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Inhibition of Collagen Intermolecular Cross-Linking by Thiosemicarbazide*

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ABSTRACT: Thiosemicarbazide (TSC) reacts at acid pH with embryonic calf skin collagen to yield a product which contains 2 moles of thiosemicarbazide/mole of collagen. This collagen derivative polymerizes normally from solution at neutral pH but the fibrils readily dissolve in the cold, similar to those of lathyritic collagen. After gel filtration or CM-cellulose chromatography denatured thiosemicarbazide collagen shows little loss of TSC, the latter being distributed among the α and β components. Comparing the TSC–collagen and normal collagen no decrease in cross-linked components is observed. Difference spectra suggest that the TSC is bound to the collagen as thiosemicarbazone derivatives.

The 2 moles of TSC are found on the "A" fragment obtained after removal of 25% of the molecule at the "B" end with tadpole collagenase. Digestion of the native protein with trypsin or chymotrypsin removes most of the TSC, which is present in two different groups of fragments of several thousand molecular weight. Lathyritic chick embryo collagen produced *in vivo* by thiosemicarbazide contains negligible amounts of this lathyrogen. A significant feature of these studies is that TSC–collagen behaves like lathyritic collagen with respect to impaired intermolecular cross-linking but has no deficiency of intramolecular cross-links suggesting that the two properties are not necessarily interdependent.

The age and the solubility characteristics of various collagen fractions in tissue have often been related to their degree of inter- and intramolecular cross-linking (see review by Harding, 1965). The extractable collagen[1] from lathyritic animals is deficient in intramolecular cross-links (Martin *et al.*, 1961), and forms unstable native fibrils which dissolve in cold salt solutions (Gross, 1963). This latter characteristic indicates an impairment of intermolecular cross-linking and probably underlies the marked loss of tissue tensile strength and increased collagen solubility in lathyritic animals (Levene and Gross, 1959). All other physical and chemical features of lathyritic collagen appear normal, implying the presence of a subtle molecular alteration.

Recently, attention has focused upon the presence of aldehyde-like compounds in collagen (Rojkind *et al.*, 1964) and their potential role in cross-linking. All of the lathyrogens are capable of forming addition products with aldehydes (Levene, 1962) but it has been clearly demonstrated that lathyritic collagen does not contain significant amounts of bound lathyrogen (Orloff and Gross, 1963).

Recent work suggests that one type of intramolecular cross-link may develop simultaneously with the conversion of specific lysines in collagen to aldehyde derivatives (Bornstein *et al.*, 1966). In addition, lathyritic collagen was found to lack these carbonyl compounds but to possess the specific precursor lysyl residues.

In our studies of the binding of the lathyrogen, thiosemicarbazide, to collagen we noted a marked inhibition of intermolecular cross-linking of fibrils formed from TSC–collagen similar to that seen for lathyritic collagen fibrils. This report describes these phenomena and discusses the implications.

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[1] Abbreviations are TSC, thiosemicarbazide; TCA, trichloracetic acid; TC^A, tropocollagen "A" fragment; TC^B, tropocollagen "B" fragment; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

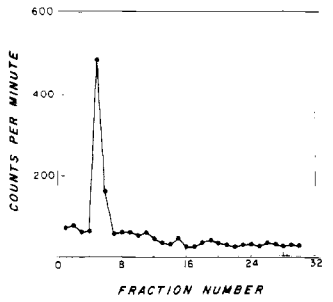

FIGURE 1: Gel filtration of 10 mg of TSC–gelatin at 40°, on a calibrated 0.9 × 60 cm column of Biogel P-4 in pyridine–acetate, pH 4.5. Previous calibration showed various proteins emerged in fractions 5–7.

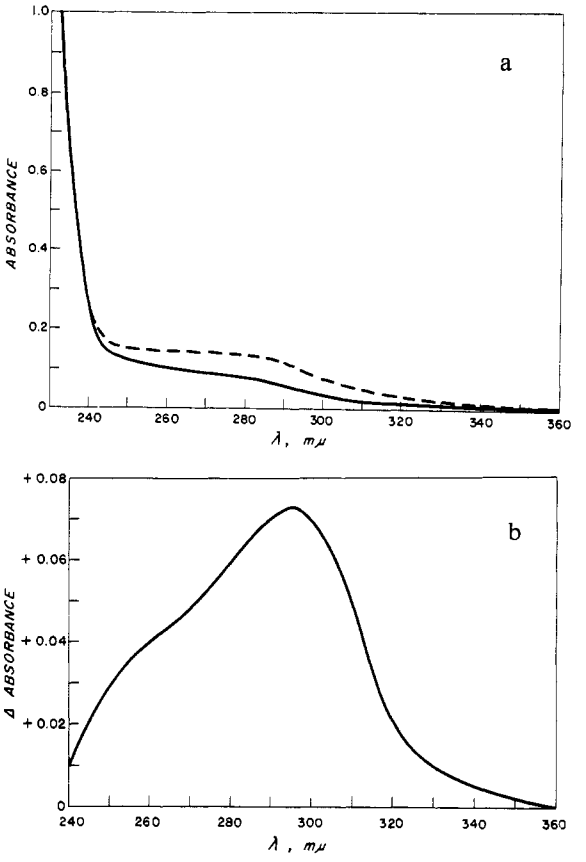

FIGURE 2: Absorption spectra and difference spectrum of collagens. (a) —— normal collagen and ---- TSC–collagen at room temperature in 0.4 M NaCl, 0.01 M Tris–HCl, pH 7.6. (b) Direct measurement of the difference spectrum. Collagen concentration, determined by hydroxyproline content, was identical in both solutions.

Materials and Methods

Collagen was obtained from sliced embryonic calf skin by pooling three sequential 0.1 N acetic acid extracts. The protein was purified by the trichloroacetic acid–ethanol method of Gross (1958) and the lyophilized product was found to contain 13% hydroxyproline, based on dry weight. Amino acid composition, ultracentrifugation, and acrylamide gel electrophoresis all indicated the collagen was free of detectable contaminants.

Lathyrism was produced in 14-day-old chick embryos by placing 5 mg of thiosemicarbazide containing 50 μc of [^{35}S]thiosemicarbazide in 0.1 ml of H_2O onto their chorioallantoic membranes; this dose caused all the clinical features of lathyrism (Levene and Gross, 1959). Two days after injection, 10-dozen decapitated embryos were homogenized in an equal volume of ice-cold 1 M NaCl. The homogenate was stirred at 4° overnight, strained through cheese cloth, and centrifuged at 13,200*g* for 1 hr. Collagen was precipitated from the supernatant by adding solid NaCl to 2.5 M and was collected by centrifugation, redissolved in phosphate buffer, pH 7.6, Γ/2 0.4, and subsequently purified by alternate precipitations at high and low salt concentrations for a total of three cycles. Following purification by passage through two cycles of the TCA–ethanol procedure 300 mg of collagen was obtained.

The reaction of collagen with thiosemicarbazide was performed according to the method of P. Gallop and M. Rojkind,[2] using [^{35}S]thiosemicarbazide of specific activity 0.22 μc/μmole. Briefly, the reaction conditions consist of mixing an ice-cold 0.2% collagen solution in 0.5 M acetic acid with a 7500-fold molar excess of thiosemicarbazide (dissolved in an equal volume of ice-cold H_2O) followed by stirring for 1 hr at 0°. The mixture was dialyzed extensively *vs.* 0.05 M acetic acid at 4°. Subsequent purifications were accomplished by the methods mentioned above.

Thiosemicarbazide obtained from Distillation Products Industries was found to contain a trace of colloidal sulfur which was filtered from the freshly prepared solution just prior to use. [^{35}S]Thiosemicarbazide (201 mc/mmole) was a product of the Radiochemical Centre and was found to be radiochemically pure by thin layer cochromatography with carrier thiosemicarbazide. The thin layer chromatography was carried out on plastic sheets coated with silica gel, using either 1-butanol–acetic acid–H_2O or ethanol–NH_4OH as developing solvents. A single spot was detected by ninhydrin spray, and passage of the plate through a radioactivity scanner showed all the radioactivity to be concentrated at this spot.

All quantitative radioactivity measurements were done in Bray's solution (1961) using a liquid scintillation spectrometer. The collagen preparations were hydrolyzed in 6 N hydrochloric acid prior to radioactivity measurements, an aliquot part of the hydrolysate being used for hydroxyproline measurement (Bergmann and Loxley, 1963). Collagen content was based on the hydroxyproline content of 13% described above. All values were corrected for radioactive decay by use of

[2] Personal communication.

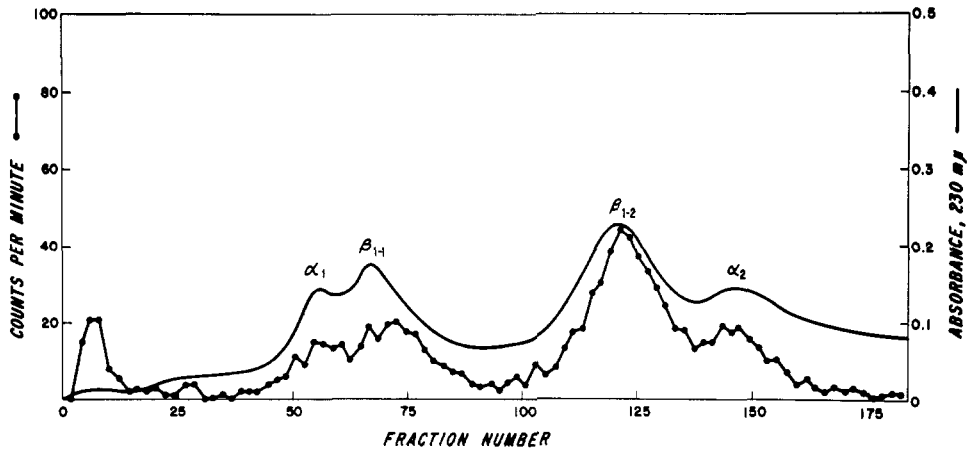

FIGURE 3: CM-cellulose chromatography of 50 mg of TSC–collagen. Samples (0.5 ml) from every other fraction were measured for radioactivity.

standard tables and for quenching by recounting all samples after addition of internal standards.

Chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corp., and trypsin, TPCK-treated to free it of chymotrypsin, was obtained from California Biochemical Corp. A 0.2% solution of collagen in potassium phosphate buffer, pH 7.6, Γ/2 0.4, was incubated at 20° for 16 hr with one-fifth its weight of enzyme. At the end of the incubation, the solution was cooled to 0° and the collagen was precipitated by addition of ice-cold absolute ethanol to a final volume of 20%. After 20 min, the collagen was collected by centrifugation. Control experiments using [^{14}C]glycine-labeled collagen showed that all of the protein precipitated under these conditions.

Collagen fibril formation and the degree of intermolecular bonding of the fibrils were tested as previously described (Gross, 1963). Acrylamide electrophoresis was carried out by the method of Nagai *et al.* (1964) as modified by T. Sakai and J. Gross (unpublished data) and carboxymethylcellulose chromatography was done according to Piez *et al.* (1963). Spectral absorption studies were performed using a Cary recording spectrophotometer. Difference spectra of the collagen solutions were obtained by direct measurement using two closely matched 1-cm light path quartz cuvets. The resultant curve was corrected for minor variations in the base-line absorbancy of the difference spectrum obtained from the cuvets containing solvent alone. All controls were carried out upon collagen which underwent the same procedures as TSC–collagen except for exposure to thiosemicarbazide.

Results

Binding of Thiosemicarbazide to Collagen in Vitro—A variable amount of thiosemicarbazide was bound to the collagen after the initial extensive dialysis (*vs.* 27 × 10^3 volumes), as many as 80 moles/mole of collagen in one case, suggesting that some nonspecific adsorption occurs. Addition of nonradioactive TSC to the dialysis fluid did not alter this adsorption. Slight adsorption was also found to occur between denatured hemoglobin and TSC under similar conditions, *ca.* 0.02 mole/mole. Three preparations of thiosemicarbazide collagen were made on different occasions. All contained 2 moles/mole of collagen, remaining at this level despite repeated sequential purification steps (Table I).

TABLE I: Binding of Thiosemicarbazide to Collagen.[a]

Sample	TSC/Collagen (mole/mole)[b]
Reaction mixture	7500
Dialyzed collagen	3.1
Repurified collagen, TCA–ethanol × 1[c]	2.2
Repurified collagen, ethanol × 2[d]	2.1
Repurified collagen, prepn 2	2.3
Repurified collagen, prepn 3	1.9

[a] For reaction conditions and purification procedures see text. [b] Moles of TSC/mole of protein, mol wt 300,000. [c] Dialyzed collagen passed through one cycle of the TCA–ethanol purification procedure. [d] Collagen from *c* precipitated twice by ethanol.

To further determine the degree of affinity between thiosemicarbazide and collagen, the protein was denatured by warming at 40° for 15 min and was passed through a calibrated gel filtration column (Figure 1). All the radioactivity appeared in the fractions contain-

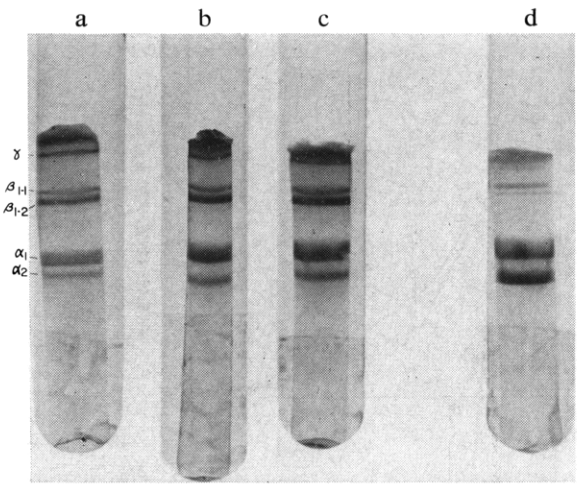

FIGURE 4: Acrylamide electrophoresis of (a) normal calf skin collagen, (b) and (c) TSC–collagen (two different preparations), and (d) lathyritic chick embryo collagen produced *in vivo* by TSC.

ing protein; these fractions were then pooled, lyophilized, analyzed, and found to contain 1.8 moles of thiosemicarbazide/mole of collagen. Occasionally, prolonged exposure (days to weeks) of either TSC–collagen or gelatin to acid conditions (pH 3–4) would result in the loss of 1 mole of thiosemicarbazide from the protein.

Thiosemicarbazones have intense absorption maxima in the ultraviolet region (Evans and Gillam, 1943) suggesting that spectrophotometric comparisons might be informative. As shown in Figure 2a the TSC–collagen spectrum is unlike the control. The difference spectrum (Figure 2b) clearly shows an asymmetric curve with a peak at 295 mμ and an inflection point at 265 mμ. The ϵ_{295} is 18.6×10^3 assuming 1 mole of thiosemicarbazone is responsible for this peak. Thiosemicarbazide alone or added to a solution of normal collagen, at a ratio of 2 moles/mole at neutral pH, yielded a single absorption maximum at 235 mμ.

To obtain more information about the affinity and site of thiosemicarbazide binding, the denatured collagen was subjected to chromatography on carboxymethylcellulose (Figure 3). As shown, the thiosemicarbazide is distributed over each of the collagen subunits although some TSC has emerged from the column prior to the emergence of the protein. Two other notable features of this chromatograph are the absence of a usual early absorbance peak and the presence of a high content of β components in this particular collagen preparation. Chromatography of the control collagen yielded an identical pattern of subunit composition, further substantiated by acrylamide electrophoresis (Figure 4).

The molar content of TSC bound to the collagen chains obtained from CM-cellulose chromatography is shown in Table II. The nonintegral numbers probably reflect cleavage of TSC due to the prolonged exposure to elevated temperature and acid pH. This cleavage may be catalyzed by CM-cellulose because TSC–gelatin lost only 10% of its thiosemicarbazide after 20 hr at 40°, pH 4.5. Interestingly, the content of TSC in the α1 components is half that of the α2 components and that of the β chains is almost twice that of the α2 fraction. More direct studies were carried out by treating isolated, purified, normal α1 and β_{12} components (shown to be homogeneous by acrylamide electrophoresis) with TSC. Following gel filtration, 1 mole of α1 contained 0.5 mole of TSC and 1 mole of β_{12} contained 1.4 moles of TSC. Difference spectra showed an asymmetrical curve for α1-TSC with a maximum at 266 mμ and an inflection at 292 mμ. The β_{12}-TSC spectrum was symmetrical with a peak at 308 mμ.

TABLE II: Binding of Thiosemicarbazide to Collagen Subunits.[a]

Sample	TSC/Subunit (mole/mole)
α1	0.17
α2	0.32
β_{11}	0.48
β_{12}	0.53

[a] The three central fractions of each peak in Figure 3, comprising 9 ml, were dialyzed and lyophilized prior to assay. Molecular weights of 100,000 and 200,000 were assumed for the α and β chains, respectively, and their hydroxyproline contents (Schleyer, 1962) were used to calculate the binding ratio.

Physical Effects of Thiosemicarbazide Binding. The rate of formation of collagen fibrils from solutions of TSC–collagen on warming to 37° and the degree of fibril cross-linking as a function of incubation time were measured. Figure 5 shows that the kinetics of fibril precipitation from solution were unchanged from those of the normal, but that the stability of the fibrils of thiosemicarbazide collagen was markedly diminished when assayed by cooling the suspension. The amount of collagen solubilized, as determined by the change in opacity, was confirmed by measuring the hydroxyproline content of the hydrolyzed supernatant fluid after centrifugation of the cooled gels. The thiosemicarbazide/collagen molar ratio was identical in the supernatant fluids and precipitates in all samples. Electron microscopy of the TSC–collagen fibrils showed the native-type structure, similar in appearance to the controls. In repeated experiments, the fibrils of TSC–collagen reproducibly dissolved upon cooling but it was found essential to use freshly prepared normal and TSC–collagen solutions to obtain such reproducibility.

The effect of thiosemicarbazide on fibril stability could not be simulated by adding it to solutions of collagen at neutral pH just prior to precipitation (Figure 6). The molar ratios of thiosemicarbazide to collagen ranged from 2/1 to 2000/1 in this experiment, yet no

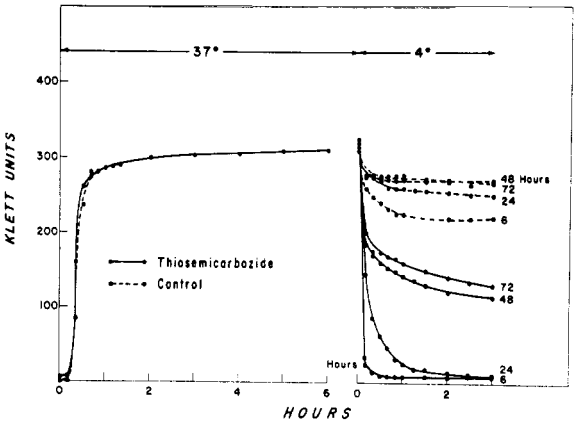

FIGURE 5: Effect of TSC binding to collagen upon fibril formation and dissolution, --- control collagen, and ——, TSC–collagen. "Hours" refers to the duration at 37°. The conditions were: 0.15% collagen solutions in 0.4 M NaCl, 0.01 M Tris–HCl, pH 7.6, initially at 4°, and previously centrifuged at high speed to remove any undissolved protein. Samples (2 ml), in duplicate, in Klett microtubes were placed in a 37° bath and the opacity monitored periodically in a Klett colorimeter using a no. 54 filter. After the time periods shown, the duplicate tubes were cooled in an ice-water bath and the degree of collagen fibril dissolution was followed by opacity measurements.

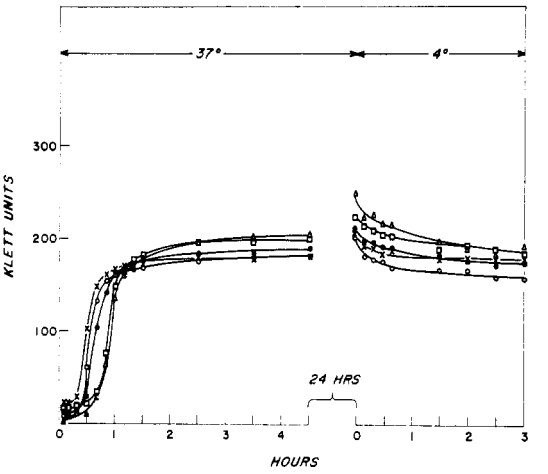

FIGURE 6: Effect of addition of TSC to normal collagen just prior to fibril precipitation. ○, control; Δ, 10^{-2} M TSC; □, 10^{-3} M TSC, ●, 10^{-4} M TSC, ×, 10^{-5} M TSC. Conditions are the same as Figure 5, except for addition of TSC.

effect was evident save for slight retardation of fibril formation at the higher TSC concentrations. Free thiosemicarbazide was stable under these conditions as determined by chromatographic examination of a portion of the supernatant fluid in two different solvent systems.

Localization of the Thiosemicarbazide. Inasmuch as thiosemicarbazide was bound to collagen as thiosemicarbazone derivatives and inhibited intermolecular cross-linking in the native-type fibril it seemed likely that specific binding sites might be involved. For further localization of such sites the TSC–collagen was incubated with tadpole collagenase (Gross and Nagai, 1965) which severs the native collagen molecule into two conformationally intact fragments, TC^A and TC^B, these pieces constituting 75 and 25% of the molecular mass, respectively (T. Sakai and J. Gross, unpublished data). The TC^A and TC^B fragments were separated by ammonium sulfate precipitation and virtually all of the thiosemicarbazide was found on the "A" fragment (Table III).

Isolation of smaller fragments of collagen containing TSC was accomplished by incubation of the native protein with either trypsin or chymotrypsin followed by precipitation of the unattacked portion of the molecule. Table IV indicates that these proteases significantly reduced the TSC content of the collagen. The released thiosemicarbazide was associated with two distinct peaks representing fragments of several thousand molecular weight as estimated by experiments using gel filtration (Figure 7). In addition to radio-

TABLE III: Distribution of Thiosemicarbazide on the Collagen Molecule.[a]

Sample	TSC/Fragment (mole/mole)
TC^A	2.4
TC^B	0.1

[a] TSC–collagen (10 mg) was incubated with purified tadpole collagenase at pH 7.6, 20°, for 16 hr. The fragments were separated by $(NH_4)SO_4$ precipitation. The hydroxyproline content of each fragment (K. A. Piez, personal communication) was used to calculate the binding ratio.

TABLE IV: Thiosemicarbazide Content after Protease Treatment.[a]

Sample	TSC/Collagen (mole/mole)
TSC–collagen	2.3
TSC–collagen + trypsin	1.5
TSC–collagen + chymotrypsin	0.5

[a] TSC–collagen (10 mg) was incubated with one-fifth its weight of enzyme at pH 7.6, 20° for 16 hr. The collagen was precipitated by ethanol and its TSC content determined.

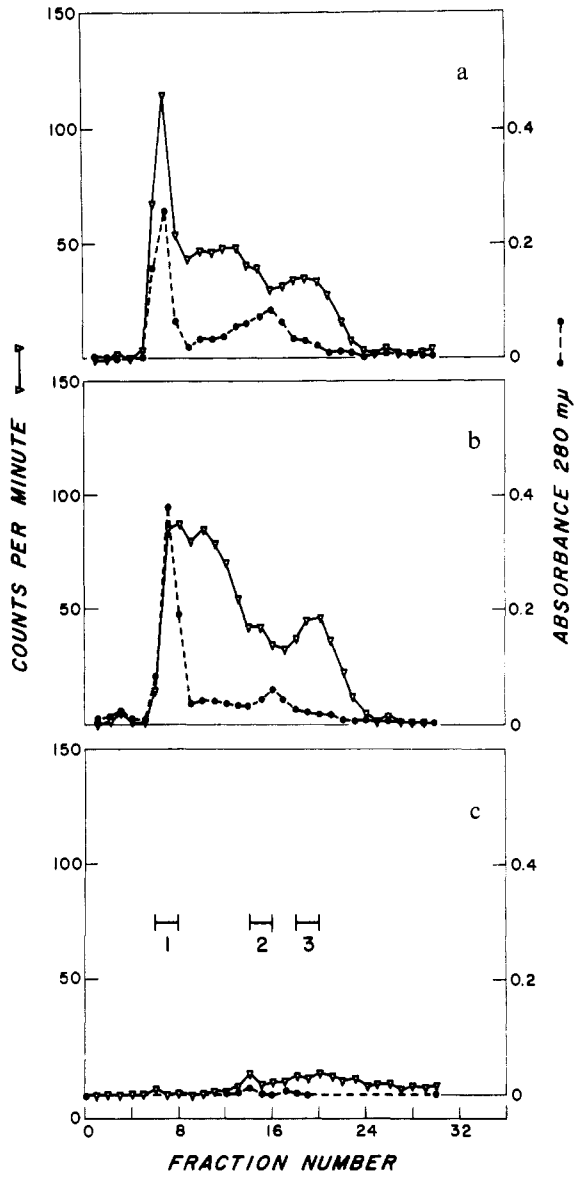

FIGURE 7: Gel filtration of the lyophilized supernatant fluids obtained from the experiment in Table III, a, trypsin, b, chymotrypsin, c, control. Filtration done at room temperature on a 0.9 × 60 cm column of Biogel P-4 in pyridine–acetate, pH 4.5. The numbered lines indicate the calibrated regions of (1) gel exclusion, (2) salts, and (3) free thiosemicarbazide.

activity, the optical density at 280 mμ was monitored to detect the location of thiosemicarbazones or the added proteases. Some free thiosemicarbazide or small peptides containing TSC were detected during the gel filtration, appearing as a peak at the area of emergence of reference TSC.

Lathyritic Collagen Produced by Thiosemicarbazide. In order to determine whether thiosemicarbazide causes experimental lathyrism by binding to collagen *in vivo*, the protein was isolated from [^{35}S]thiosemicarbazide-injected chick embryos. It contained 0.02 mole of TSC/mole of protein, was deficient in β components (Figure 4), and reconstituted fibrils dissolved on cooling.

Discussion

Although varying amounts of thiosemicarbazide may partially adsorb to collagen or other proteins, the consistent finding of 2 moles of TSC/mole of collagen in three different preparations after repeated purification steps indicates that a distinct product is formed. Moreover, this binding most likely is covalent because: (1) gel filtration of the denatured protein fails to remove the TSC; (2) limited digestion of the native protein with tadpole collagenase, trypsin, or chymotrypsin yields fragments containing TSC; (3) exposure of collagen to TSC at neutral pH neither produces thiosemicarbazone spectra nor has any effect on fibril stability. This latter point is consistent with the finding that acid pH catalyzes the condensation of carbonyl compounds and the analog of TSC, semicarbazide (Jencks, 1959).

The exact nature of the chemical bond between thiosemicarbazide and collagen cannot be determined by our studies but the absorption spectra strongly suggest that thiosemicarbazones are being formed by reaction with carbonyl groups in collagen. These groups seem to be both α,β-unsaturated, with a peak near 295 mμ, and saturated with a peak near 265 mμ (Evans and Gillam, 1943).

The presence of 2 moles of TSC/mole of calf skin collagen implies that either one or two of the three α chains in the collagen molecule are able to bind TSC. That two chains bind is supported by the finding that the TSC content of the α1 fraction is half that of α2 fraction although there are 2 moles of α1/mole of α2. This is substantiated by the experiment in which isolated, normal α1 was found to bind 0.5 mole of TSC/mole of protein. The same result also implies that the α1 fraction may be composed of two different types of polypeptide chains, as shown for codfish skin collagen (Piez, 1965) and suggested by cleavage studies of rat skin collagen (Bornstein and Piez, 1965). In addition, since there are 2 moles of TSC/collagen, molecule α2 must contain 1 mole of TSC/mole of protein.

The greater content of TSC in the β components compared with the α fractions indicates that there is no loss of carbonyl groups during intramolecular cross-linking, in contrast with a proposed aldol condensation mechanism (Bornstein *et al.*, 1966). Indeed, the β_{12} content of 1.4 moles of TSC/mole of protein, close to the statistically expected value of 1.5, denotes that the α1 chains which do not contain carbonyl groups are capable of forming β components. Furthermore, the spectral studies suggest that prior to intramolecular cross-linking the carbonyl compounds may convert from a saturated to an unsaturated configuration.

The absence of an ultraviolet absorbing peak which usually occurs early in the CM-cellulose chromatograph may reflect the degree of purity of the initial collagen preparation, since this peak was found during chromatography of less pure preparations. In addition,

the embryonic calf skin collagen used in this study contains a high proportion of β components, not ordinarily found in acid-extracted calf skin collagen (Schleyer, 1962).

Two moles of "aldehyde" per collagen molecule provides one more than the minimum number required to form a continuous, covalently linked polymeric network between adjacent collagen molecules. Such a network may account for many of the properties of older collagenous structures (Veis, 1964), including those produced *in vitro* (Gross, 1964). Since the molecules are largely in quarter-stagger array in the native fibril without abutting end to end (Hodge and Petruska, 1963), the carbonyls probably do not react with each other but may couple to other amino acid side chains projecting from the helical core.

The formation of fibrous aggregates from collagen solutions *in vitro* seems to largely depend upon electrostatic interactions (see review by Wood, 1964) and is unaffected by TSC binding as shown by the kinetics of fibril formation (Figure 5) and by the finding of normal fibrillar structure in the electron micrographs. The present work suggests that once the molecules have aggregated into the correct packing form, other factors are involved in intermolecular bonding. These include carbonyl groups which may be in close proximity to "receptor" sites located *ca.* one-fourth, one-half, and three-fourth's the distance along the molecule, with interaction occurring to form a more stable fibrous aggregate. If Schiff bases are produced, these may eventually become "fixed" by reduction. Reactions not involving carbonyl groups probably occur also, as both lathyritic (Gross, 1963) and thiosemicarbazide collagen fibrils (Figure 5) very slowly become insoluble with prolonged aging. Furthermore, the thiosemicarbazide collagen which slowly becomes insoluble does not lose its TSC. In bone collagen, noncovalent forces may be primarily involved in intermolecular cross-linking (Glimcher *et al.*, 1965).

Using semicarbazide under similar conditions, Wood (1963) found that the treated collagen formed fibrils less readily than the control although the fibrils ultimately formed were of similar stability. He also noted upon prolonged incubation of semicarbazide collagen fibrils at 37° that there was decreased shift of subunit composition toward higher molecular weight components than in the controls. The relation of these findings to the present work is not clear.

Neither thiosemicarbazide nor β-aminopropionitrile (Orloff and Gross, 1963) attach to lathyritic collagen *in vivo* indicating that they produce such collagen by indirect means. Although TSC–collagen produced *in vitro* mimics lathyritic collagen with respect to impaired intermolecular cross-linking, this effect seems to result from masking of carbonyl groups already in the collagen rather than inhibiting their formation (Bornstein *et al.*, 1966).

The intramolecular cross-linking of rat collagen seems to occur simultaneously with the appearance of "aldehydes" derived from specific lysine residues (Bornstein *et al.*, 1966), but the precise chemical nature of the cross-links is unknown. The evidence presented here suggests that thiosemicarbazide binds to carbonyl groups in similar regions because: (1) they are located on the "A" fragment of collagen and can be removed by chymotrypsin. (2) Both a saturated and unsaturated "aldehyde" seem to be present. If similar carbonyl groups participate in both inter- and intramolecular cross-linking it will be of interest to determine their precise location and relationships. The present results suggest two major groups of peptides are involved, one excluded by gel filtration and the other partially entering the gel. Since the exclusion point of Biogel P-4 is *ca.* 3800 mol wt these findings imply that the peptides have molecular weights of at least several thousand.

We conclude that thiosemicarbazide firmly attaches to collagen *in vitro*, inhibiting intermolecular bonding without affecting the content of intramolecular cross-links, showing that in certain circumstances the function of these two types of bonds may be sharply dissociated. Such separation is also found in adult chicken bone collagen which contains few cross-linked chains yet forms extremely stable fibers (Glimcher *et al.*, 1965). Thus, there appears to be no direct relationship between inter- and intramolecular cross-linking, suggesting that the abnormal solubility properties of lathyritic collagen are not simply caused by the lack of β components.

Acknowledgment

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The Reactivity toward *N*-Bromosuccinimide of Tryptophan in Enzymes, Zymogens, and Inhibited Enzymes*

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ABSTRACT: The oxidation of tryptophan by *N*-bromosuccinimide (NBS) in α-chymotrypsin, acetylchymotrypsin, diisopropylphosphoryl- (DIP) chymotrypsin, *N*-*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone- (TPCK) inhibited chymotrypsin, and chymotrypsinogen A was investigated over a pH range of 4.0–7.0.

The native enzyme possessed an additional 2–3 moles of NBS-reactive tryptophan at pH 5.5–6.0 compared with the inhibited enzymes or the zymogen. Acetylchymotrypsin after deacylation behaved exactly like native chymotrypsin in its oxidizability by NBS. This suggests that the difference in reactivity of bound tryptophan is the result of a conformational change. Much smaller differences in reactivity were observed in the pH range 5.5–6.0 between trypsin, DIP-trypsin, and trypsinogen; however, a large difference was noticeable between trypsin and its complex with the inhibitor from beef pancreas, where at pH 5.0 two tryptophan equivalents in the complex were protected from oxidation by NBS. The nature of the pH effect in the reaction of NBS with proteins and the use of NBS in effecting selective modifications of proteins are discussed.

The use of *N*-bromosuccinimide (NBS)[1] as a specific reagent for the modification of tryptophan in proteins has led to two major applications: one is a rapid and convenient spectrophotometric method for determining the tryptophan content of a protein (Patchornik *et al.*, 1958); the other provides a means of cleaving the tryptophyl peptide bond (Patchornik *et al.*, 1958, 1960; Ramachandran and Witkop, 1959, 1964; Witkop, 1961).

The former application is based on the large decrease in absorbance at 280 mμ accompanying the transformation of the indole to the oxindole chromophore. For most tryptophan-containing proteins this oxidation is instantaneous and quantitative at acidic pH values. The second application utilizes an excess of reagent, also at low pH, for the cleavage of the tryptophyl peptide bond in yields ranging from 30 to 70%. In this cleavage the amide carbonyl of the tryptophan participates in the opening of the postulated labile indole bromonium intermediate. This procedure has been used to determine or audit the identity of the residues following tryptophan. The comparatively low yield of the cleavage reaction has so far limited the utility of this procedure to spot-checking of protein sequences.

Recently, NBS has seen a further application in the preparation of oxidized proteins, in which some or all tryptophan residues have been modified (Hayashi *et al.*, 1964, 1965; Davidson and Westley, 1965; Viswanatha and Lawson, 1961; Okada *et al.*, 1963; Lokshina *et al.*, 1962; Green, 1963). Such selective modifications have provided useful information on the involvement of tryptophan in enzymic processes and in the interaction of proteins with other proteins and small molecules.

Many of these oxidative modifications, as we have

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[1] The following abbreviations were used in this paper: NBS, *N*-bromosuccinimide; DIP, diisopropylphosphoryl; TPCK, *N*-*p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone; PTI, pancreatic trypsin inhibitor.