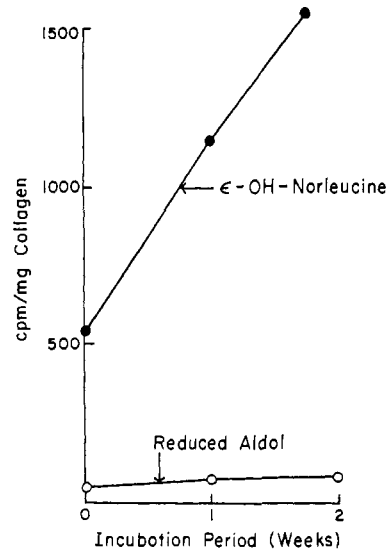


FIGURE 6: Changes in the concentrations of reduced α-aminoadipic semialdehyde and reduced aldol product in the supernatant obtained after *in vitro* aging of NSS collagen from 0 to 2 weeks, cooling at 4° overnight, and centrifuging.

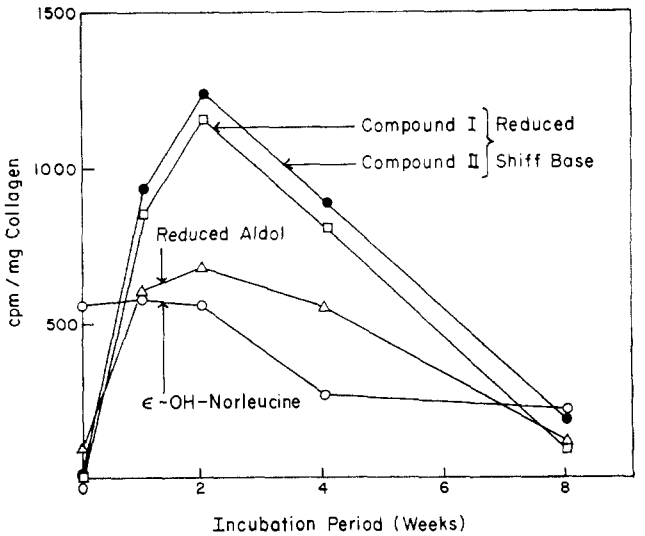


FIGURE 7: Changes in the concentrations of reduction products of $\alpha$-aminoadipic semialdehyde, aldol, and Schiff base in the residue remaining after centrifugation of NSS collagen aged *in vitro* at 37° and cooled at 4° overnight. The values at time zero are those of unincubated NSS collagen.

Each fraction was hydrolyzed separately with 3 N HCl or 2 N KOH, as described in the Experimental Section. It was found during these studies that with acid hydrolysis a part of $\epsilon$-hydroxynorleucine appears as $\epsilon$-chlorononorleucine and a large percentage of reduced aldol product is destroyed. On the other hand, with alkali hydrolysis the recovery of reduced Schiff-base products was low. Similar observations have been made by Lent *et al.* (1969) in the case of elastin and Kang *et al.* (1969) and Paz *et al.* (1969) in the case of collagen.

When the residue which remained after cooling was treated with $NaBT_4$ and analyzed, the presence of reduced $\alpha$-aminoadipic semialdehyde was still apparent (Figure 7). The amount of $\epsilon$-hydroxynorleucine remained constant during the first 2 weeks, while the amounts of reduced aldol condensation product exhibited a marked increase. Although no radioactive peaks corresponding to reduced Schiff bases were present in the supernatant or in the original unincubated samples, the rapid formation of a Schiff base during incubation at 37° was quite evident in the residue. After reaching a maximum at 2 weeks the uptake of tritium slowed down, reaching a minimum value at 8 weeks, similarly to all the other reducible components present in this fraction.

## Discussion

The enzymatic conversion of lysine in collagen and elastin to peptide bound $\alpha$-aminoadipic semialdehyde (allysine) has been shown by Pinnell and Martin (1968). Aldehydes from the N-terminal peptides, $\alpha_1$-$CB_1$ and $\alpha_2$-$CB_1$ isolated by CNBr treatment of collagen, react to form an intramolecular cross-link (Bornstein and Piez, 1966). The aldol condensation product of two allysine residues can be isolated after reduction with $NaBH_4$ and alkali hydrolysis of collagen, and has been shown to elute with the neutral amino acids as a post-leucine peak (Kang *et al.*, 1969). Spontaneous generation of intramolecular cross-links in collagen has been reported earlier (Deshmukh and Nimni, 1969; Schiffmann and Martin, 1970). This aging process causes collagen to become almost completely insoluble in cold 0.45 M NaCl (pH 7.0) after 2 weeks and insoluble in 0.5 M acetic acid or 0.2 M cysteamine (pH 7.0) after 6–8 weeks (Deshmukh and Nimni, 1969). It should be pointed out that this insolubilization seems to occur faster *in vitro* than *in vivo*. In 1-year-old rats one can find significant amounts of insoluble collagen with a high aldehyde content in contrast to that syntehsized *in vitro*, which relatively rapidly becomes depleted of aldehydes. The slower maturation seen *in vivo* may be a reflection of its constant turnover, a factor which is particularly significant in a constantly growing animal such as the rat.

In the present experiments the aldehyde increase associated with the *in vitro* aging was restricted to the collagen that could be redissolved in 0.45 M NaCl at 4°. This material when reacted with MBTH showed the presence of simple aldehydes, which agrees with the fact that when denatured it consists almost exclusively of $\alpha_1$ and $\alpha_2$ chains. The residue (insoluble in 0.45 M NaCl but soluble in 0.5 M acetic acid) is enriched in $\beta$ components (50–60%) together with aldehydes of the $\alpha,\beta$-unsaturated type. Addition of D-penicillamine, INH, $\beta$APN, or EDTA to the incubation media inhibited the formation of new aldehydes. A twofold molar excess of copper over the concentration of D-penicillamine partially restored aldehyde synthesis.

There are some significant differences between this set of experiments and those reported by previous investigators. Whereas Pinnell and Martin (1968) added lysyl oxidase obtained from embryonic chick cartilage and Rucker *et al.* (1970) added lysyl oxidase from bovine aorta our experiments were carried out with nothing but "purified" collagen in the media. *In vitro* aging was carried out at pH 7.0, and at a higher ionic strength than that normally encountered in the extracellular space. Collagen will form native-type fibrils in 0.45 M NaCl which are more readily redissolvable by cooling than those made up in 0.15 M NaCl. This, therefore, becomes a practical advantage for studying the synthesis and interaction of aldehydes in such a time-dependent process as is the maturation of collagen. Since even under these nonideal conditions collagen seems to mature at a faster rate than *in vivo*, it is evident that regulating mechanisms must be present in the animal to slow down the process of cross-linking.

Two obvious explanations can be attempted in order to explain our findings. One is that our preparations of NSS, purified as previously described by repeated reprecipitations by dialysis and by salting out steps, still contained significant amounts of tightly associated or nonseparable lysyl oxidase. Another alternative is that the oxidative deamination of lysine is nonenzymatic, catalyzed by divalent cations or other cofactors present in trace amounts in the media, and that the inhibitors used are functioning as chelating agents. Recent experiments by Fowler *et al.* (1970) have shown that the $\epsilon$-amino group of $\alpha$-*N*-acetyllysine or $\alpha$-*N*-trifluoroacetylhydroxylysine can be oxidatively deaminated nonenzymatically in the presence of pyridoxal 5-phosphate and $Cu^{2+}$ ions. Although such a mechanism was postulated to occur in collagen, their experimental data do not substantiate their claim. The presence of radioactive peaks eluting as dihydroxynorleucine and hydroxynorleucine following reduction of collagen previously incubated with $CuSO_4$ and pyridoxal for 72 hr at 38° does not prove that the aldehyde precursors were not already present in the collagen before incubation. Use of reduced collagen as a substrate or quantitation of the increase of such components is necessary.

In attempting to answer these questions, an enzymatic extract from the skin of weanling rats was prepared by the method of Pinnell and Martin (1968). When this crude extract

was added to the incubation mixture its stimulatory effect could be clearly seen, judged by the tritium release from the C-6 position of lysine from biosynthetically labeled collagen substrate. Nevertheless, mixtures containing heat-inactivated enzyme, and no enzyme at all, still exhibited significant activity, indicating that the phenomena observed was at least in part independent of the addition of enzymatic extract. βAPN at a relatively high concentration (0.1 M) further inhibited tritium release.

When radioactive lathyritic collagen was used as a substrate the activity at all times was about half of that encountered with normal collagen and addition of an enzymatic extract from normal rat skin has a relatively greater enhancing effect on this collagen than on normal collagen. These findings are in essence similar to those of Siegel *et al.* (1970). These investigators found no enzymatic activity in extracts from lathyritic animals and have indicated the possibility of an irreversible association of βAPN with lysyl oxidase. Our data in this connection would, therefore, substantiate our postulate that a tightly bound lysyl oxidase may be present in our collagen preparations, which in the case of lathyritic collagen is irreversibly inactivated. Five lysyl and hydroxylysyl residues per molecule of native tropocollagen have been found to exhibit a particularly high affinity for forming Schiff bases with pyridoxal phosphate (Page and Benditt, 1969), and it is reasonable to expect that these particular sites may be preferred targets for deamination. Further work is still necessary to clarify this situation. Aldehyde formation in the extracellular space is a slow process, particularly as it relates to aldehydes which are formed in the helical region of tropocollagen during maturation and which also give rise to peptide-bound α-aminoadipic semialdehyde upon reduction (Deshmukh and Nimni, 1971). The relative contributions of enzymatic and nonenzymatic synthesis to the physiological process of cross-linking required further understanding of the mechanisms involved. Siegel *et al.* (1970) have clearly demonstrated the requirement of $Cu^{2+}$ ions and have shown that $Fe^{2+}$ and $Co^{2+}$ could partially restore the lysyl oxidase activity after dialysis. The presence of oxygen enhanced the activity but oxidative deamination of lysine still occurred at a very significant rate under an atmosphere of nitrogen.

It is possible that lysyl oxidase is required for the biosynthesis of aldehydes near the N-terminal region of collagen. This is a relatively rapid process, which may have to occur before tropocollagen assembles into its final position in a fiber. On the other hand, aldehydes have been shown to form in the helical portion of the molecule during maturation and their location on the CNBr peptides has recently been reported (Deshmukh and Nimni, 1971). This process is relatively much slower and could be nonenzymatic, particularly in view of the difficulty that an enzyme would have to penetrate the interstice of the fiber for the purpose of modifying specific lysine residues.

The fraction of collagen that can be redissolved in cold 0.45 M NaCl (pH 7.0) after incubation at 37° shows progressively increasing amounts of ε-hydroxynorleucine detectable after reduction with $NaBT_4$. This could only be monitored during the first 2-weeks incubation, since beyond this time no significant amounts of collagen redissolve after cooling. The magnitude of these changes parallels those of MBTH-reactive aldehydes and total tritium uptake after reduction. The amount of radioactivity which eluted as a reduced aldol condensation product did not change and was consistent with the fact that the concentration of β components in this soluble fraction is never greater than 10%.

When the residues were reduced with $NaBT_4$ they showed significant amounts of reduced Schiff-base components, already identified by Bailey and coworkers (1970) and by Tanzer *et al.* (1970) as lysinonorleucine and hydroxylysinonorleucine. The concentration of these Schiff-base components as well as that of aldol condensate increased during the first 2-weeks incubation. At this time additional aldehydes were still forming and proceeding to form Schiff bases. It should be emphasized that whereas peptide bound α-aminoadipic semialdehyde was already present in significant amounts in the initial NSS collagen preparation, the other species were absent except for small amounts of aldol condensation product. After incubation at 37° is begun, the proper staggering of the tropocollagen molecules not only offers the opportunity for intermolecular Schiff-base formation but seems to favor the formation of intramolecular cross-links through the aldol condensation mechanism. After the second week the concentration of all these reducible components begins to decline and reaches a minimum after week 8 of incubation. At this time most of the collagen has become insoluble not only in 0.45 M NaCl at 4°, but also in cold 0.5 M acetic acid. It is then when the nonreducible cross-links which still remain to be identified begin to emerge, giving rise to true insoluble collagen, a form of collagen characterized by an increasing number of heat- and acid-stable intermolecular cross-links (Heikkinen *et al.*, 1964). Our present findings differ from those obtained by Franzblau *et al.* (1970) during their studies on the *in vitro* formation of intermolecular cross-links, probably due to differences in the materials used since these investigators studied acid-soluble collagen from chick skin. Using rat skin NSS collagen we do not detect the formation of a new reducible intermediate, quite basic in nature and which they found to elute as a "posthistidine" peak.

Other approaches, besides those involving fixation with $NaBH_4$ seem to be necessary to further understand and identify these cross-links in rat skin collagen which both *in vivo* and *in vitro* become stabilized by a mechanism that does not seem to require or resemble $NaBH_4$ reduction.

## References


Bailey, A. J., Peach, C. M., and Fowler, L. J. (1970), *Biochem. J. 117*, 819.

Bornstein, P., Kang, A. H., and Piez, K. A. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 417.

Bornstein, P., and Piez, K. A. (1966), *Biochemistry 5*, 3460.

Chou, W. S., Savage, J. E., and O'Dell, B. L. (1969), *J. Biol. Chem. 244*, 5785.

Deshmukh, A. D., and Nimni, M. E. (1969a), *Biochem. Biophys. Res. Commun. 35*, 485.

Deshmukh, K., and Nimni, M. E. (1968), *Biochim. Biophys. Acta 154*, 258.

Deshmukh, K., and Nimni, M. E. (1969b), *Biochem. J. 112*, 397.

Deshmukh, K., and Nimni, M. E. (1971), *Biochemistry 10*, 1640.

Fowler, L. J., Peach, C. M., and Bailey, A. J. (1970), *Biochem. Biophys. Res. Commun. 41*, 251.

Franzblau, C., Kang, A. H., and Faris, B. (1970), *Biochem. Biophys. Res. Commun. 40*, 437.

Heikkinen, E., Mikkonen, L., and Kulonen, E. (1964), *Exp. Geront. 1*, 31.

Kang, A. H., Faris, B., and Franzblau, C. (1969), *Biochem. Biophys. Res. Commun. 36*, 345.

Lent, R. W., Smith, B., Salcedo, L. L., Faris, B., and Franzblau, C. (1969), *Biochemistry 8*, 2827.

Levene, C. I., and Gross, J. (1959), *J. Exp. Med. 110*, 771.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Miller, E. J., and Fullmer, H. M. (1966), *J. Exp. Med. 123*, 1097.
Page, R. C., and Benditt, E. P. (1967a), *Proc. Soc. Exp. Biol. Med. 124*, 454.
Page, R. C., and Benditt, E. P. (1967b), *Proc. Soc. Exp. Biol. Med. 124*, 459.
Page, R. C., and Benditt, E. P. (1967c), *Biochemistry 6*, 1142.
Page, R. C., and Benditt, E. P. (1969), *Science 163*, 578.
Paz, M. A., Blumenfeld, O. O., Rojkind, M., Henson, E., Furfine, C., and Gallop, P. M. (1965), *Arch. Biochem. Biophys. 109*, 548.
Paz, M. A., Lent, R. W., Faris, B., Franzblau, C., Blumenfeld, O. O., and Gallop, P. M. (1969), *Biochem. Biophys. Res. Commun. 34*, 221.
Pinnell, S. R., and Martin, G. R. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 708.
Rojkind, M., and Juarez, H. (1966), *Biochem. Biophys. Res. Commun. 25*, 481.
Rucker, R. B., Roensch, L. F., Savage, J. E., and O'Dell, B. L. (1970), *Biochem. Biophys. Res. Commun. 40*, 6.
Schiffmann, E., and Martin, G. R. (1970), *Arch. Biochem. Biophys. 138*, 226.
Siegel, R. C., Pinnell, S. R., and Martin, G. R. (1970), *Biochemistry 9*, 4486.
Tanzer, M. L., Mechanic, G., and Gallop, P. M. (1970), *Biochim. Biophys. Acta 207*, 548.
Weissman, N., Shields, G. S., and Carnes, W. H. (1963), *J. Biol. Chem. 238*, 3115.

# Nuclear Residual Proteins from Goose Erythroid Cells and Liver*

K. R. Shelton† and J. M. Neelin

ABSTRACT: A method has been devised for extraction of nuclear residual proteins in phenol as two successive fractions at pH 8.4 and 9.4. Residual proteins from goose liver, mature erythrocytes, and regenerating erythroid cells, differing widely in synthetic activities, were compared by gel electrophoresis in sodium dodecyl sulfate. Both fractions from avian liver are heterogeneous, resolving, respectively, into at least 20 bands each, while the fractions from erythroid cells appeared to be somewhat less heterogeneous. Besides significant quantitative differences in many bands, some proteins appeared to be qualitatively tissue specific. Differences between mature and regenerating erythroid cells were limited to relatively minor components and to variable amounts of coextracted hemoglobin.

Biological functions have been ascribed to only a few nonhistone nuclear proteins, but relatively more residual protein[1] is found in metabolically active tissues with greater contents of chromosomal RNA, but with constant amounts of histone (Dingman and Sporn, 1964; Marushige and Ozaki, 1967). The level of template capacity (Seligy and Neelin, 1970) and the specificity of transcription *in vitro* (Gilmour and Paul, 1969) are affected by residual proteins on the chromatin. Regions of disperse chromatin, relatively rich in nonhistone proteins (Frenster, 1965; Arbuzova *et al.*, 1968), synthesize RNA more actively than condensed chromatin. These studies imply that residual protein may play a role in genetic activity.

Furthermore, nonhistone chromosomal proteins are generally more active than histones in incorporation of amino acids (Daly *et al.*, 1952; Stellwagen and Cole, 1969) and phosphate (Langen, 1968; Gershey and Kleinsmith, 1969), and the level of such metabolism appears to be related to nuclear activity (Turkington and Riddle, 1969; Kleinsmith *et al.*, 1966). Synthesis of specific residual proteins has been shown to respond to gene-activating stimuli (Teng and Hamilton, 1969; Shelton and Allfrey, 1970).

In view of these suggestive observations, it was of interest to apply new methods of residual protein solubilization and resolution (Shelton and Allfrey, 1970) to a selection of cell populations in order to permit an assessment of protein heterogeneity and specificity, and to open the way for studies of the metabolism of specific components. The cells chosen were those of goose liver, normal blood, and regenerating blood. Liver is a mixed population of cells, predominantly hepatocytes, most of which are synthesizing protein, RNA, and DNA. Almost all of the cells of normal avian blood after removal of the "buffy coat" are mature, nucleated erythrocytes which do not divide or make DNA, and which synthesize little protein or RNA (Cameron and Prescott, 1963; Scherrer *et al.*, 1966). The regenerating blood used herein usually contains 10–30% erythroblasts and some reticulocytes as well as mature cells and ghosts (G. H. M. Adams and J. M. Neelin, unpublished observations); longer intervals after injection of phenylhydrazine (Adams and Neelin, 1970) diminish erythroblasts and increase reticulocytes. Such cells show a range of activities as reflected by cell and nuclear volumes (Mathias *et al.*, 1969), transport functions (d'Amelio and

* Contribution No. 11950, from the Biochemistry Laboratory, National Research Council of Canada, Ottawa, Canada. *Received December 18, 1970.*

† Present address: Department of Biochemistry, Medical College of Virginia, Health Sciences Center, Virginia Commonwealth University, Richmond, Va. 23219; a National Research Council of Canada Postdoctoral Fellow, 1969–1970.

[1] The venerable term, "residual protein," is used herein to define the nonhistone nuclear proteins which are not extracted by isotonic saline and dilute acid.