CONCERNING SOME SPECIAL STRUCTURAL FEATURES OF THE COLLAGEN MOLECULE

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ABSTRACT A summary of results and ideas concerning special features of the covalent structure of collagen is presented. Our recent data on the nature of aspartyl ester pairs in collagen cross-linking and speculations about how these may function in fiber maturation are discussed. An important new aldehydic component associated with the cross-link sites has been detected and is under investigation. A model of collagen based upon 4 subunits of 25,000 to 30,000 per α component has been developed. These subunits appear to be held together in a linear array by three pairs of ester bonds. A more detailed picture of the distribution of "crystalline" and "amorphous" regions along the tropocollagen molecule has been proposed primarily based on the results of analyses of collagen peptide fractions obtained with collagenase. Other topics such as γ-glutamyl bonds in collagen and results with carbonyl group-detecting reagents which demonstrate the presence of small amounts of α-keto acid groups are briefly considered.

In his lucid presentation Dr. Gross has defined the nature of the tropocollagen macromolecule and provided the ground work for the present discussion of certain special chemical features of the molecule which have great bearing on its configurations. We shall consider the evidence that the fundamental unit of collagen, the tropocollagen molecule, consists of particular arrangements of subunits joined by special covalent bonds.

The work I am about to review includes the efforts of many colleagues of Dr. Sam Seifter and me, in particular, those of Dr. Olga Blumenfeld concerning the nature of cross-links in collagen (1), those of Dr. Mercedes Paz dealing with the carbohydrate and aldehyde components of collagen, and of Dr. Carl Franzblau in relation to the occurrence of γ-glutamyl linkages in the polypeptide chains of collagen (2, 3).

Several years ago, Dr. Sam Seifter, Dr. Edward Meilman, and I observed that exposure of denatured tropocollagen (which contains α and β subunits) to such reagents as hydrazine or hydroxylamine under mild conditions results in further fragmentation of the molecule to species of approximately 20,000 to 30,000 molecular weight (4). Fig. 1 illustrates typical results obtained with denatured collagens

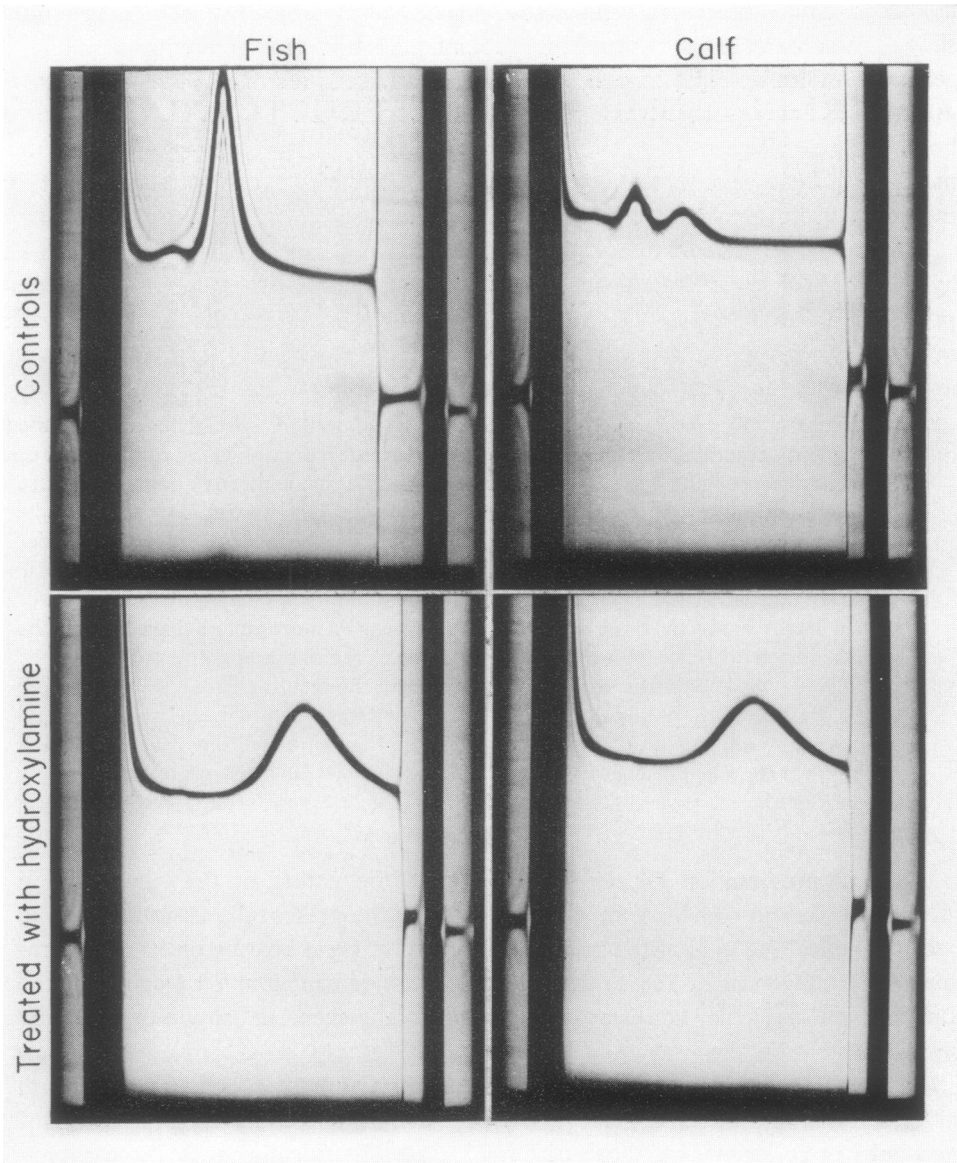


FIGURE 1 The sedimentation patterns of parent gelatins obtained from calf and fish tropocollagen as influenced by treatment for 90 minutes with 1 M hydroxylamine at pH 10.0. The photographs were taken 100 minutes after the full rotor speed of 59,780 RPM was obtained.

of calf skin and carp swim bladder (ichthyocol). The gelatins, before treatment, are seen to be composed chiefly of α units of molecular weight approximately 100,000 (S_{20} value of approximately 3.3). The other peak represents the β unit of approxi-

mately 200,000 molecular weight (S_{20} value of 5.2). The calf skin preparation shows a small amount of a third, or γ, component. It is also apparent that calf skin gelatin has a higher ratio of β to α than does ichthyocol gelatin. Upon incubation at 40° with hydrazine or hydroxylamine at pH values from 9 to 10, the S_{20} values of both fish and calf gelatins drop to approximately 1.9. This value, corresponding to a molecular weight of approximately 30,000, does not diminish even after much longer treatment with the nucleophilic agent.

Coincident with cleavage of denatured collagen by hydroxylamine or hydrazine, hydroxamic acid or hydrazide groups repectively appear bound to the protein components. For both species of collagen, it is clear that the number of hydroxamic acid or hydrazide groups appearing is constant and reproducible, amounting to about 6 per α unit of the collagen molecule. Because of the greater sensitivity of the method for measuring acid hydrazide groups, further studies of the nature of bonds cleaved by nucleophilic agents were conducted with hydrazine.

A kinetic study was made of the course of the action of 1 M hydrazine on denatured calf collagen at pH 10 at 40°. The results are shown graphically in Fig. 2, and

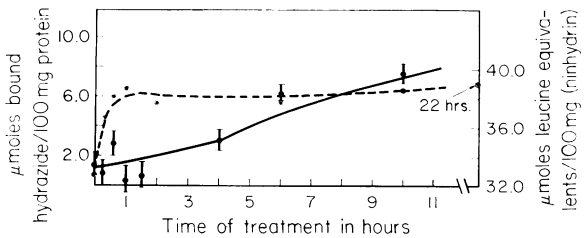


FIGURE 2 Kinetics of the reaction of calf procollagen with hydrazine at pH 10.0, 1 per cent calf procollagen, 1 M hydrazine, at 40°. (- - -) bound hydrazine. (———) leucine equivalents (ninhydrin)

indicate that the formation of bound acid hydrazide groups leveled off at approximately 6 groups per α component. Similar results were obtained when denatured ichthyocol was used. In either case no significant increase of amino groups, measured by the ninhydrin reaction, was discernible in the early stages of treatment with hydrazine (the large background of ninhydrin reactivity being due to ϵ-amino groups of lysine in the protein). Thus, during that time of reaction in which hydrazine cleaved certain of the cross-linking bonds, little if any scission of peptide bonds occurred. In other experiments in which hydroxylamine was used as the nucleophilic agent similar results were obtained.

An attempt was now made to obtain and separate peptides containing the acid hydrazide groups, since these could then reveal the nature of the acyl participant in the cross-links. An enzymatic digest of hydrazine-treated gelatin was prepared using the highly specific bacterial collagenase. This enzyme, which hydrolyzes -X-gly- bonds in peptide sequences containing -pro-X-gly-pro- (in which X is almost any

amino acid (5, 6)), on the average splits every fifth peptide bond in collagen. (In the so called crystalline regions of the molecule the enzyme splits every third peptide bond resulting in a large number of tripeptides, and in the so called non-crystalline or amorphous regions the enzymatic scission is not so extensive and results in a group of peptides which contain from 10 to 20 residues.) We calculated that approximately 2 per cent of the total number of peptides resulting from this digestion would bear an acid hydrazide group involving an acyl function previously concerned with cross-linking. We were now faced with the difficult task of isolating the hydrazide-containing peptides from among the large number of peptides which were not marked in this manner.

Dr. Blumenfeld (1) proceeded to study the separation of the peptides using a wide variety of materials customarily employed in selective elution chromatography. It soon was apparent that peptides bearing the acid hydrazide function were difficult to elute from usual Dowex 50 columns and, on the other hand, were not retained on columns of DEAE or CM cellulose. Eventually it was found that phosphocellulose could be used for the absorption and elution of the acid hydrazide–containing peptides, but a special device had to be introduced in order to make the separation specific. The reasons for the nature of this device are as follows.

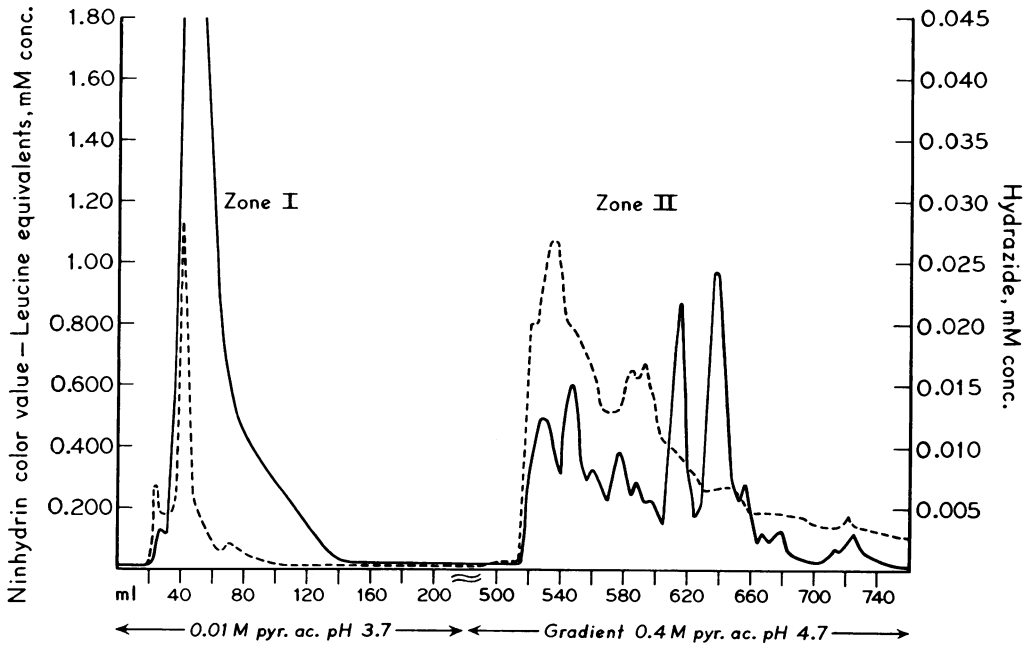


FIGURE 3 Chromatography of collagenase digest of hydrazine-treated gelatin from ichthyocol on a 0.9 × 30 cm phosphocellulose column at 25°. (——) ninhydrin, (- - - -) hydrazide. The quantity of peptides placed on the column corresponded to 100 mg of protein.

Fig. 3 presents an elution pattern of peptides obtained using the phosphocellulose column. Two zones of peptides, marked 1 and 2 respectively, are evident. Zone 1 contained 10 per cent of the total acid hydrazide groups in the digest and 50 per cent of the total amino groups measurable with ninhydrin. Zone 2 contained 90 per cent of the total acid hydrazide groups and 50 per cent of the total amino groups. Rechromatography of zone 2 total fractions on a second column of phosphocellulose resulted in no further separation of peptides characteristic of zone 1. It was therefore necessary to devise a method of modifying the acid hydrazide–containing peptides so that rechromatography could effect a separation from peptides in the fraction which were not marked with hydrazide. Accordingly, the aggregate peptides of zone 2 were reacted with *o*-benzaldehyde sodium sulfonate, so that the hydrazide–containing peptides were converted selectively into peptides containing benzhydrazones substituted with strongly charged sulfonic acid groups. The treated zone 2 peptides were now rechromatographed on phosphocellulose columns with the results shown in Fig. 4. The peptide-hydrazones were not retained and in this manner were separated from the bulk of peptides which did not contain hydrazide functions.

Amino acid analyses of the aggregate fraction containing peptides marked with the hydrazone function were conducted and proved most informative. As seen in Table I, such preparations obtained either from calf skin collagen or ichthyocol

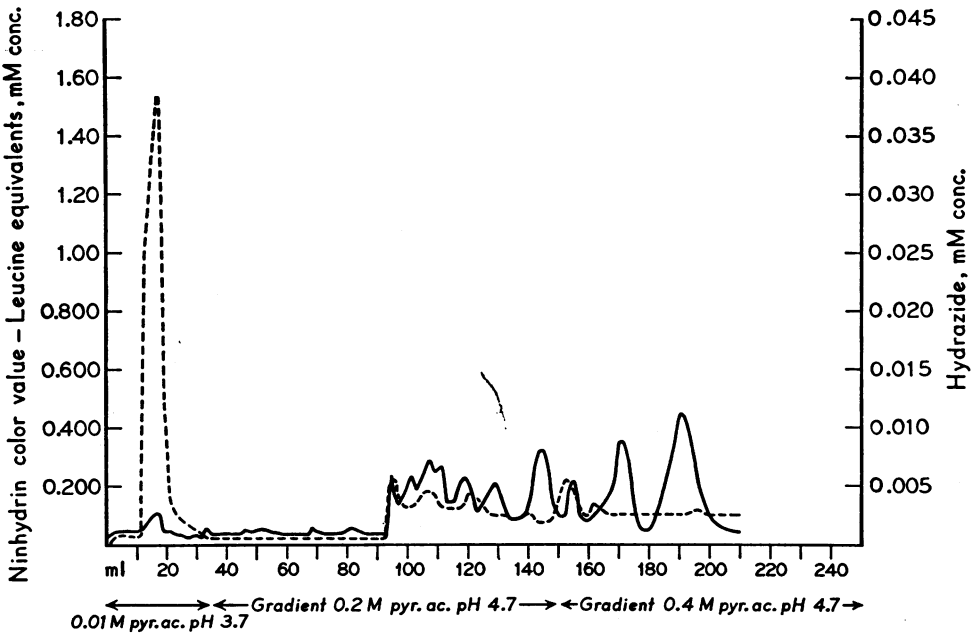


FIGURE 4 Rechromatography of peptides of zone II on 0.9 × 30 cm phosphocellulose column at 25° after reaction with *o*-benzaldehyde sodium sulfonate. (——) ninhydrin, (- - - -) hydrazone.

TABLE I

COMPOSITION OF HYDRAZONE-CONTAINING PEPTIDES FROM ICHTHYOCOL AND CALF SKIN COLLAGEN

Component	Hydrazone-containing peptides from ichthyocol	Hydrazone-containing peptides from calf skin gelatin
	residues per 100 residues	*residues per 100 residues*
Glycine	38.8	36.0
Proline	8.8	6.8
Hydroxyproline	0	0
Alanine	12.0	12.0
Aspartic acid	20.2	17.6
Glutamic acid	4.6	6.7
Threonine	1.8	1.7
Serine	3.6	7.4
Isoleucine	0.9	1.1
Leucine	3.2	3.6
Valine	4.5	0
Phenylalanine	0	1.0
Tyrosine	0	0
Methionine	0	0
Lysine	2.3	3.1
Arginine	0	0.7
Histidine	0	1.1
Hydroxylysine	0	0
Hydrazone or hydrazide	19.9	17.4

revealed equivalent amounts of aspartic acid and hydrazone, while the number of equivalents of glutamic acid was considerably less. The evidence was cogent that the cross-links of denatured collagen disrupted by treatment with hydrazine involved the carboxyl groups of aspartic acid residues.

The amino acid analysis showed the aggregate peptides to have other interesting features. Glycine, as in the parent molecule, still occurred as 1 out of 3 total residues. Further, when the aggregate fraction was treated with fluorodinitrobenzene, subsequent analysis revealed an amount of amino terminal glycine almost equivalent to the amount of proline, and constituent peptides with an average chain length of approximately 10 amino acid residues. Ultimately the peptides were shown to contain no hydroxyproline and to have but 1 proline residue per peptide; the latter occurred at the amino terminus following the terminal glycine, NH_2gly-pro-.

From knowledge of the specificity of collagenase, it is expected that in the digest every peptide greater than a tripeptide would have at least 2 proline residues for every amino terminal glycine residue (NH_2gly-pro————pro-X), the sole exception being a terminal peptide. Peptides arising from amino ends of collagen (NH_2 ————pro-X) and those arising from carboxyl ends (NH_2gly-pro———— COOH) would have only 1 proline. The peptides isolated from the digest and found to contain the bound hydrazone groups resembled carboxyl terminal peptides.

The isolated peptides contained 1 proline and 2 aspartic acid residues for each amino terminal glycine. It was then apparent that the cross-links involving aspartyl residues occurred in pairs separated by no more than 7 residues. We then established that the cross-links were most probably ester in nature, and involved the α-carboxyl group of one aspartyl residue and the β-carboxyl of the second aspartyl residue.

I shall not describe the detailed chemical procedures used to arrive at these conclusions as they have been published elsewhere (1). We performed a Lossen rearrangement on hydroxylamine-treated gelatin and lithium borohydride reduction on untreated gelatin. These studies provided the evidence that half the number of esters were through β-carboxyl groups and half through α-carboxyl groups.

As mentioned above, there are approximately 6 cross-links per α component or 18 per tropocollagen molecule. We believe these bonds to be esters occurring in pairs near carboxyl terminal portions of polypeptide chains. To reiterate, half that number involves α-carboxyl groups of aspartyl residues and the other involves β-carboxyl groups of aspartyl residues.

Dr. Blumenfeld has been studying the kinetics of cleavage of these esters by nucleophilic agents at different pH values. As mentioned previously, at pH 10 cleavage with hydrazine yields a value of 6 bound hydrazide equivalents per α component. However, at pH 8, in a somewhat slower reaction, the value of measured bound "hydrazine" is approximately 9. In the same reaction as shown in Fig. 5, Dr.

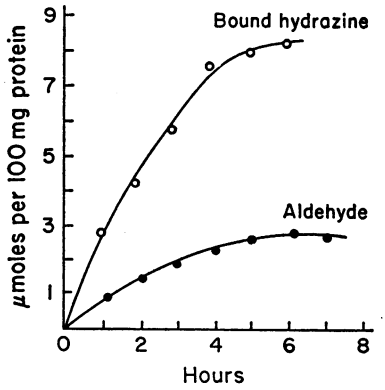


FIGURE 5 Kinetics of reaction of ichthyocol (0.7 per cent) with hydrazine at pH 8.0, 40°C, 3 M hydrazine (at this pH value the effective concentration of NH_2NH_2 is about 1 M). At selected intervals samples were removed, adjusted to pH 3.0, and exhaustively dialyzed against distilled water at 5°C. Analyses for total protein-bound hydrazine, bound aldehyde (Sawicki *et al.* procedure (7)), and Kjeldahl protein nitrogen were carried out.

Paz, employing the aldehyde procedure of Sawicki *et al.* (7), was able to measure the exposure of 3 aldehyde groups for the 6 esters cleaved. Thus, the value of 9 bound hydrazine equivalents obtained at pH 8, could be accounted for by 6 acyl hydrazide groups resulting from cleavage of the esters and 3 aldehyde hydrazone groups. The rate of aldehyde exposure was the same as the rate of ester cleavage indicating their interdependence, and it would appear that the aldehyde function itself is involved in the ester linkage network.

In Fig. 6 we present a schematic conception of the tropocollagen molecule. Each

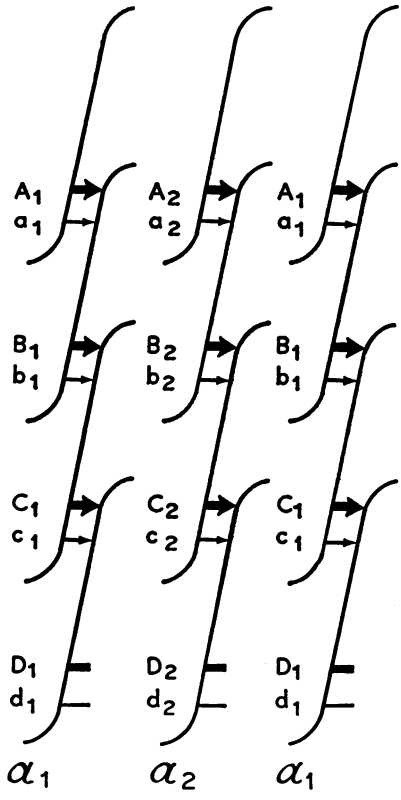


FIGURE 6 Schematic diagram of a tropocollagen molecule 15 A × 3000 A, 360,000 molecular weight. There are two α_1 strands, and one α_2. Each α strand (120,000) is conceived as a linear array of 4 subunits averaging 30,000 molecular weight and joined by ester pairs. The different chemical nature of the ester pairs is depicted by the heavy and light arrows.

strand represents an α unit. From the results of Dr. Piez and Dr. Gross (8, 9), we know that there are 2 α_1 strands and one α_2 strand. In our scheme we have considered that each α unit is composed of 4 subunits linked along the strand into a network by pairs of ester bonds. On the average each subunit is assumed to have a molecular weight of approximately 30,000. The subunits are joined together by pairs of esters at terminal positions; in the figure heavy and light arrows are used to indicate that some esters occur through the α-carboxyl group of aspartyl residues and others through the β-carboxyl group.

The experimental justification of this picture obviously requires that the proposed subunits be separated and characterized. One should be able to achieve this separation by the application of various column techniques to a solution of hydrazine-treated tropocollagen. As of now, we can merely say that although the average size of subunits is approximately 30,000, the individual subunit molecular weights could vary from 10,000 to 60,000.

For a moment let us reconsider Fig. 1. The sedimentation patterns of the calf and fish gelatin controls show both α and β units, but the amount of β in the calf is much higher than in the case of fish. Some γ component, not seen in fish gelatin, is evident

in the calf preparation. However, in either type of collagen the total complement of esters is about 6 per 1000 amino acid residues. In Fig. 7 we present a model illustrating a hypothetical scheme for maturation and aging of collagen. A progressive transesterification is depicted which, in time, could give rise to β, γ, and intermolecularly

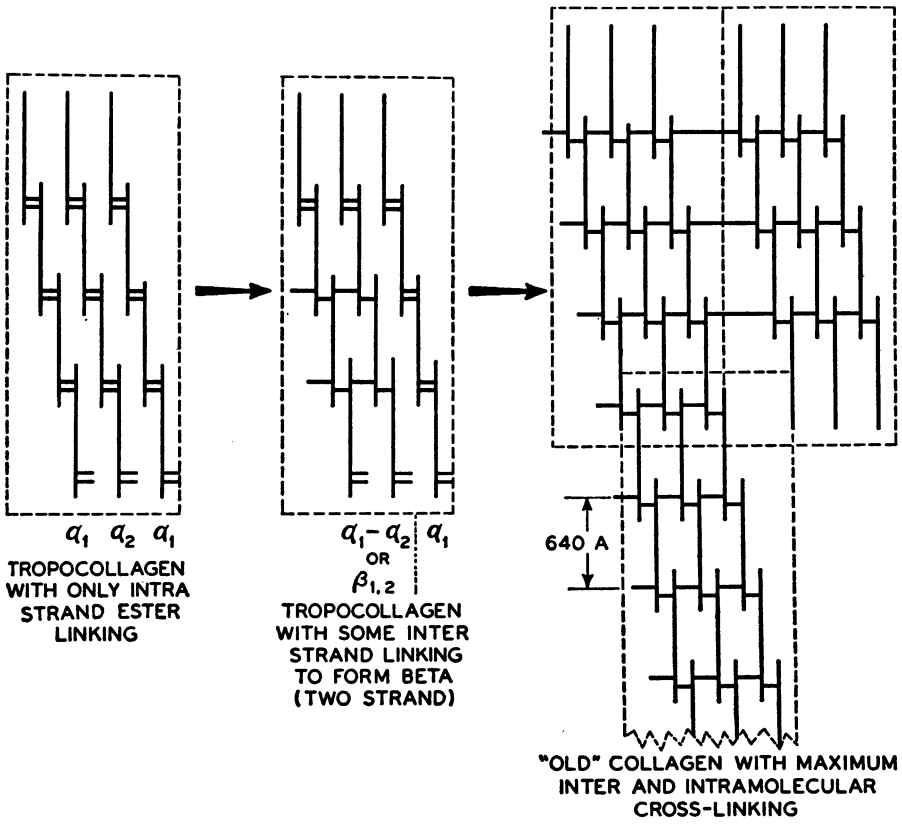
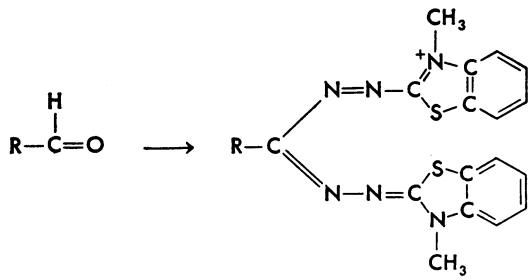


FIGURE 7 Schematic diagram to represent the maturation and aging in collagen fibers. The tropocollagen is represented as described in Fig. 6. The exploration of how increased interstrand cross-linking and intermolecular cross-linking may occur without increase in the number of ester bonds is illustrated by this hypothetical transesterification process.

cross-linked collagen without any increase in the total number of ester bonds. The pairing and duplex nature of the cross-linking aspartyl esters suggest that one type of ester may be fixed and the other type of ester amenable to transesterification.

Problems of eminent concern are the nature of the alcohol participants in the ester groups and the character and mode of linkage of the aldehyde function. The purest preparations of tropocollagen yield low but consistently significant hexose values. In particular, an anthrone procedure has been employed for quantitative purposes. Grassmann and his coworkers (10) and Gross *et al.* (11) have demonstrated

the occurrence of both glucose and galactose in tropocollagen preparations, and the former have shown the hexose to be destroyed by periodate. Dr. Paz in our laboratory has found that the periodate treatment of tropocollagen at pH 8.0 leads to destruction of anthrone-reacting hexoses and production of protein-bound glyoxal. These results indicate probably O-glycosidic attachment of hexose to the protein and a free 2,3-dihydroxy structure for bound carbohydrate. Results of Dr. Paz employing a number of colorimetric procedures including treatment with periodate and thiobarbiturate suggest the absence of sialic acids but indicate the possible presence of a 2-deoxy type sugar. Such sugars on treatment with periodate and thiobarbiturate give rise to a chromogen with an absorption maximum at 530 mμ. In order to understand her results we assumed, as a working hypothesis, that a bifunctional carbohydrate was involved in the collagen cross-links; *e.g.*, a component with an aldopyranose structure on one end and a 2-deoxyfuranose structure on the other. A wide variety of experimental observations would be explained by this construction, but a component of this nature could not be isolated. Recently, Dr. Blumenfeld has isolated the hexoses from ichthyocol and by use of glucose oxidase found 1.3 equivalents of glucose and 1.9 of galactose per 1000 amino acid residues. The nature of the aldehydic component released on deesterification of ichthyocol has not yet been ascertained. However, it has been isolated as an intensely blue formazan-like derivative:



From chromatography on paper and on thin layers of silica gel, Dr. Paz has shown that the formazan derivative isolated is not that of formaldehyde, acetaldehyde, aldol, glycolaldehyde, glyoxal, malonic dialdehyde, erythrose, ribose 5-phosphate, or 2-deoxyribose 5-phosphate. Pyranose structures are unreactive in this modification of the aldehyde procedure of Sawicki *et al.* (7). However, free aldehydes and certain reactive furanose structures can give this reaction.

Concerning other features of the tropocollagen molecule, some work of Dr. Carl Franzblau will be cited (2, 3). From a collagenase digest of ichthyocol Dr. Franzblau isolated a fraction of larger peptides. These were enriched in contents of ester and hexose, and the ratio of ester to hexose remained approximately 2:1. He also found that the contents of tyrosine maintained almost a 1:1 relationship (on the basis of equivalents) to hexose measured by the anthrone reaction. Thus the ester groups,

carbohydrate, and tyrosine are associated with larger peptides in collagenase digest.

From an interpretation of analyses of many peptides from a collagenase digest of ichthyocol, we have constructed a schematic composite picture of the tropocollagen molecule and its subunits (Fig. 8). Initially we present the segmental long spacing (SLS) of collagen of Dr. Schmitt, Dr. Gross, and Dr. Highberger (12). According to them this structure is composed of tropocollagen units packed in perfect register in a polar structure. Embodying the concept of Dr. Piez *et al.* (8, 9), each tropocollagen unit then consists of 3 polypeptide strands of which 2 are α_1 and the other α_2. Each of these strands is then divided into subunits with molecular weight of 30,000, giving 4 units per strand. The subunits within each strand are connected in a network of ester linkages.

From the analyses of peptides apparently arising from crystalline or amorphous regions we can infer the occurrence of certain sequences within one hypothetical subunit of 30,000 molecular weight. "Amorphous" regions contain polar amino acids, take up electron stains, and appear as bands in the electron microscope. "Crystalline" regions consist of sequences of non-polar amino acids and appear as interbands. Starting with a polar region ————pro-X, there follows a crystalline region of approximately 21 residues $(\text{gly-pro-X})_7$. These regions of the molecule assume a polyproline II type of helical structure. Another polar region then interposes and this is followed by another crystalline region of about 21 residues. This alternation of polar and non-polar regions repeats about 4 times, until the carboxyl terminus is reached. Aspartyl residues in this portion bear the ester cross-links. Dr. Blumenfeld's analysis suggest occurrence of at least 4 subunit chains which terminate with carboxyl groups. Each of the C-terminal regions has 2 aspartic residues no more than 7 residues apart. We have assumed that a diester linkage occurs involving the carbohydrate, and that the carbohydrate moiety makes O-glycosidic attachment to an adjacent subunit. We have pictured the carbohydrate as a hexose attached to a second carbohydrate of undetermined character. The latter in fact may be the source of aldehyde discussed above. On the adjacent subunit we have placed a tyrosine residue close to the insertion point of the O-glycosidic bond. This placement was inferred from the fact that peptides bearing the aspartyl hydrazide functions, isolated after cleavage of esters with hydrazine, did not contain tyrosine, indicating that tyrosine is in the vicinity of the cross-link but does not participate in it. The phenolic hydroxyl groups (numbering 3 per 1000 amino acid residues) in collagen can be titrated spectrophotometrically and accordingly are free.

You will note that 1 peptide of the carboxyl terminal peptides contains 1 lysyl residue. It is interesting that the ϵ-amino group of this lysine residue did not react with fluorodinitrobenzene. This lysyl residue perhaps is that occurring in the peptide described by Mechanic and Levy (13, although we have no evidence to this effect. This residue would acount for 1 of the 25 lysyl residues per 1000 amino acid residues in collagen.

"SEGMENT LONG SPACING" (SLS) FORM
SOLUBILIZATION
TROPOCOLLAGEN
2800 A°
15A°
α 1
α 2
α 1
Polar region)-Pro-X
(Gly-Pro-X)
n=7
Gly-Pro-(Polar region)-Pro-X
(Gly-Pro-X)
n=7
Gly-Pro-(Polar region)-Pro-X
Gly-Pro-(Polar region)
4
NON-DIALYZABLE PEPTIDES "AMORPHOUS" (BAND)
RAPIDLY DIALYZABLE "CRYSTALLINE" INTERBAND
NON-DIALYZABLE PEPTIDES "AMORPHOUS" (BAND)
RAPIDLY DIALYZABLE "CRYSTALLINE" INTERBAND
NON-DIALYZABLE PEPTIDES "AMORPHOUS" (BAND)
Gly-Pro (Gly4 Glu2 Asp1 Lys1 Ala2 Ser1X) Pro-X
εNH-X
-Gly-Pro
(Gly3, Ala1, Lys1, Leu1, Glu1)
(Gly3, Ala1, Ser1, Glu1)
(Gly3, Ala1, Ser1, Val1)
(Gly3, Ala1, Val1, Thr1)
-Asp-Asp
COOH
Hexose
OH
(unknown carbohydrate)
Tyr

Earlier we stated that we have been unable to detect sialic acid in tropocollagen. However, protein-bound keto acid groups or unsaturated aldehydes can be demonstrated. Thus, ichthyocol or calf collagen treated with 2,4-dinitrophenylhydrazine in 1 N HCl for a few minutes at room temperature, precipitated, washed with acetone, dialyzed, and examined spectrophotometrically, consistently shows the presence of about 2 keto acid dinitrophenylhydrazone groups per tropocollagen molecule. In Fig. 9, the spectra of the hydrazone of protein-bound keto acid groups are compared with that of the 2,4-dinitrophenylhydrazone of pyruvic acid. The results of Levene (14) with 2,4-dinitrophenylhydrazine may owe their origin to these keto acid groups or to unsaturated aldehydes.

In addition to 2,4-dinitrophenylhydrazine other hydrazine reagents can be used to demonstrate the keto acid. A more interesting one is apresoline, which is 2-hydrazinophthalazine. This drug is of interest to researchers in the collagen field because it can induce an experimental arthritis in guinea pigs and also in some human beings receiving it as a hypotensive agent. Under mild, neutral conditions apresoline reacts readily with these carbonyl groups of collagen. In Fig. 9 we compare the spectra of protein-apresoline compound with that of the pyruvic acid–apresoline derivative. It may be speculated that apresoline enters collagen at these sites and the possibility could then exist for an autoimmune reaction to the modified protein.

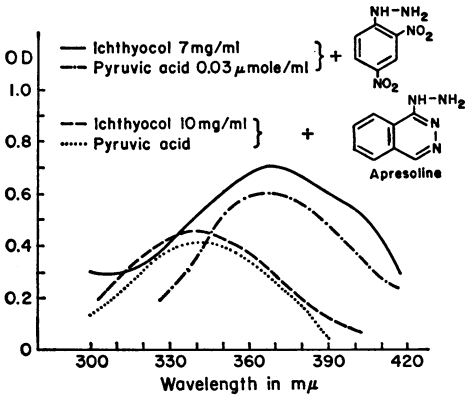


FIGURE 9 The spectra of ichthyocol after reaction at 25°C with certain carbonyl reagents resemble those of the corresponding α keto acid derivatives. This corresponds to about 2 keto acid groups per tropocollagen molecule (360,000). This figure also indicates that the drug apresoline (2-hydrazinophthalazine) can react directly with tropocollagen.

FIGURE 8 Schematic diagram of tropocollagen illustrating the alternation of "crystalline" (interbands) and "non-crystalline" (bonds) regions in a hypothetical 30,000 subunit. This diagram is based on results with the enzyme collagenase which will degrade crystalline regions mainly into tripeptides and other regions into larger peptides. The organization of the subunits into α strands is illustrated by the networks involving the aspartyl ester pairs on one subunit in diester linkage with a hexose residue which is O-glycosidically linked to an adjacent subunit. The Y component (unknown carbohydrate) represents the origin of the aldehyde in tropocollagen. The approximate composition of 4 carboxyl terminal peptides involved in the subunit is represented. The 1 lysine residue in one of these peptides has some type of blocked ϵ-amino groups (ϵ-NH-X)

Collagen contains a large number of γ-glutamyl peptide bonds, which occur along the backbone polypeptide chains. Dr. Franzblau has shown that at least 30 per cent and perhaps as much as 50 per cent of the glutamyl residues in collagen are not in the usual α peptide linkage but occur rather in γ-glutamyl linkage (2, 3). As one might expect, γ-glutamyl linkages are found in the amorphous regions of the molecule. Recently, Dr. Marcos Rojkind has isolated γ-glutamyl peptides from a collagenase digest of ichthyocol. The structural significance of such bonds is that they provide 2 additional points of free rotation along the polypeptide backbone of collagen. Accordingly, at these points the chains can assume a wide variety of conformations.

In conclusion, we have touched briefly on many areas in the special chemistry of collagen. Much which has been presented is fact, and much is fancy. We hope that the hypotheses which guide our work will stimulate discussion and further research by others. At least it may justify the interest and many years of labor that we have devoted to this fascinating protein.

REFERENCES

1. BLUMENFELD, O. O., and GALLOP, P. M., The participation of aspartyl residues in the hydroxylamine- or hydrazine-sensitive bonds of collagen, *Biochemistry*, 1962, **1**, 947.
2. FRANZBLAU, C., Unusual linkages in collagen, Ph.D. Dissertation, Albert Einstein College of Medicine, Yeshiva University, New York, 1962.
3. FRANZBLAU, C., GALLOP, P. M., and SEIFTER, S., The presence of γ-glutamyl peptide bonds in collagen, *Biopolymers*, 1963, **1**, 79.
4. GALLOP, P. M., SEIFTER, S., and MEILMAN, E., Occurrence of "ester-like" linkages in collagen, *Nature*, 1959, **183**, 1659.
5. SEIFTER, S., GALLOP, P. M., and FRANZBLAU, C., Some aspects of collagenolytic action, *Tr. New York Acad. Sc.*, 1961, **23**, series 2, 540.
6. GALLOP, P. M., and SEIFTER, S., Features of primary structure and unusual linkages in the collagen molecule, *in* Collagen, (N. Ramanathan, editor), New York, Interscience Press Inc., 1962.
7. SAWICKI, E., HAUSEN, T. R., STANLEY, T. W., and ELBERT, W., The 3-methyl-2-benzothiazolone hydrazone test, *Anal. Chem.*, 1961, **33**, 93.
8. PIEZ, K. A., LEWIS, M. S., MARTIN, G. R., and GROSS, J., Subunits of the collagen molecule, *Biochim. et Biophysica Acta*, 1961, **53**, 596.
9. PIEZ, K. A., EIGNER, E. A., and LEWIS, M. S., The chromatographic separation and amino acid compositions of the subunits of several collagens, *Biochemistry*, 1963, **2**, 58.
10. GRASSMANN, W., HOFMANN, V., and WOLF, K., Electron microscope and chemical studies of the carbohydrate groups of collagen, *in* Connective Tissue, (R. E. Tunbridge, editor), Council International Organization of Medical Science Symposium, Oxford, B. H. Blackwell Ltd., 1957, 157.
11. GROSS, J., DURNSHA, B., and GLAZER, N., Comparative biochemistry of collagen, *Biochim. et Biophysica Acta*, 1958, **30**, 294.
12. SCHMITT, F. O., GROSS, J., and HIGHBERGER, J. H., *in* Fibrous Proteins and their Biological Significance, *Symp. Soc. Exp. Biol.*, 1955, **9**, 148.
13. MECHANIC, G. L., and LEVY, M., An ε-lysine tripeptide obtained from collagen, *J. Am. Chem. Soc.*, 1959, **81**, 1889.
14. LEVENE, C. I., Studies on the mode of action of lathyrogenic compounds, *J. Exp. Med.*, 1962, **116**, 119.