COLLAGEN REDUCTION BY SODIUM BOROHYDRIDE: EFFECTS OF RECONSTITUTION, MATURATION AND LATHYRISM

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Reconstituted native type collagen fibrils become crosslinked upon reduction with sodium borohydride, probably due to the conversion of intermolecular Schiff bases into secondary amines (Tanzer, 1967, 1968). In contrast, treatment of collagen or gelatin solutions with sodium borohydride does not produce crosslinking while exposure of non-striated reconstituted fibrils to $NaBH_4$ has a slight crosslinking effect. Under the conditions employed in all of these experiments about one mole of tritium is incorporated per mole of collagen when NaB^3H_4 is used. Complete acid hydrolysis of the radioactive collagen followed by ion-exchange chromatography and scintillation spectrometry produced the elution patterns shown below. The diverse effects of fibril reconstitution, collagen maturation and lathyrism upon the specific activity and the nature of the radioactive products is described. We find that the physical state of the collagen during reduction qualitatively and quantitatively affects the radioactive products, that there is only a quantitative difference between normal and lathyritic collagens and that there may be a progressive change in the chemical nature of collagen aldehydes during maturation.

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EXPERIMENTAL

Acid extracted embryonic calf skin collagen was from the previous preparation (Tanzer et al, 1966); normal and lathyritic guinea pig skin collagens of progressive degrees of maturation were obtained by sequential extractions with 0.15M NaCl, 0.45M NaCl, 1.0M NaCl and 0.1 M acetic acid. These collagens have been previously shown to progressively increase in β component content (Tanzer, 1968). Native type reconstituted collagen fibrils were formed by incubating 0.2% (w/v) collagen solutions in 0.16M NaCl, pH 7.5 at 37°. Non-striated fibrils were formed under similar conditions except that 1.0 M NaCl was used as the solvent. These preparations were mixed with a 1/2 volume of warm sodium phosphate, pH 7.5, of suitable ionic strength, just prior to the addition of sodium borotritide. Collagen solutions, 0.2% (w/v) in sodium phosphate, pH 7.5, u = 0.4 were incubated at 37° for 15-20 minutes prior to reduction and did not usually form aggregates under these conditions; aggregated samples were not used. All preparations received a 500 fold molar excess (relative to the amount of protein) of sodium borotritide in 20-30 μl of 0.01N NaOH and incubation was continued for 5 minutes. The collagen fibrils were harvested by centrifugation at 20° while the collagen solutions were cooled to 4° and the protein was precipitated by the addition of ice cold ethanol to a final concentration of 20% (v/v). The harvested preparations were dialyzed exhaustively and were then dried by lyophylization. The samples were hydrolyzed at 110° in 6N HCl for 22 hours and the hydrolysates were dried by rotary evaporation following which they were redissolved in a small amount of water. An aliquot portion of the solutions was used for the determination of specific activity while the remainder was subjected to ion exchange chromatography (Piez and Morris, 1960). The chromatographic system was routinely calibrated by determining the elution positions of the common amino acids. The column effluent was collected in 2 ml fractions and 0.2 ml of each fraction were placed in

a scintillation vial containing 10 ml of Bray's solution and 5% (w/v) cab-o-sil. Radioactivity was measured in a liquid scintillation spectrometer.

RESULTS

Comparison of the elution patterns of the calf skin preparations (Fig. 1) showed that the physical state of the collagen during reduction affected the nature of the radioactive products. The most likely explanation for these results is that the substances eluting at 250-300 ml (Fig. 1A) are the crosslinks which develop in the reconstituted native type fibrils. Accordingly, the compounds eluting at 180-220 ml (Fig. 1C) would be the precursor aldehydes (reduced to alcohols) which give rise to the crosslinks. Furthermore, the reciprocal relationship between these sets of compounds seen for the non-striated fibrils (Fig. 1B) is compatible with the observation that, in these fibrils, crosslinking occurs only to a slight extent. The presumptive crosslinks are homogeneous upon paper chromatography and paper electrophoresis and have been isolated as their dinitrophenyl derivatives. The results indicate that they are not identical with lysinonorleucine, a crosslink of elastin (Franzblau, 1965).

In the hope of obtaining a simpler chromatographic pattern than that of Figure 1, collagens of different degrees of maturity obtained from a single batch of guinea pig skin were exposed to sodium borotritide. The results are shown in Table I and Figure 2. It can be seen that, as found for calf skin collagen, the uptake of tritium is similar upon comparing collagen solutions and reconstituted fibrils. Furthermore, the different salt and acid collagens incorporated similar amounts of tritium, implying that their reducible aldehyde content under the conditions used, did not vary with maturation. In contrast, the elution patterns of these samples showed distinct quantitative and qualitative differences (Fig. 2). The chromatographic pattern of the 0.1 M acetic acid

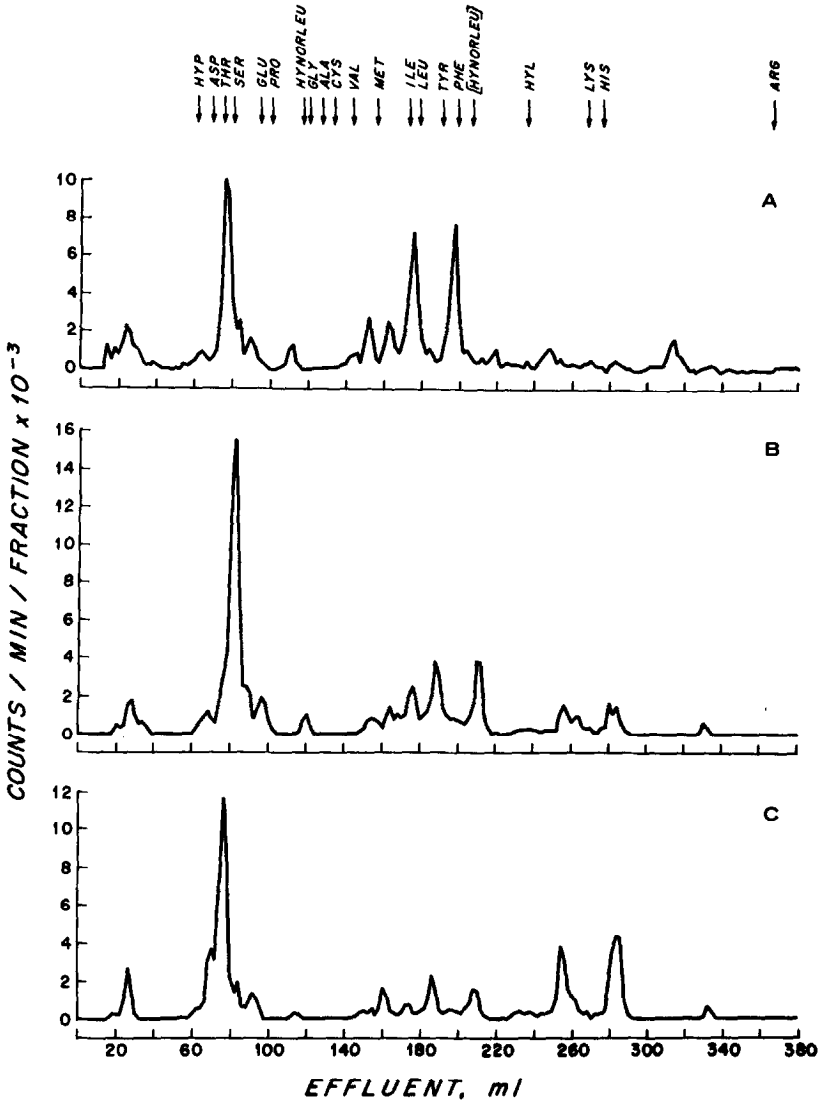

Figure 1 Elution patterns of the hydrolysates of borotritide treated calf skin collagens. (A) Collagen in solution, (B) Reconstituted non-striated fibrils, (C) Reconstituted native type fibrils.

collagen is more complex than that of the 0.45 M NaCl collagen and partially resembles the analogous calfskin pattern in Figure 1A. The pattern of the 0.15 M NaCl collagen was similar to Figure 2A while the

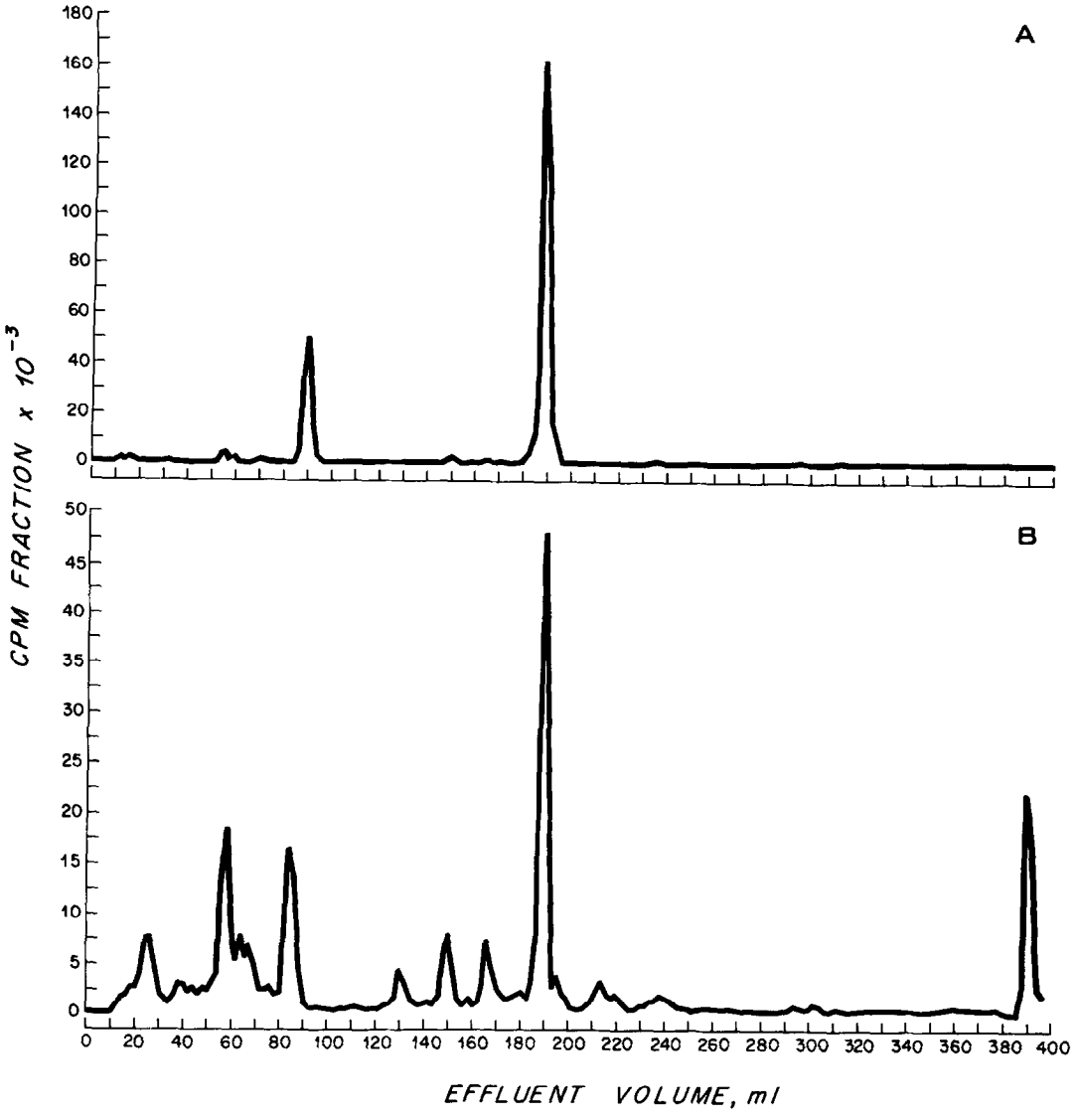

Figure 2 Elution patterns of the hydrolysates of borotritide treated guinea pig skin salt and acid collagens. (A) 0.45M NaCl collagen in solution, (B) 0.1M acetic acid collagen in solution.

pattern of the 1.0 M NaCl collagen was intermediate between Figure 2A and 2B. Perhaps there is a progressive alteration in the chemical nature of collagen aldehydes with maturation, accounting for the

TABLE I

Tritium Uptake of Salt and Acid Collagens

Sample[a]	Solution	Fibrils
	dpm/mg. collagen $X10^{-6}$	
0.15 M NaCl	1.23	0.97
0.45 M NaCl	1.25	1.03
1.0 M NaCl	1.59	1.34
0.1 M Acetic Acid	1.28	1.11

a The salt and acid designations refer to the solvents originally used for sequential extraction of the collagens from guinea pig skin.

diminution of some components coincident with the appearance of others while the tritium incorporation remains constant. The material eluting at 80-90 ml in Figure 2A has been tenatively identified as hydroxynorleucine and the substance eluting at 180-190 ml is probably the hydrolysis artifact of hydroxynorleucine (Gallop et al, 1968).

Comparison of 0.15 M NaCl extracted, borotritide-treated normal and lathyritic guinea pig collagens showed that these preparations had specific activities of 7740 cpm/μM of hydroxyproline and 4980 cpm/μM of hydroxyproline respectively. Both chromatographic patterns were similar to Figure 2A; the two peaks of the lathyritic pattern were each about 1/2 the height of the two peaks of the control pattern when identical quantities of protein hydrolysate were subjected to chromatography. Control studies showed that the normal and lathyritic collagens consisted almost entirely of ∝ chains and that the reconstituted lathyritic fibrils dissolved much more readily than the normal fibrils.

Thus, no qualitative differences were apparent upon comparison of the aldehyde components of normal and lathyritic collagens; therefore the instability of the reconstituted lathyritic fibrils may simply reflect the diminished aldehyde content.

DISCUSSION

Collagen has been shown to undergo changes in its primary structure during maturation as reflected in the development of crosslinks (Butzow and Eichhorn, 1968), in the conversion of lysines to aldehyde derivatives (Piez, et al, 1966; Schneider et al, 1967), and in the enrichment of α, β unsaturated aldehydes (Blumenfeld and Gallop, 1966; Piez, 1966). The present results suggest that during maturation progressive changes occur in the chemical nature of the aldehydes in collagen. Several of the known aldehydes are found in the protein as fractions of residues per mole (Gallop et al, 1968), suggesting the occurrence of a pathway involving aldehyde intermediates. In as much as we have not identified the aldehydes which participate in intermolecular crosslinking in vitro, direct comparison with the known aldehydes can not be made. It is clear, however, from Figure 1, that only some of the aldehydes in the protein are involved in forming the crosslinks in reconstituted fibrils. This result may reflect either specificity in the chemical nature of the aldehydes or specificity in their location in the protein.

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