Discussion

On a preparative scale the enzymatic synthesis of 3-hydroxyuric acid suffers from the fact that dilute solutions of it, like those of uric acid (Griffiths, 1952; Stahl, 1969) are susceptible to air oxidation. A total synthesis of it required unusual conditions for cyclization of the imidazolone ring to avoid simultaneous deoxygenation at N-3.

In a biological assay the low incidence of tumors at a relatively high dose (Table I) indicates that 3-hydroxyuric acid and its major metabolites, presumably *N*-hydroxyallantoins produced by the action of uricase, are quite weak oncogens.

A small amount of the highly oncogenic 3-hydroxyxanthine is produced by the action of xanthine oxidase *in vitro*. The knowledge that similar reduction products of purine *N*-oxide derivatives are produced *in vivo* (Stöhrer and Brown, 1969b), and that xanthine oxidase activity is present in subcutaneous tissues (Myles and Brown, 1969), suggests that the tumors observed are most probably due to a small amount of 3-hydroxyxanthine arising *in vivo* from the 3-hydroxyuric acid.

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Modification of Chromatin with Acetic Anhydride*

Robert T. Simpson

ABSTRACT: Acetylation of rabbit liver chromatin with increasing excesses of acetic anhydride leads to increasing modification of tyrosyl and lysyl residues of chromatin proteins, reaching a limiting value at the acetylation of 54 amino acid residues/100 base pairs. Only slight alterations in the thermal stability of chromatin, no detectable alterations in the conformations of protein or DNA, and no dissociation of chromatin proteins from the nucleoprotein complex occur consequent to the modification reaction. Only a portion (*ca.* 25%) of the lysyl residues of the proteins of chromatin is acetylated, while all the tyrosyl residues appear to react. Polyacrylamide gel electrophoresis demonstrates that some lysyl residues of all the three main histone fractions react with the acylating agent in the native chromatin complex. All histone molecules are acetylated, and the modification reaction appears to possess a high degree of specificity in terms of the numbers of lysyl residues available for modification for each individual histone class. This modification should facilitate the localization of those lysyl residues of histones which bind to DNA.

The compositions of the proteins of chromatin are such that an approximate equivalence exists between the positively charged basic groups of the histones and nonhistone proteins, on the one hand, and the negatively charged phosphates of DNA, on the other, suggesting that the nucleoprotein might exist as a stoichiometric complex, with all the basic amino acids electrostatically linked to DNA phosphate (Bonner *et al.*, 1968). Other evidence has, however, made such a suggestion less likely. Thus, a significant proportion of the basic groups of chromatin can be titrated in native nucleoprotein (Walker, 1965). Furthermore, a large portion of the phosphate groups are accessible to interaction with calcium (Simpson and Sober, 1970), cationic dyes (Simpson, 1970), or polylysine (Itzahki, 1970; Clark and Felsenfeld, 1971).

Recently, primary structure investigations of the several classes of histones have generated a surprising result, that the basic groups of these proteins are neither randomly arranged nor repetitively spaced throughout the length of the peptide chain, but rather are preferentially localized in large segments of high concentration of basic residues. Thus, the amino-terminal regions of the arginine-rich (FIV) (DeLange *et al.*, 1969; Ogawa *et al.*, 1969) and the slightly lysine-rich (FII) (Iwai *et al.*, 1970; Hnilica *et al.*, 1970) histones are much more basic than the carboxyl-terminal regions of these molecules. In the case of the lysine-rich histones, partial sequence studies also indicate asymmetry in basic residue distribution, although, in this case, the carboxyl-terminal portion of the molecule is the more highly basic (Bustin and Cole, 1969, 1970). Taken together, these observations have suggested that the basic regions of the histones might serve as a DNA binding region, lying in close proximity to the DNA phosphate backbone, while the relatively less basic regions of the histones are less firmly attached to the nucleic acid, perhaps indeed, free in solution to interact with other proteins or

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smaller molecules. To date, there has been apparently no evidence to either confirm or refute this hypothesis.

The present study describes initial attempts to ascertain the distribution of the basic groups in histones and nonhistone chromatin proteins which are not electrostatically linked to DNA phosphate. Chemical modification with a group selective reagent has been employed as an approach to this problem. The results demonstrate that a significant proportion of the basic groups of chromatin proteins is available for modification in the native nucleoprotein complex. All the histone classes as well as nonhistone proteins are modified.

Experimental Section

Chromatin was isolated from mature New Zealand white rabbit liver as previously described (Simpson, 1970). The mass ratio of protein to DNA for the samples employed in the present studies was 1.9–2.2 to 1. Nonhistone protein accounted for 35–45% of the total protein content. Calf thymus chromatin was isolated from frozen tissue (Pel-Freeze Biologicals) by repeated washing with 0.075 M NaCl–0.024 M EDTA (pH 8.0) as described by Zubay and Doty (1959). The mass ratio of protein to DNA for this chromatin was 1.8. All chemicals used were reagent grade. [^{3}H]Acetic anhydride was obtained from New England Nuclear Corp. as a 20% solution in benzene. The specific radioactivity of the reagent as utilized was approximately 2×10^6 dpm/μmole.

Acetylation of chromatin was performed by addition of the appropriate excess of acetic anhydride to a vigorously stirred solution of chromatin at a DNA concentration of 1 mg/ml, in 0.1 M sodium borate (pH 9.0) at 0°. The reaction was allowed to proceed for 20 min, several times the expected half-life for hydrolysis of the reagent under these conditions. Excess acetate was removed by prolonged dialysis against three to four changes of 50–100 volumes of 0.0001 M sodium borate (pH 9.0) at 4°. The results of the modification experiments are expressed as amino acid residues modified per 100 base pairs of chromatin DNA.

Concentrations of DNA and protein were determined, and circular dichroism measurements made as previously detailed (Simpson, 1970). ^{3}H was determined by using a Beckman LS-250 counter and Aquasol (New England Nuclear Corp.) as scintillation fluid.

Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate was performed as described by Weber and Osborn (1969) with the modifications that electrophoresis was carried out in 0.03 M sodium phosphate buffer (pH 7.5) and the gels were stained with 1% aniline blue black and destained by soaking in 40% ethanol–7% acetic acid containing a small amount of Dowex 1. Protein distribution was determined by scanning the gels in a Zeiss PMQ II spectrophotometer with chromatogram scanning attachment and a log recorder. Unstained gels were fractionated in 1-mm sections using an AutoGelDivider (Savant Corp.) for determination of radioactivity. Electrophoresis of acetylated chromatin proteins, and control histone (Worthington Corp.) at acidic pH values was performed using the modifications of Shirey and Huang (1969) to the acid–urea polyacrylamide system of Panyim and Chalkley (1969).

Results

A detailed evaluation of the localization of the free or reactive basic groups of the proteins of chromatin can be obtained by selective chemical modification of the nucleoprotein

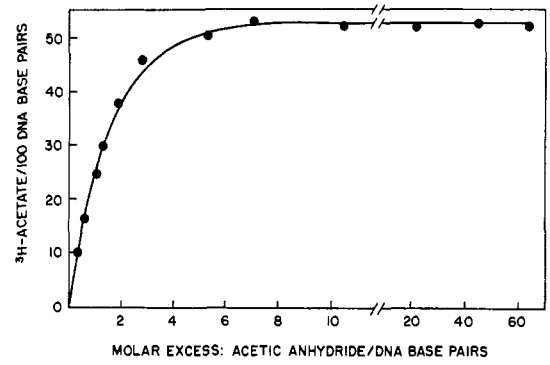

FIGURE 1: Acetylation of rabbit liver chromatin. Chromatin, at a DNA concentration of 1 mg/ml in 0.1 M sodium borate, pH 9.0, 0°, was reacted with increasing excesses of acetic anhydride. After prolonged dialysis to remove excess reagent and products, the incorporation of ^{3}H-labeled acetate was determined.

complex. Under the conditions employed for the current study, acetic anhydride forms stable derivatives primarily with ϵ-amino groups of lysyl residues, α-amino groups, and phenolic hydroxyl groups of tyrosyl residues. Acetylation of a variety of proteins with acetic anhydride has demonstrated the virtually quantitative modification of the free lysyl and tyrosyl residues of the proteins with little in the way of side reactions (Riordan and Vallee, 1967).

Reaction of increasing amounts of acetic anhydride with rabbit liver chromatin at a DNA concentration of 1 mg/ml leads to an increasing incorporation of labeled acetate, which appears to reach a limit at a value of 54 molecules of acetate/100 base pairs of DNA of chromatin, at a molar excess of acetic anhydride to DNA base pairs of 6 (Figure 1). Increasing the concentration of the modifying agent by 10-fold leads to no further incorporation of acetate. Modification at a ratio of 6 moles of acetic anhydride/DNA base pair was adapted as standard for further experiments. Under similar conditions for the modification, acetylation of calf thymus chromatin led to incorporation of 45 molecules of acetate/100 DNA base pairs, presumably reflecting its lower protein content.

The conformation of proteins and DNA in acetylated chromatin was examined by circular dichroism. No decrease in the helix content of the protein components is apparent from the circular dichroism spectrum in the 200- to 230-nm region. The decreased ellipticity at 270–280 nm of the DNA of chromatin, when compared to free DNA (Permogorov *et al.*, 1970; Shih and Fasman, 1970; Simpson and Sober, 1970) is preserved unaltered in the acetylated nucleoprotein complex.

Acetylation does lead to a slight alteration in the thermal denaturation profile of chromatin (Figure 2). The modified chromatin melts at a temperature some 4° lower than that of the native material. However, the melting profile is still broad, characteristic of the native protein–nucleic acid complex, in contrast to the much sharper and lower thermal transition of free DNA (Figure 2). Further, none of the proteins of chromatin appear to be dissociated from the complex consequent to the modification reaction, inasmuch as all the acetate is sedimented by centrifugation for 18 hr at 40,000 rpm, 4°. All of the isotopic label is retained by an Amicon XM-300 membrane on pressure dialysis, a similar indication of the integrity of the acetylated chromatin complex.

As noted above, acetylation would be expected to modify both lysyl and tyrosyl residues of chromatin proteins. The lysyl modification is quite stable, while the acetyl ester link-

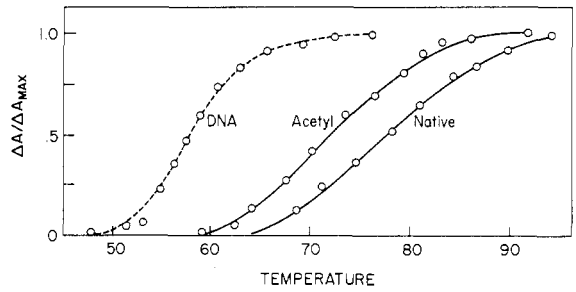

FIGURE 2: Thermal denaturation of DNA and acetylated and native rabbit liver chromatin. Samples were dialyzed to equilibrium *vs.* 0.001 M sodium borate (pH 8.7) and thermal denaturation profiles determined in a Zeiss spectrophotometer at 260 nm. Ten minutes equilibration was allowed at each temperature. The results are normalized to the maximal absorbancy change.

age to the phenolic hydroxyl of tyrosine is split by either dilute alkali or hydroxylamine. When acetylated chromatin, containing 54 molecules of acetate/100 base pairs is incubated at pH 11.0 and 25° for 1 hr and then dialyzed to remove the hydrolyzed label, the remaining acetate covalently bound to chromatin proteins corresponds to a content of 35 molecules of acetate/100 base pairs of DNA. This difference, 19 molecules of acetate, is the appropriate value for the modification of all of the tyrosyls of the chromatin proteins detected by amino acid analysis, consistent with the spectrophotometric titrations of Walker (1965). For calf thymus chromatin, similar incubation and dialysis led to a decrease from 45 to 31 molecules of acetate per 100 DNA base pairs.

Initial attempts to localize the acetylated lysyl residues utilized polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Acetylated chromatin was dissociated at a DNA concentration of 0.6 mg/ml in 5 M guanidine hydrochloride–0.001

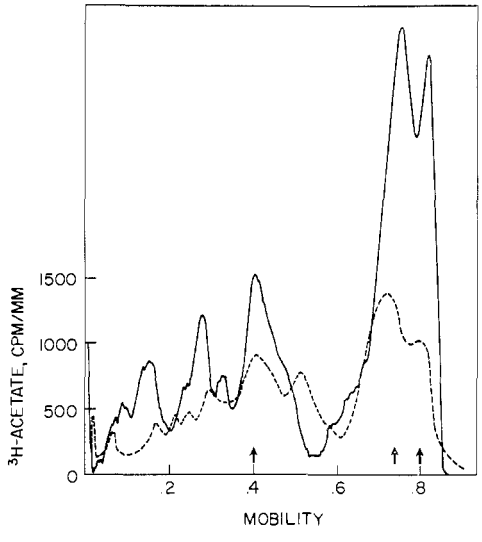

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of acetylated rabbit liver chromatin. Electrophoresis and gel division were carried out as described in the Experimental Section. Absorbancy due to the protein stain (———) is plotted on an arbitrary scale. [³H]Acetate was determined in several experiments and the results averaged (----------). Migrations are relative to the movement of the bromophenol blue tracking dye. The positions of migration of authentic FI (lysine-rich) FII (slightly lysine-rich), and FIV (arginine-rich) histones are indicated by the arrows from left to right, respectively.

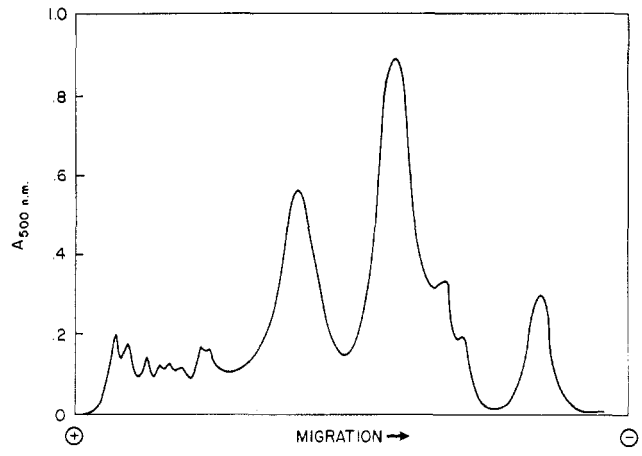

FIGURE 4: Acidic urea polyacrylamide gel electrophoresis of acetylated chromatin proteins. Electrophoresis was carried out as in the Experimental Section. Absorbancy due to the protein stain is plotted *vs.* the distance of electrophoretic migration. Native FII and FIV histones migrate further than the most cathodic band shown in this electrophoretic separation.

M Tris·Cl (pH 7.8) and DNA sedimented by centrifugation at 30,000 rpm for 24 hr at 20°. Such dissociation and sedimentation removes more than 95% of the proteins from the DNA of chromatin. Less than 5% of the isotope sediments with DNA in such experiments, presumedly this is contained in nondissociated proteins. The supernatant containing the modified proteins of chromatin was dialyzed exhaustively *vs.* 0.0001 M sodium borate (pH 8.7) and lyophilized. After dissociation with 1% sodium dodecyl sulfate and 1% β-mercaptoethanol, the lyophilized proteins were analyzed by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate.

The results of electrophoresis of such an acetylated chromatin sample are detailed in Figure 3. With one exception, the nonhistone proteins of these chromatin preparations migrate more slowly than the lysine-rich histones, and no nonhistone proteins migrate with mobilities equal to those of histones (S. Levy and R. T. Simpson, unpublished observations). Rather extensive modification of the nonhistone proteins of chromatin has occurred. Acetylation of all three histone fractions is shown by the peaks of isotope incorporation coincident with the migration positions of authentic FI, FII, and FIV histones as indicated. The degree of modification of the lysine-rich histone, FI, appears to be greater than that of the slightly lysine-rich histone, FII, or the arginine-rich histone, FIV. Of more interest in the current study, however, is the observation that some lysyl residues of *all* the histone fractions are available for acylation, and hence are presumably not bound to DNA under the conditions of the modification. The basic groups of proteins which are titratable in the native complex (Walker, 1965) thus derive from contributions of all histone classes, and not from the partial binding of any single group of histones.

Sodium dodecyl sulfate electrophoresis separates molecules only on a size basis, and thus it does not indicate whether some histone molecules of a particular class are more markedly substituted than others of that group. Electrophoresis of acetylated chromatin proteins on acidic urea polyacrylamide gels, however should clarify this point for each class of histones. When acetylated chromatin proteins are subjected to such an electrophoretic separation, no protein bands are observed at the migration positions of authentic histones. Thus

all the histone molecules of chromatin are acetylated. New protein bands, not observed in native chromatin proteins, are present at more anodic positions (Figure 4). Two of these bands are simple, while the middle band contains three components, as has been observed for native histones (Panyim and Chalkley, 1969), although the proportions of the three components observed in this middle band are altered relative to those observed for control histone. Similar results are obtained with acetylated calf thymus chromatin. There is no indication of the presence of varying degrees of modification of the various histones (*e.g.*, band spreading, or new bands in addition to those expected), suggesting that the modification reaction has indeed been specific, acetylating only a limited, specific group of lysyl residues in each histone class.

Discussion

Although the knowledge of the macromolecular architecture of cell components is required for full understanding of their function, only recently has such detailed study of these components been attempted. Perhaps the best example of the success of such an approach is the currently expanding knowledge of the structure and function of the bacterial ribosome (*cf.* Kurland, 1970). Characterization of the protein and nucleic acid components of the ribosome, coupled with knowledge of their mode of assembly and the availability of the proteins for chemical modification, has generated insight into the functional and structural properties of this complex organelle (Kurland, 1970; Craven and Gupta, 1970). In this study of chromatin structure, methodology similar to that recently employed in the study of the ribosome has been utilized.

It has become apparent that significant proportions of the acidic groups of DNA and the basic groups of chromatin proteins are not electrostatically linked to one another, but rather are free to interact with solution components (Walker, 1965; Simpson and Sober, 1970; Simpson, 1970; Itzahki, 1970; Clark and Felsenfeld, 1971). To help in understanding the structure of the chromatin complex, it is desirable to be able to localize in the primary sequence of the histones those basic groups which are and are not bound to DNA-P. Thus, the current study was designed to determine whether certain specific histones are entirely salt linked to DNA, while others contribute all the free basic groups; or alternatively, is there evidence to suggest that each of the histone classes are only partially electrostatically bound to DNA, and that each histone possesses nonbound basic residues. Of particular importance, the method employed allows not only investigation of this primary question but also permits localization of the modified groups in the histone sequences.

Acetylation of chromatin with acetic anhydride proves to be a mild modification, reaching a stable limiting value of incorporation, and leading to only minor alterations in the stability of the chromatin complex. Approximately 75% of the lysyl residues of the chromatin proteins do not react with the acylating agent, indicating their removal from the ambient medium, presumably through the formation of electrostatic bonds with DNA-P.[1] In contrast, most of the lysyl residues of globular proteins react readily with acylating agents. All the lysyl residues of the slightly lysine-rich and arginine-rich histones can be modified by acetic anhydride in the free proteins (Iwai *et al.*, 1970; DeLange *et al.*, 1969).

All of the proteins of chromatin, specifically, all the histone fractions, are modified to some degree by acetic anhydride, even though no physical changes indicative of alterations in the conformation of the complex could be detected nor dissociation of the proteins of the complex could be demonstrated. The fact that all histone fractions were modified demonstrates that no histone fraction is completely electrostatically linked to DNA. Further, none of the histones present in acetylated chromatin migrate on acid urea gels with velocities equal to those of native histones. Thus, within each individual class of histone, all the molecules are partially acetylated. New bands are seen for acetylated chromatin proteins on such an electrophoretic separation and these bands are indicative of a high degree of homogeneity among their component protein molecules. The calculations of Panyim and Chalkley (1969) have indicated that a charge difference of 1 or 2 per histone molecule should be detectable on such an electrophoresis. The absence of new bands in excess of the modified histone bands, and the lack of band spreading, together suggest that the stoichiometry of reactive, free lysyl residues for each individual histone class is remarkably restricted. These results strongly imply that certain, particular, lysyl residues in the primary structure of any individual group of histone molecules may be accessible to modification, and hence suggest a somewhat unexpected specificity in the interaction between these basic proteins and DNA in native chromatin.

The most provocative extension of this study is the possibility of critically examining the hypothesis that the basic regions in the primary structure of the histones constitute DNA binding regions, while the relatively nonbasic regions do not bind to DNA. Localization of the modified groups in the primary sequence of the histones is currently in progress. Coupled with other information concerning the topological localization of the proteins of chromatin (Simpson, 1970), such experiments should allow the generation of more detailed models of the nucleoprotein complex, chromatin.

Acknowledgment

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[1] Formation of electrostatic linkages between basic protein residues and acidic residues of the histones or nonhistone proteins might also mask reactivity of lysyl residues to acetylation. Such a possibility seems unlikely however, in view of the freely reversible titration of all the acidic groups of chromatin proteins in the native complex (Walker, 1965).

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Structure of Rat Skin Collagen α1-CB8. Amino Acid Sequence of the Hydroxylamine-Produced Fragment HA1*

Gary Balian, Eva Marie Click, and Paul Bornstein†

ABSTRACT: Hydroxylamine cleavage of α1-CB8, a large CNBr-produced peptide obtained from the α1 chain of rat skin collagen, yielded two fragments. The amino acid sequence of the 99 residues in the NH_2-terminal fragment, HA1, has been determined. In keeping with other collagen sequences, glycine was found as every third amino acid and hydroxyproline was limited to the third position in the repeating triplet Gly-X-Y. However, hydroxylation of several prolyl residues in this position was incomplete, indicating that the phenomenon of partial hydroxylation extends to prolyl residues far removed from the NH_2 terminus of the α chain. A pronounced clustering of charged residues, which correlates well with the band pattern observed in electron micrographs of segment long-spacing aggregates of α1-CB8, was found in HA1. The data obtained in this study, together with other published sequences, further suggest that certain amino acids, notably leucine and phenylalanine, are distributed in a nonrandom fashion in positions X and Y of the collagen triplet.

The determination of the primary structure of α1-CB8, a large fragment obtained by CNBr cleavage of the α1 chain of rat collagen, was undertaken to provide detailed information regarding the chemistry of collagen. It is expected that the analysis of a collagen sequence of this magnitude (282 amino acids) will (1) assist in relating the structure of the molecule to its primary function as a self-aggregating unit in fiber formation; (2) augment existing knowledge of the distribution of specific amino acids in the collagen triplet, Gly-X-Y, and provide information regarding possible sequence homologies which may reflect duplication of genetic material; (3) elucidate further the pattern of hydroxylation of proline (Bornstein, 1967) and lysine (Butler, 1968; Bornstein, 1969a); (4) provide additional correlations between the distribution of charged amino acids and the band pattern in electron micrographs of SLS[1] aggregates of collagen (von der Mark *et al.*, 1970).

α1-CB8 constitutes about 25% of the length of the α1 chain of rat collagen and is located in the middle of the first half of the chain (Piez *et al.*, 1969; Rauterberg and Kühn, 1968). In the first paper in this series (Bornstein, 1970) the fractionation and amino acid compositions of the tryptic peptides of α1-CB8 were reported and the nature and mechanism of cleavage of a hydroxylamine-sensitive bond were described. The hydroxylamine-sensitive bond, thought previously to be an example of a nonpeptide bond linking intra-α-chain subunits (Gallop *et al.*, 1967), was shown to consist of a cyclic imide which formed by cyclization of an asparaginyl side chain with the subsequent (glycyl) amide group in the polypeptide chain (Bornstein, 1969b, 1970). Elucidation of the nature of the hydroxylamine-sensitive bond radically altered its significance. However, use was made of the specific cleavage of α1-CB8 by hydroxylamine to separate two large fragments, HA1 and HA2, which accounted for the amino acid composition and molecular weight of the starting material.

The availability of a point of cleavage roughly one-third of the length of α1-CB8 from the NH_2 terminus greatly simplified the determination of its amino acid sequence. The primary structure of the NH_2-terminal fragment HA1, consisting of 99 amino acids, is reported in this communication. The determination of the structure of HA2 (183 amino acids) is in progress and will be reported separately (G. Balian, E. M. Click, M. Hermodson, and P. Bornstein, in preparation).

Materials and Methods

Preparation of α1-CB8 and Cleavage with Hydroxylamine. α1-CB8 was prepared from salt-extracted lathyritic rat skin collagen (Butler *et al.*, 1967; Bornstein, 1970). Acid-extracted normal rat skin collagen was also used as a source for some

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[1] Abbreviations used are: SLS, segment long spacing; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; DFP, diisopropyl fluorophosphate.