than by subtotal splenectomy. The spleen, therefore, may be of greater importance in the production of normal γ-globulin than lymphoid tissue in other parts of the body.

A study by Andreasen and coworkers (*9*) is not in accordance with our investigation. They found that the serum protein fractions were within normal range after subtotal extirpation of lymphoid organs, including the spleen. The time of observation, however, was much shorter in their study than in ours. This may explain the discrepancy in results.

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Intermolecular Cross-Linking of Collagen and the Identification of a New Beta-Component

Abstract. *Extraction of skin with 5M guanidine after salt and acid extraction yields a gelatin fraction which contains a greater proportion of double-chain (β) components than can be accounted for by intramolecular cross-linking of collagen molecules. This fraction also contains a new β-component, identified as the dimer of α2 and designated β_{22}. This dimer must be formed by intermolecular cross-linking since each collagen molecule contains only one α2 chain. Thus, direct evidence is presented for the occurrence of both inter- and intramolecular cross-linking by what appears to be a single continuous process.*

Chromatographic studies of denatured collagen show that the collagen monomer contains three chains, two of which (the α1 chains) are apparently identical while one (the α2 chain) is different in amino acid composition (*1, 2*). With time, covalent cross-links form between the chains to produce β-components (β_{12} is a covalently bonded dimer of the α1 and α2 chains; similarly β_{11} is a dimer of two α1 chains) (*3*). The rate and extent to which this occurs is dependent on the tissue and species (*2*).

Table 1. Partial amino acid composition of the component β_{22} compared with that of α1 and α2 chains in human skin collagen.

Amino acid	Residues per 1000 residues		
	α1	α2	β_{22}
Hydroxyproline	91	82	83
Proline	135	120	118
Valine	21	33	31
Isoleucine	7	15	14
Leucine	20	30	31
Histidine	2	10	11

It is believed that cross-linking is of importance to the structure and function of connective tissue since in at least one known pathological condition, lathyrism (*4*), interference with cross-linking results in a profound disorder of connective tissue. However, chromatographic studies reported thus far relate directly only to intramolecular cross-linking whereas it would appear that the process of intermolecular cross-linking is the one which imparts to collagen fibers properties important to the function of connective tissues. It has been necessary to assume, in the physiologic interpretation of these studies, that the two processes are related.

We present evidence that the process of cross-linking in skin collagen results in the formation of intermolecularly bonded double-chain components (and presumably, later, higher aggregates) which are identical or analogous to those resulting from intramolecular cross-linking.

Skin from several mammalian species was extracted at 5°C with 1*M* sodium chloride and then five times with 0.5*M* acetic acid to remove most of the extractable collagen. The residue was then extracted with 5*M* guanidine, *p*H 7.5, at 5°C for 24 hours. After clarification by filtration, the guanidine extract was dialyzed exhaustively against water and the resulting precipitate was lyophilized as a suspension. The results of hydroxyproline analysis indicated that the preparation contained about 75 percent collagen that was present as gelatin as a result of the denaturing action of guanidine (*5*). In the rat this fraction represented about 20 percent of the total skin collagen. Samples of the lyophilized material were dissolved by warming at 45°C for a few minutes in potassium acetate buffer (0.075 ionic strength), *p*H 4.8, for ultracentrifugation, or in sodium acetate buffer (0.06 ionic strength), *p*H 4.8, for chromatography (*2*). The samples did not dissolve completely but hydroxyproline analysis showed that most of the insoluble protein was noncollagenous.

Sedimentation velocity patterns in the ultracentrifuge demonstrated that the guanidine-extracted gelatin consisted largely of double-chain β-components. Small amounts of the α- and the triple-chain γ-components were also present (Fig. 1). For comparison a sample of denatured acid-extracted collagen containing about 50 percent β-component is included. In these patterns the ratio of β- to α-components appears to be smaller than it actually is because of the effect described by Johnston and Ogston (*6*).

The presence of large amounts of β-component was confirmed by chro-

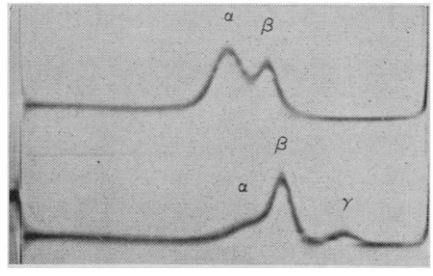

Fig. 1. Sedimentation patterns of heat-denatured collagen from human skin extracted with acid (upper pattern, wedged cell) and then extracted with 5*M* guanidine (lower pattern, standard cell). Centrifugation performed in 12-mm cells at 59,780 rev/min in 0.15*M* potassium acetate at *p*H 4.8 and 40°C; phase plate angle 65°C, sedimentation from left to right, exposure at 106 minutes.

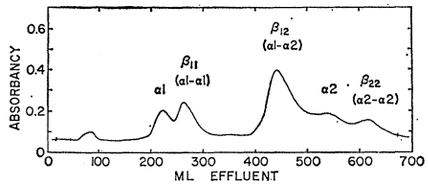

Fig. 2. Elution pattern of approximately 60 mg of denatured collagen from human skin extracted with 5*M* guanidine after salt and acid extractions. The denatured collagen was chromatographed on carboxymethylcellulose at 40°C. Absorbancy was measured at 230 mμ.

matography of guanidine-extracted samples from rat, guinea pig, and human (infant) skin. Measurements of the areas under the peaks demonstrated that these samples consistently contained 70 to 80 percent β-components. Acid-extracted collagens from the same species usually contain 50 to 60 percent but never more than 67 percent β-components. In addition to the expected peaks representing the α1 and α2 single chains and β_{12} and β_{11} double chains, a small peak consistently appeared after that of the α2 chain (Fig. 2). Protein from this peak was chromatographed a second time. The isolated protein had the same sedimentation coefficient and the same mobility during acrylamide-gel electrophoresis (*7*) as the β_{11}- and β_{12}-components. Its amino acid composition was identical to that of the α2 chain (Table 1). It must therefore be the covalently linked dimer of the α2 chain and is designated β_{22} (*8*). Its chromatographic behavior is consistent with this identification. The small amount of γ-component seen in the ultracentrifuge has been tentatively identified on chromatographs as part of the leading edge of the β_{12} peak (Fig. 2).

Since each collagen molecule consists of three chains, a purely intramolecular cross-linking process would yield a maximum of 67 percent β-components. In addition, a dimer of two α2 single chains could not form since the preponderance of evidence indicates that there is only one α2 chain per molecule (*2*). For these reasons we conclude that guanidine-extracted gelatin is obtained in part from intermolecularly cross-linked collagen.

That guanidine preferentially extracts this fraction can be explained as follows. Neutral salt and dilute acid solutions do not denature collagen in the cold. Therefore, these solvents tend to extract single collagen molecules but not covalently linked aggregates. However, 5*M* guanidine is a strong denaturing agent and could extract β-components from aggregates by first unfolding the chains. Preliminary results of isotope-incorporation experiments indicate that the intermolecularly cross-linked fraction extracted with guanidine does not necessarily represent collagen which is biologically older than the acid-extractable fraction. That is, inter- and intramolecular cross-linking may occur concurrently as part of a single process.

Bakerman and Hersh (*9*) have also found a collagen fraction that contained largely β-components when examined in the ultracentrifuge, but their conclusion that the collagen molecule must therefore contain a whole number of β-components does not necessarily follow if intermolecularly cross-linked components were extracted. For example, two collagen molecules could give rise to three β-components as a result of one intermolecular and two intramolecular cross-links.

Our results emphasize that the usual extraction procedures do not solubilize intermolecularly cross-linked collagen. Since the absolute and relative amounts of inter- and intramolecular cross-linking would appear to be important determinants of the properties of collagenous tissues, a knowledge of both the type and degree of cross-linking present is necessary for the interpretation of studies on the relationship of collagen structure to function.

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Eserine and Amphetamine: Interactive Effects on Sleeping Time in Mice

Abstract. *The sleeping time of mice given pentobarbital was found to be significantly shortened by the injection of eserine or amphetamine. When both eserine and amphetamine were given with pentobarbital the sleeping time, though much shortened, was significantly longer than with the addition of amphetamine alone. Though possessing an analeptic effect of its own, eserine appears to antagonize the analeptic effect of amphetamine.*

Amphetamine has been shown to antagonize the electroencephalographic synchronization produced by barbiturates (*1*) and to have strong analeptic effects on laboratory animals injected with pentobarbital (*2*). Eserine (physostigmine), though antagonistic to barbiturate-produced electroencephalographic synchronization (*1*), is usually considered to have no, or very little, effect on behavior. Since both drugs have been demonstrated to have an effect on the mesodiencephalic reticular formation, which in turn is known to have an influence on an animal's state of arousal, it was considered of interest to determine whether eserine would antagonize pentobarbital in some behavioral parameter as it does electroencephalographically and whether it

Table 1. Effect on sleeping time of injecting pentobarbital (PB), 60 mg/kg; amphetamine (AA), 2 mg/kg; and eserine, 0.1 mg/kg.

Group	Injections			Sleeping time* (min)
	1st	2nd	3rd	
1	PB	Saline	Saline	86 ± 3.8
2	PB	AA	Saline	52 ± 1.8
3	PB	Eserine	Saline	59 ± 2.7
4	PB	AA	Eserine	61 ± 2.6

* Mean ± standard error.