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Elastin Content of the Aorta from the Quantitation of the Desmosines

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ELASTIN CONTENT OF THE AORTA FROM THE QUANTITATION OF THE DESMOSINES

by

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VITA

H. Patrick Covault was born St. Patrick's Day, 1952, in Dayton, Ohio. He suffered the usual childhood diseases, such as grade school and high school, and finally graduated from Wright State University in 1975 with a B.S. in biology. Continuing his education at Wright State, he graduated with a M.S. in biological chemistry in 1977.

Following his acceptance to Loyola University of Chicago as a Ph.D. applicant in the Department of Biochemistry and Biophysics in 1977, he joined the laboratory of Dr. Albert Dietz, at the V.A. Hospital, Hines, IL.

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LIST OF ABBREVIATIONS

A	Absorbance
AAA	Automated amino acid analysis
AU	Absorbance units
ΔA	Change in absorbance
BAPN	β-Aminopropionitrile
BSA	Bovine serum albumin
CTP	Connective tissue protein
Des	Desmosine
EIA	Enzyme-immunoassay
EMIT^{R}	Enzyme multiplied immunoassay technique
FFDW	Fat-free dry weight
G-6-PDase	Glucose-6-phosphate dehydrogenase
Glu	Glutaraldehyde
HETP	Height equivalent to theoretical plates
Ide	Isodesmosine
N	Theoretical plates
OPA	ortho-Phthaldialdehyde
RIA	Radioimmunoassay
R_s	Resolution factor
UV	Ultra-violet

Two roads diverged in a yellow wood,
And I sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could...

...Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference.

-Robert Frost

DEDICATED TO MY PARENTS

HARRY E. and MARY C. COVAULT

CHAPTER I

INTRODUCTION

Elastin is one of the major connective tissue proteins and is present in virtually every organ of the body. It is unique in that it is a protein with both elasticity and tensile strength. Elastin is found in both "yellow" and "white" connective tissue (tissue with a high content of elastin is designated as yellow, while tissue with a high content of collagen is designated as white). It is the functional protein component of the elastic fiber and displays an amorphous, highly refractive, generally wavy appearance under light microscopy. Upon stretching of the elastic fibers, the waviness disappears and increased birefringence is displayed. These properties are attributed to a parallel arrangement of the elastin polypeptide chains within the elastic fiber upon stretching [1].

Specific stains for elastin have long been in use in light microscopy. Classic stains for elastin are Verhoeff's hematoxylin, resorcin-fuchsin, and orcein [1,2]. Other stains used to visualize elastic fibers include Nile blue sulfate, basic fuchsin, osmic acid, Sudan black, and Mallory's aniline blue stain, which distinguishes between elastin and collagen [3,4]. Electron microscopy stains, such as uranyl acetate and lead citrate, allow the visualization of the surrounding microfibrillar component, while elastin itself remains mostly unstained [1]. Thus, the ability of mature elastic tissue to take up light microscopy stains resides either in the presence of unreacted aldehydic groups within the elastic matrix or in the strong hydrophobic nature of the protein [5,6].

From microscopy and biochemical studies, it has been determined that elastic tissue is comprised of water (approximately 70%), a microfibrillar protein component, lipids, carbohydrates, and the protein elastin [7,8]. It has been observed that elastin can be found wherever elasticity and tensile strength are required, primarily in structures which display high deformation under small loads and complete recovery after removal of that load. Therefore, elastin is in great abundance in the ligamentum nuchae of ungulates, the vertebral ligamenta flava, aorta, lung, and pulmonary arteries of all primates [1]. Specialized forms of elastic tissue include elastic cartilaginous tissue, such as the ear and epiglottis. Bronchi, skin, adipose tissue, and loose connective tissues are included as elastic tissue even though low in elastin content [1] (Table 1).

A unique property of elastin is its almost complete resistance to methods normally used to solubilize other proteins. This was first observed by Richard and Gies who subjected elastin to boiling water which served to gelatinize and remove collagen [9]. These investigators noted elastin's resistance to alkali degradation and its non-polar nature, which was attributed to a high carbon to oxygen ratio. Finally, they demonstrated that elastin peptides aggregated upon heating. This coacervation, which represents a form of precipitation, is believed due to a high content of non-polar amino acids in the elastin molecule.

Using x-ray diffraction studies, Lloyd and Garrod observed structural similarities between elastin and lightly vulcanized rubber in that both lack molecular orientation [10]. It was determined that elastin is not a true rubber since it requires water as a lubricant. Elastin consists of randomly coiled polymer chains which are joined together by

TABLE 1

ELASTIN CONTENT OF CONNECTIVE TISSUES FROM DIFFERENT ANIMALS

Species	Aorta	Lung	Uterus	Ear	Ligament
Human	47.1	24.0	6.2		
Bovine	48.2	6.0			66.0
Porcine	45.0	24.6	1.0		
Dog	40.0	15.7	0.9		
Rat	40.6	13.4	1.5		
Chick	58.5	12.0	0.9		
Rabbit	17.4	6.0	0.8	4.0	
Guinea pig	25.0	4.2			
Hamster	22.0	2.4			

Elastin content is expressed as percentage of the salt-extracted, freeze-dried tissue.
Whole lung excluding major pulmonary vessels and bronchi.
From Starcher and Gallone (101)

cross-links into an extensible three-dimensional network. The cross-links are the derived amino acids, desmosine and isodesmosine, identified by Partridge and co-workers [11,12]. These cross-links are responsible for the characteristic physical properties of the elastin molecule, i.e., its rubber-like elasticity and almost complete insolubility.

The catabolic turnover rate of elastin is low relative to other proteins. Therefore, changes in tissue content as well as structural composition of elastin is altered principally by de novo syntheses [1,13]. Perhaps the most dramatic changes in elastic tissue and elastin have been observed in the arteries, lungs, and skin of various species with age. A "brittleness" in these organs with age has also been observed. This observation may be related to elastin content [1,9,13,15,18,21,22].

Elastin has been implicated in several disease states (Table 2). It has been suggested that changes in elastin may play a role in alterations which occur in blood vessels during degenerative arterial disease, as it is common to see alterations in the microscopic elastic network of these vessels during the disease state [1,9,13]. There is some evidence that certain pulmonary disorders, such as emphysema, may be associated with changes in the elastin content of the lung [1,9,19,20]. Finally, elastin may be involved in certain hereditary skin diseases [1,9,23].

Thus it would be advantageous to know the amount and composition of elastin in normal conditions in order to understand the various pathological processes involved in the mentioned disease states. However, this information is lacking in that the major source of data pertaining to the amount of elastin is derived from gravimetric determinations on the residue remaining following the extraction of minced or milled samples.

TABLE 2

EFFECTS OF DISEASE ON ELASTIN AND RELATED FEATURES

Disease	Histologic Abnormality	Functional Abnormality
Atherosclerosis	Fragmented arterial elastic lamellae with calcium and lipid deposits and intimal thickening.	↑ Vessel stiffness, vascular occlusion and tears.
Emphysema	Fragmented elastic fibers preservation of microfibrils despite destruction of amorphous elastin.	↑ Compliance of the lung at low lung volumes.
Pseudoxanthoma elasticum	Fragmented elastic fibers, granular deposits in place of amorphous elastin.	↑ Compliance of skin and fragile vessels.

From Sandberg et. al. (1)

TABLE 2 (CON'T)

EFFECTS OF DISEASE ON ELASTIN AND RELATED FEATURES

DISEASE	BIOCHEMICAL ABNORMALITY	ETIOLOGIC DEFECT
ATHEROSCLEROSIS	↓ELASTIN, ↑COLLAGEN CONTENT AND ↑CALCIUM AND LIPID CONTENT OF ELASTIN ↑POLAR AMINO ACID AND CROSS-LINKS IN ELASTIN.	POSSIBILITIES: RESPONSE TO INJURY; MONOCLONAL ORIGIN OF PLAQUES; CLONAL SENESCENCE.
EMPHYSEMA	EARLY ↑ELASTIN CONTENT AND ↑ELASTIN SYNTHESIS. LATE: ↑ELASTIN SYNTHESIS AND POSSIBLY ↑ELASTIN DEGRADATION. LATER: NORMAL ELASTIN CONTENT: ↑POLAR AMINO ACIDS OF ELASTIN AND POSSIBLY ↓PROTEOGLYCANS.	POSSIBILITY: IMBALANCE BETWEEN ELASTASE AND INHIBITOR SYSTEMS, PERHAPS IN RESPONSE TO EXOGENOUS AGENTS OR GENETIC FACTORS.
PSEUDOXANTHOMA ELASTICUM	POLYIONIC DEPOSITS ON ELASTIC FIBERS.	SPECULATION: POLYIONIC DEPOSITS (POSSIBLY GLYCOPROTEINS) ATTRACT CALCIUM AND CAUSE ELASTIN DAMAGE.

The determinations are, at best, tedious and somewhat non-reproducible and subject to various problems including sample degradation [14,24-26]. In the gravimetric procedures the extraction conditions are varied, and so are the results. These extractions include: 1) extraction with 1% sodium chloride followed by several cycles of autoclaving [14,27,28]; 2) extraction with hot 0.1 mol/L sodium hydroxide [14, 18,28-31]; 3) extraction with formic acid [14,32,33]; and 4) extraction with 5 mol/L guanidine followed by treatment with collagenase [14,34].

A methodology for the study of elastin currently under development focuses on the two cross-linking amino acids, desmosine (Des) and isodesmosine (Ide). These amino acids have only been found in the protein elastin and in the non-elastin protein in egg shell membranes [35-38]. Thus, their quantitation may be used as an index of the elastin content of tissue, just as hydroxyproline is used as an index of the amount of collagen present in a tissue [39,49].

Although various chromatographic methods have been developed for the purification and quantitation of Des and Ide [37,41-48], few studies employ amino acid quantitation as an index of elastin content [39], the chromatographic procedures being tedious, cumbersome, and limited in their scope of application. The few studies utilizing the desmosines for the estimation of elastin employed automated amino acid analysis, a technique which is both costly and time consuming.

FUNCTIONAL ASPECTS OF ELASTIN

Many of the physical properties of the elastin molecule resemble those of the rubber molecule [50]. However, elastin is not believed to be

a largely random network of cross-linked chains, like rubber. In contrast it is believed to be a network of cross-linked chains containing significant ordered structure [51]. Several models have been proposed to explain its functional aspects, as well as its high degree of organization [50-56].

Partridge envisioned elastin as tetrahedrally packed, globular protein subunits, with the hydrophobic residues turned inward with the cross-links protruding from the surface [50]. Using this model, Weis-Fogh and co-workers suggested the functional properties occur by polar-apolar interactions, as the hydrophobic core of the globular subunits are exposed to an aqueous environment upon stretching [52].

Another model, the random chain, is supported by evidence suggesting that the elastin chains are in rapid Brownian motion [53,54]. This model is also supported by classical thermodynamic considerations [53,54].

A third model, the "oiled-coil", has been suggested by Gray and co-workers [55]. They claim that the regions of the elastin molecule which contain the cross-links are separated by regions composed predominantly of glycine, proline, and valine. They view these regions as a "broad left handed coil" with the hydrophobic residues on the inside and the hydrophilic residues on the outside. Upon extension of the molecule, the coil would be opened, which would expose the hydrophobic groups to a more polar environment. This configuration would be thermodynamically unfavorable and the molecule would tend to return to its original state.

A fourth coiled model is suggested from the observation that the repeating sequences in the elastin molecule predominantely represent a single secondary structural feature, the β-turn [56].

COMPONENTS OF ELASTIN BIOSYNTHESIS

The biosynthesis of elastin is a complex process that includes: synthesis of a microfibrillar component by fibroblasts; synthesis of an elastin precursor, tropoelastin; and finally, the formation of the mature elastin fiber by the action of lysyl oxidase.

Microfibril Component:

The non-elastin component of the elastic fiber is the microfibril [13]. This glycoprotein component is referred to as a structural protein, but its exact function has not been determined [1]. These fibers, which are 10 to 20 nm in diameter, are synthesized by either the fibroblasts or the smooth muscle cells [49,57,58]. This protein lies adjacent to either the fibroblast or the smooth muscle cell (which also produce elastin) responsible for its synthesis. The microfibrils are laid in bundles parallel to the long axis of the developing elastin fiber. These fibers may represent the first immature elastin fibers, and as the elastin matures, the microfibrils are found in the interstices and around the periphery of the elastin fiber. There has been some evidence that, in certain areas, these microfibrils display periodicity and take on a beaded appearance [1]. The amino acid content of the microfibrils is dissimilar to that of elastin as evidenced by the presence of histidine, methionine, and cystine, and the lack of Des and Ide. Also, the non-polar to polar amino acid ratio differs from that of elastin [13]. The microfibrils have been shown to contain at least two glycoprotein constituents, designated MFP 1 and MFP 2 [59]. MFP 1 is a collagenous glycoprotein of 150,00 daltons while

MFP 2 is a non-collagenous glycoprotein of 300,000 daltons [59].

Tropoelastin:

Concomitant with the synthesis of the microfibrils is the synthesis of tropoelastin, or soluble elastin [58]. Tropoelastin is synthesized from the same fibroblasts or smooth muscle cells as the microfibrils, and was first isolated by Smith and co-workers from the aortas of copper-deficient swine [60]. Since this discovery other native soluble elastin molecules have been isolated from the aorta of various species including copper-deficient chicks [61], lathyritic chicks [62,63], and from the ligamentum nuchae of copper-deficient calves [64].

Tropoelastin is composed of a single polypeptide chain of approximatly 800 residues (68,000-72,000 daltons) [65,66]. At present only 70% of the sequence is known, but it is clear that the majority of the residues are non-polar amino acids, such as glycine, proline, alanine, valine, phenylalanine, isoleucine, and leucine. The small number of polar amino acid residues, such as aspartic acid, glutamic acid, arginine, and lysine lend solubility to the molecule.

The amino acid content of a tropoelastin preparation is given in Table 3. Tropoelastin usually contains little or no histidine. It also lacks methionine, and other sulfur-containing amino acids. Arginine is present in minimal amounts while the remainder of the molecule consists largely of apolar amino acids. For these reasons, the purity of tropoelastin is sometimes measured by the absence of histidine and methionine in protein hydrolysates [67]. Hydroxyproline, a major component of collagen (40%), is found in relatively small amounts (5%) in the tropoelastin

TABLE 3

AMINO ACID COMPOSITION OF SOLUBLE AND MATURE

(INSOLUBLE) ELASTIN FROM THE PIG

Amino Acid	Soluble Elastin	Mature Elastin
Glycine	245	256
Alanine	187	181
Proline	91	90
Hydroxyproline	7	8
Valine	103	92
Isoleucine	14	14
Leucine	41	41
Tyrosine	14	12
Phenylalanine	24	25
Arginine	4	5
Lysine	37	5
Cross-links*	Very low	25
Aspartic acid and asparagine	3	5
Threonine	10	11
Serine	8	11
Glutamic acid and glutamine	12	16
Methionine, cysteine, tryptophan and histidine	0-1	1

Human elastin has a similar composition.

Based on an assumed chain length of 800 residues.

* - Expressed as lysine equivalents. A number of intermediates are also included in this estimate. Actual lysine equivalents in desmosine and isodesmosine are eight to 10.

From Sandberg, et. al. (1)

molecule. Other hydroxylated amino acids are not found in the tropoelastin molecule [68].

All methods for the purification of the tropoelastin molecule are usually performed in the presence of proteolytic inhibitors [61-63,67,69]. This is necessary due to the susceptibility of the tropoelastin molecule to cleavage by various proteases which are often associated with the soluble elastin fractions [13,70].

Recently, there has been some discussion of the existence of a precursor of tropoelastin [71,72]. Rucker and co-workers have postulated that tropoelastin is derived from a soluble protein of approximately 95,000 daltons [71]. These investigators used aortas from copper-deficient chicks and α_1-antitrypsin at each step of purification. With this modification they were able to detect a pre-tropoelastin. Their conclusions were based on terminal amino group analysis of the molecule which they state is the same as tropoelastin. Also, the amino acid composition of the pretropoelastin is similar to that of tropoelastin. It contains a limited number of aldehydic functions and is a viable candidate for action by lysyl oxidase. The pre-tropoelastin was found in greater concentrations in the aortas from the copper-deficient chicks.

Foster and co-workers have reported the isolation of a proelastin, 120,000-130,000 daltons, from lathyritic chick aorta and second passage aortic smooth muscle cells from rabbits [72]. Using a pulse-chase technique, involving the incorporation of ^{3}H-valine and ^{14}C-proline, they observed a high molecular weight soluble protein. When chased for a period of 30 minutes to 1 hour, its concentration decreased with the concomitant appearance of the 70,000 dalton tropoelastin. Direct identification of

the high molecular weight protein as a precursor to tropoelastin was provided by amino acid analysis. The amino acid composition of the high molecular weight peak was very similar to that of tropoelastin. Foster also reported finding a soluble protein of 90,000-100,000 daltons which had also been identified by Rucker [71]. This was added evidence of the existence of a pro-elastin molecule.

These findings are controversial. Rosenbloom and co-workers found that the presumed pro-elastin contained considerable amounts of histidine while tropoelastin and elastin contained little, if any [73]. Rosenbloom used a hisitidine analog, histidinal, that would inhibit elastin biosynthesis if pro-elastin were a precursor to elastin. They found that while collagen synthesis was markedly inhibited by histidinal, tropoelastin synthesis was relatively unaffected. By using pulse-chase experiments they observed that the labeled tropoelastin was efficiently incorporated into insoluble elastin both in the presence and absence of histidinal. From these findings, Rosenbloom suggests that tropoelastin was the primary precursor in elastin biosynthesis in chick aorta.

Lysyl Oxidase:

Lysyl oxidase is the major enzyme responsible for the conversion of tropoelastin to mature elastin. This enzyme has been shown to catalyze the reaction of the peptidyl lysine residues in the tropoelastin molecule to lysinal. This reaction is necessary if the cross-links found in the mature elastin molecule are to occur. Lysyl oxidase has been detected in numerous connective tissues and smooth muscle cells in vitro. It has been shown to be involved in the oxidation of peptidyl lysine to lysinal

in both elastin and collagen biosynthesis [74]. The enzyme has been found in close association with its substrate, tropoelastin, and has an absolute requirement of copper for maximal activity [75-78].

There is also evidence that the enzyme exists in various forms [98]. Two isozymes of 59,000 and 61,000 daltons, A and B respectively, have been isolated from bovine aorta [78]. It is unclear which form predominates in vivo. Another lysyl oxidase of approximately 180,000 daltons has been isolated from bone although its function is not yet known [79].

BIOSYNTHESIS OF ELASTIN

Three components of elastin biosynthesis (microfibrils, tropoelastin, and lysyl oxidase) work in concert to form the mature elastin fiber. The general scheme is as follows: first, the fibroblasts or smooth muscle cells synthesize the microfibrils which are then deposited along the small infoldings on the surface of the cell [58]. Second, perhaps concomitant with the synthesis of the microfibrils, tropoelastin is synthesized intracelluarly on the ribsomes [58]. Once tropoelastin is synthesized, it is transported outside the cell by an unknown mechanism [58]. Here the enzyme lysyl oxidase oxidizes the ε-amino groups of the lysine residues to aldehydes. These aldehydes condense, via Schiff base reaction, to form the cross-links in the elastin molecule [58]. As the modified lysine residues condense, tropoelastin loses its solubility and aggregates around the microfibrils, possibly by electrostatic interaction between the two molecules, forming the mature elastin molecule [58].

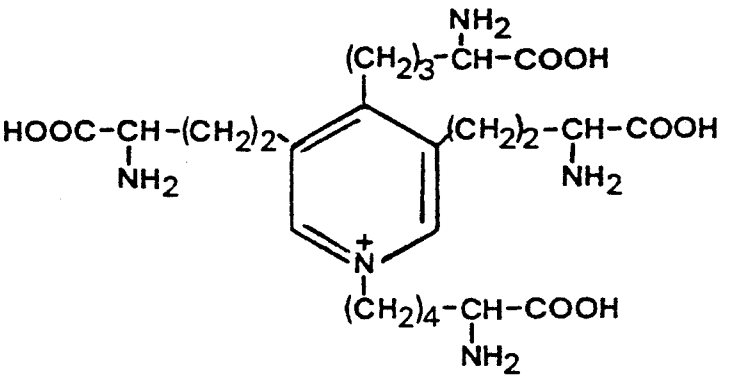
Mature Elastin:

In its mature form, insoluble elastin is composed of 4 soluble elastin subunits (tropoelastin) that are intermolecularly cross-linked into a fibrous network. Elastin, as in tropoelastin, maintains a predominance of non-polar amino acids, with less than 5% polar amino acids (Table 3). This predominance of non-polar amino acid residues seems unique to elastin and is, therefore, a good means of identification.

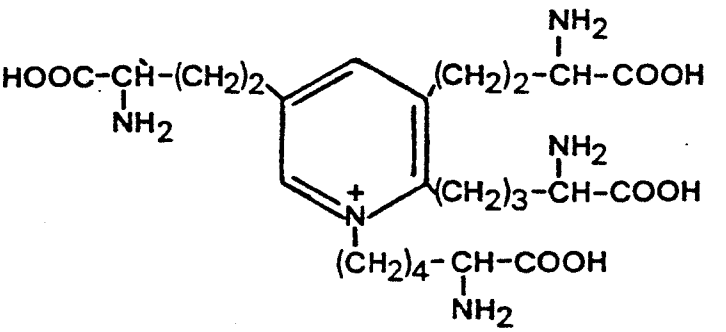
One of the main differences between tropoelastin and elastin is the greater lysine content of the former (Table 3). While the tropoelastin molecule contains up to 40 lysine residues per 1000, the elastin molecule may contain only 3 to 8. This decrease in the amount of lysine is due to a direct conversion of lysine into cross-links. As a consequence of these cross-links, a highly polymerized and insoluble protein is produced [13].

Elastin is similar to collagen in that it contains large amounts of the amino acids glycine, valine, alanine, and proline. In fact, due to the large amount of valine and proline incorporated in the elastin molecule, these amino acids, when labeled with a radioisotope, are used as an index of elastin biosynthesis [79]. Elastin contains approximately 10% as much hydroxyproline as collagen and contains no hydroxylysine. Elastin and egg shell membrane protein are unique in that they are the only proteins so far analyzed found to contain Des and Ide , which account for approximately 1% by weight (Fig. 1).

The amino acid composition of elastin has been shown to vary slightly between tissues from the same species. A slight variation has also been observed in the same tissue from various species. In both cases,



DESMOSINE



ISODESMOSINE

Fig. 1. Structure of desmosine and isodesmosine.

the variability lies mainly in the non-polar amino acids (Table 4). This variability may be due to the purification methods utilized [80,81].

Much of the elastin molecule appears to be comprised of repeating sequences in the form of tetra-, penta-, and hexa-peptide units [13]. For example, common tetra-, and penta-peptide sequences are Gly-Gly-Val-Pro and Pro-Gly-Val-Gly-Val, respectively. The repeating hexa-peptide unit common to elastin is Pro-Gly-Val-Gly-Val-Ala-. There also seems to exist a homology with respect to the N-terminal portion of the elastin molecule from unrelated species [61,62].

Another structural feature which seems common to the repeating sequences in the peptide chain is the beta turn [82]. With this beta turn present, the elastin chain is able to fold back on itself. These beta turns, when repeated several times, impart a unique structure to the elastin molecule, known as the beta spiral. This beta spiral is not known to occur in any other mammalian protein and may be important in the formation and function of the elastin fiber [1,82].

One other feature which seems to set elastin apart from other proteins is its alanine enrichment [1]. In the tropoelastin molecule, lysine residues almost always occur in pairs, and it is in these regions where alanine enrichment seems to occur. That is, the lysine pairs are separated by anywhere from 1 to 3 alanine residues and can be preceded by as many as 8 alanine residues in the sequence [83,84]. It appears that the alanine enrichment is essential for the cross-linking of the lysine residues in the molecule [1].

From studies on these restricted areas of alanine enrichment, it has been proposed that a specialized structure exists in the cross-link areas,

TABLE 4

ARTERIAL ELASTIN - COMPOSITION OF INSOLUBLE AORTA ELASTINS

Amino acid	Pig	Chick	Dog	Rabbit	Cow		Human	
					Aorta	Ligamentum	Female	Male
Gly	330	340	335	321	311	332	282	281
Ala	234	180	228	227	223	228	220	232
Val	120	177	97	101	137	138	129	126
Pro	117	131	119	125	132	117	118	112
HyPro	11	16	13	16	13	16	11	15
Ile	18	20	28	22	27	25	27	27
Leu	54	57	49	58	65	60	62	60
Try	16	13	28	28	8	6	25	23
Phe	33	21	27	22	33	29	25	25
Thr	14	22	15	12	9	10	17	18
Ser	11	13	16	7	9	10	14	16
Asp + Asn	6	6	8	15	7	8	11	16
Glu + Gln	19	19	19	14	16	16	23	30
Met*							(2)	(2)
His*	<1	2	2	<1	<1	<1	2	4
Arg	6	8	10	3	6	5	11	14
Lys	6	4	4	7	5	3	6	10
Ide	1.2	1.0	0.7	0.6	1.2	0.9	1.1	0.8
Des	1.8	1.3	1.0	0.5	2.0	1.4	1.7	1.1
LNL	0.9	0.7	0.5	0.4	0.9	1.2	0.8	0.6

From Rasmussen, et. al. (121); Based on 1000 residues.

* - Met and His may represent other amino acids which cochromatograph as histidine and methionine.

possibly an α-helical structure [85]. Thus it appears that the elastin has a dual nature: one of an extensible portion which contains the hydrophobic amino acids; and another compact region, rich in alanine, which contains the lysine residues responsible for the formation of the cross-links in the mature elastin molecule [1].

Cross-Linking Amino Acids in Elastin:

All the known cross-links found in elastin are derived from peptidyl lysine [3]. These cross-links account for elastin's low modulus of elasticity and its inherent rapid and reversible extensibility. These cross-links maintain the gross structure of the protein as well as limit the amount of extensibility under stress.

LaBella and co-workers found initial evidence for a cross-linking substance in 1963 when they reported finding a fluorescent component with a yellowish pigment in a partial hydrolysate of thoracic aorta elastin [86]. These investigators found that a single substance was responsible for the fluorescence at 405, 440, and 460 nm and that the fluorescence increased with age.

Partridge and his group were studying elastin by degradation with proteolytic enzymes [86,87]. In partial hydrolysates they found amino acids, small peptides, and an additional fraction. This fraction contained a component which had a molecular weight higher than ordinary amino acids, yet was not a polypeptide [86,87]. When this fraction was collected it was bright yellow in color and gave the characteristic blue-white fluorescence of elastic tissue. The fraction was then subjected to further hydrolysis and two peaks were resolved by ion-exchange chroma-

tography [86,87]. These two peaks were found to be very hygroscopic and upon titration each peak displayed 4 pK's [12]. When the absorbance spec- of the two compounds was determined, compound "A" displayed two absorbance maxima, 235 and 268 nm, while compound "B" displayed only one maximum at 278 nm. The spectra of compounds A and B were compared to spectra of model compounds. It was determined that the structures resembled the trimethylpyridines [11]. The structures of these compounds were elucidated through these studies, as well as infrared and nuclear magnetic resonance spectroscopy (Fig. 1). Compound A was termed desmosine while compound B was termed isodesmosine. Both compounds had a MW of 543.

Biosynthesis of Desmosine and Isodesmosine:

Partridge and co-workers were among the first to determine that the cross-links in elastin were derived from peptidyl lysine [11,12,35-37,83]. They observed that when the desmosines were oxidized with alkaline ferricyanide, the pyridinium ring was destroyed and lysine was liberated. Using pulse-chase techniques, other workers found that when ^{14}C-lysine was incubated with aorta from chick embryos for 24 hours, the label appeared in elastin, while the desmosines remained unlabelled [89,90]. Following a 7 day chase period of cold lysine, they observed that the desmosines became heavily labelled [89].

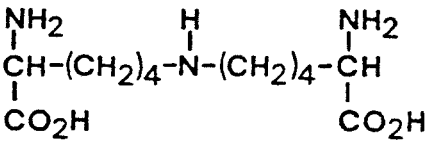
Partridge then injected rats with ^{14}C-lysine daily for a period of 10 days and isolated the desmosines and lysine from hydrolyzed aortic elastin [90]. From these studies they determined that the ratio of radioactivity of desmosine and isodesmosine to lysine was 4 and that it required approximately 17 days for this ratio to occur. Ultimately Part-

ridge suggested a biosynthetic pathway whereby 4 neighboring lysine molecules have 3 of their ε-amino groups deaminated oxidatively, and then cyclize to form a pyridinium ring [58,90].

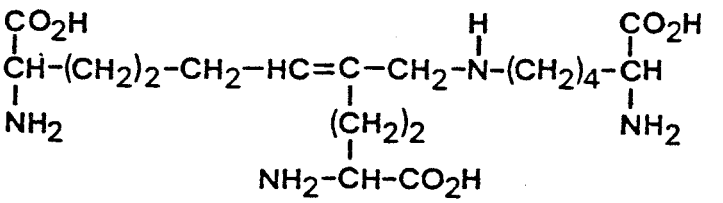
Diets deficient in copper and/or containing lathyrogenic agents, such as β-aminopropionitrile (BAPN) or semicarbazide, inhibit the synthesis of elastin. These same agents were found to inhibit the biosynthesis of the desmosines. The inhibition displayed in by a copper deficient diet is based on the fact that lysyl oxidase requires copper for activity. The inhibition by BAPN or semicarbazide is due to their binding of the aldehyde functions after the catalytic action of lysyl oxidase. Using two techniques for inhibiting desmosine and isodesmosine synthesis, O'Dell and others found other minor cross-links were possible in elastin biosynthesis [91]. They utilized aorta organ culture techniques in the presence of lathyrogenic agents with a copper-deficient medium. They observed that the rate of ^{14}C-lysine incorporation into elastin was not affected, but the rate of labeled lysine incorporation into desmosine and isodesmosine was greatly reduced. From these results they determined that other cross-links in elastin were possible [91,92].

Miller and co-workers found the condensation product of two lysine molecules in which one of the ε-amino groups is deaminated [93]. This condensation results in the formation of lysinonorleucine (Fig. 2).

Partridge found that elastin which had been reduced with sodium borohydride and hydrolyzed by acid gave a peak which overlapped with lysinonorleucine on amino acid analysis [88]. This compound was found to have an 18 carbon backbone with a molecular weight of 436 and was termed merodesmosine. It could be derived from the condensation product of 3



LYSINONORLEUCINE



MERODESMOSINE

Fig. 2. Structure of lysinonorleucine and merodesmosine.

lysine residues (Fig. 2). With further studies, it was determined that merodesmosine could result from two reactions: 1) the condensation of the Schiff base of lysinonorleucine (dehydrolysinonorleucine) with a lysine residue; or 2) the condensation of 2 aldehydic functions from lysine residues with another lysine residue. Both pathways resulted in the formation of dehydromerodesmosine which can then be reduced to merodesmosine.

In summary, the biosynthetic pathway suggested by Partridge seems the most plausible [94-97]. The key step in the pathway seems to be the oxidative deamination of the ε-amino groups of selected lysine residues, yielding α-aminoadipic acid-δ-semialdehyde. This reaction is accomplished by the action of lysyl oxidase. The aldehyde can then react in one of two ways: 1) formation of a Schiff base with a lysine residue yielding dehydrolysinonorleucine; or 2) condensation with another aldehyde via an aldol condensation. Dehydrolysinonorleucine can be further reduced to form lysinonorleucine or can form 4-hydroxy-2,3-dehyroisodesmopipyridine via combination with an aldol condensation product. This compound can then be dehydrated to form 2,3,4,5-dehydroisodesmopipyridine, which can spontaneously undergo oxidation to form isodesmosine (Fig. 3). If the aldol condensation product unites with a lysine residue to form dehydromerodesmosine, merodesmosine can be synthesized by a simple reduction. However, if the dehydromerodesmosine reacts with α-aminoadipic acid-δ-semialdehyde, 2-hydroxy-5,6-dehydrodesmopipyridine is formed, which can spontaneously dehydrate to form 2,3,5,6-dehydrodesmopipyridine. Desmosine can then be synthesized by an oxidative reaction (Fig. 3).

Studies with model aldehydes and amines suggest that after the initial enzymatic oxidation of the ε-amino groups to lysyl residues, the

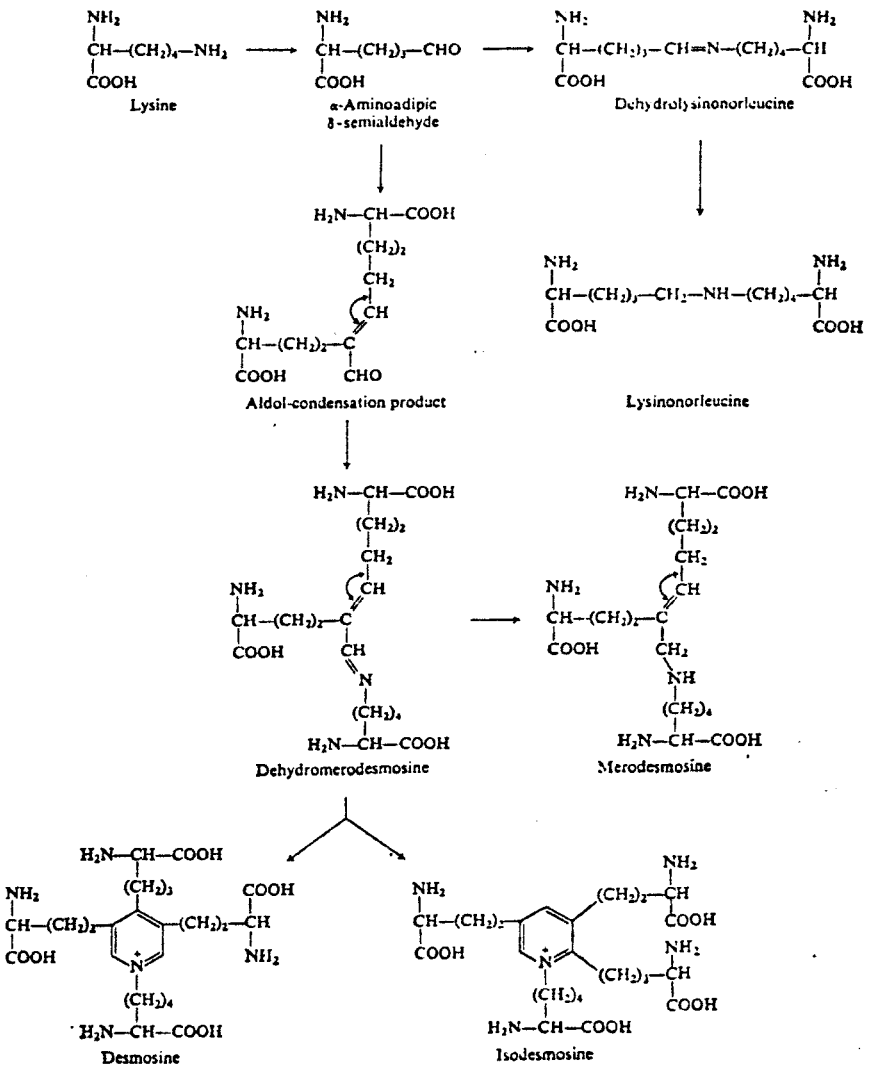


Fig. 3. Biosynthetic pathway for the synthesis of desmosine and isodesmosine, and their intermediates. From Francis, G., et al (102).

above reactions occur spontaneously [98,99].

The amino acid sequences around the cross-linking regions have been elucidated [11,100]. These areas are rich in alanine and also contain a high percentage of serine, glutamic acid, phenylalanine, and tyrosine. It was proposed by Foster and co-workers that lysines, which are preceded and followed by alanine or glycine, are oxidized, but those lysine residues followed by large hydrophobic amino acids (particularly tyrosyl residues) do not undergo oxidation [100]. Thus, the placement of the tyrosyl residues may determine the positions where cross-links are not formed [69,100].

CHAPTER 2

PROPOSAL

The aim of this research project is to devise a more rapid and accurate method for the quantitation of the desmosines and therefore the estimation of the elastin. One possible method of quantitation, proven to be both rapid and accurate for minute amounts af analytes, is the immunoassay. In all immunoassays, there is one basic reaction:

$$Ab + Ag \longrightarrow Ab\ Ag$$

where Ab is the antibody produced against a specific antigen Ag. This reaction depends on the specificity of the Ab for the Ag in forming the Ab·Ag complex. In this simple reaction, either the depletion of the Ab or Ag or the appearance of the Ab·Ag complex may be quantitated. However, this quantitation is usually difficult due to the amounts of Ab or Ag present in the reaction, and the minute amount of Ab·Ag complex formed. To aid in the quantitation, a label is introduced into the assay system.

The classical label for such immunoassays is a radioisotope which can be attached to the Ag. In this case the reaction for a radioimmunoassay (RIA) is as follows:

$$Ab + Ag + Ag^* \longrightarrow Ab{\cdot}Ag^* + Ab{\cdot}Ag + Ag + Ag^*$$

where Ag* is the labeled antigen. Due to the effect of mass action, the amount of Ag* bound to the Ab is proportional to the concentration of the unlabeled Ag. Thus, by varying the amount of Ag, and maintaining the Ab and Ag* at constant concentrations, a standard curve for the Ag may be constructed. The remaining necessity in RIA involves the separation of

the Ab•Ag* complex from the Ag*. Because this step involves a physical separation (double antibody; activated charcoal; solid phase separation), RIA is considered a heterogenous immunoassay technique.

While RIA is a sensitive and accurate assay it has its drawbacks. One, for example, is the fact that the assay employs a radioisotope which must be handled and disposed of properly. Secondly, RIA's tend to have a short storage life and are expensive.

Due to these drawbacks, a different type of immunoassay has been developed. This assay relies on the basic immunoassay reaction, but the label is an enzyme rather than a radioisotope. Instead of determining the activity of the isotope, enzyme activity is a measure of the Ag in the assay. This technique, developed by Syva, Palo Alto, Ca., is called an enzyme multiplied immunoassay technique (EMIT®), and relies on an artificially produced phenonmenon by which the enzyme activity can be controlled by any desired substance [103].

In this system, the enzyme is activated by minute quantities of Ag, and this activity is proportional to the concentration of the Ag present. This type of assay is developed by first conjugating the Ag or hapten (compound of less than 1000 daltons, which by itself does not elicit an antigenic response from the host) to an enzyme [103]. The conjugation is regulated such that the conjugation site is in the proximity of the reactive site of the enzyme [103]. Finally, specific Ab are produced against the Ag or hapten (Hp).

With the addition of the enzyme substrate to the reaction mixture which contains the Ab and the enzyme-Ag (Hp) conjugate, no activity is detected. This inhibition is thought to be due to the inability of the

substrate to reach the active site of the enzyme due to physical blocking of the site by the Ab [103]. If, however, free Ag (Hp) is added to the reaction mixture, there will be competitive binding between the Ab and the enzyme-Ag (Hp) conjugate or the free Ag (Hp), allowing the enzyme to react with its substrate, thereby displaying activity proportional to the amount of free Ag (Hp) present. As the concentration of the free Ag (Hp) increases, the Ab binds more of the free Ag (Hp) allowing more of the enzyme to react with its substrate, further increasing its activity. By varying the concentration of the free Ag (Hp), a standard curve may be constructed of the concentration of the free Ag (Hp) vs. enzyme activity. This type of assay is termed a homogenous immunoassay because no physical separation step is required.

Several enzymes are employed with this type of assay, including egg white lysozyme (EC 3.2.1.17), and glucose-6-phosphate dehydrogenase (G-6-PDase EC 1.1.1.49).

Thus, the first part of this research project is to apply this type of assay to quantitating desmosine, and isodesmosine. First, they are conjugated to a carrier protein such as bovine serum albumin (BSA). Once conjugated, antibodies to both desmosine and isodesmosine can be produced using rabbits. Concomitant with the conjugation of the desmosines to BSA, desmosine and isodesmosine are conjugated to several enzymes, including lysozyme and glucose-6-phosphate dehydrogenase. Once these preliminary steps are achieved, an enzyme immunoassay system is possible for the quantitation of the desmosines.

Another potential method for the quantitation of the desmosines from elastin is amino acid analysis via high performance liquid chromatography

(HPLC). Several methods now exist for amino acid analysis by HPLC utilizing several different separation techniques [104-106]. These techniques include the derivatization of the amino acids with a fluorogenic compound followed by reverse phase HPLC separation [104], separation of underivatized amino acids by ion-suppression [105], or the separation of the amino acids by ion-exchange HPLC [106]. Another possible means of separating the amino acids is by ion-paired HPLC, which in essence creates a pseudo-ion-exchange column and also employs some of the characteristics of a reverse phase column.

High performance liquid chromatography is a technique whereby a complex mixture can be readily separated and each component quantitated. While its fundamental theories were drawn from gas liquid chromatography (GC), HPLC has supplanted GC in many areas of analytical work, especially in the analysis of non-volatile or thermally labile compounds.

An HPLC system usually consists of a pump, a column, and a detector. Of these three, the column is the most important for it is the column which actually does the separating of the various components of the mixture. It achieves this separation by the interaction of the compound with both the stationary phase (column packing) and the mobile phase (eluting solvent). This interaction varies according to the stationary phase and the mobile phase employed in the separation.

The interaction of the compound with the various phases on the column can be quantitated by the various parameters, k', α, R_s, and N. The capacity factor, k', is a measure of the retention of the compound on the column for that particular system. It can be calculated by the equation:

$$k' = \frac{V_1 - V_0}{V_0}$$

where V_1 is the retention volume of the compound of interest; V_0 is the void volume of the column.

The separation factor, α, is a measure of the ability of the chromatographic system to separate two components of a mixture, and is defined as the ratio of the k' of the two components:

$$\alpha = \frac{k'_2}{k'_1}$$

where k'_2 is the capacity factor for the longer retained component.

The resolution factor, R_s, expresses the separation of the two peaks and includes the average peak width as well as the peak to peak separation. It is calculated as follows:

$$R_s = \frac{V_2 - V_1}{1/2(w_1 + w_2)}$$

where w_1 and w_2 are the width of the two peaks at the baseline. Baseline resolution of the two peaks would correspond to an R_s value of 1.5 [119].

Finally, the theoretical plate number, N, which is a term used for comparative purposes, describes the efficiency of the column. The efficiency is calculated as follows:

$$N = (t_r/w)^2$$

where t_r is the retention time of the peak and w is the width (cm) of the peak at baseline. With this number the efficiency of two different columns can be compared for a particular compound providing all other operating parameters remain constant.

As stated earlier, there are different types of HPLC. These include

adsorption or liquid-solid chromatography, partition or liquid-liquid chromatography, and ion-exchange chromatography. In adsorption chromatography, the components to be separated are bound to the surface of the column packing, and this bonding is dependent on the functional groups on the molecule. The common adsorbants are silica gel and alumina. In adsorption chromatography the stationary phase is polar while the mobile phase is non-polar.

In ion-exchange HPLC, retention is the result of two simultaneous processes: 1) the distribution of compounds between the mobile phase, usually polar, and the organic stationary phase; 2) reaction with the ionic sites within the stationary phase. These ionic sites usually consist of $R\text{-}SO_3^-$ groups for cation exchange columns or $R\text{-}N(CH_3)_3^+$ groups for anion exchange columns. Classically, ion-exchange columns have been utilized for amino acid analysis.

Partition chromatography relies on the partitioning of the solute between two phases, mobile and stationary, and the functionality of the solute for separation. This type of chromatography is the most widely used method for HPLC analysis. Partition chromatography is divided into two types, normal and reverse phase. In normal phase, so named due to historically being the first used, the stationary support is coated (bonded) with a polar moiety, while the mobile phase is relatively non-polar. Typically, normal phase supports are bonded to cyano (CN), amine (NH_2), or hydroxyl (OH) compounds.

In reverse phase chromatography, the stationary phase support is bonded, via siloxanes, to non-polar groups, usually C_2 to C_{18} alkanes. Of these, the most widely utilized is the octadecylsilane (ODS). The

retention mechanism for reverse phase HPLC is more complex than classical forms. In reverse-phase HPLC, the mobile phase is of higher polarity than the stationary phase; thus polar substances tend to elute prior to less polar. This is just opposite to the results obtained from a normal phase column.

There are 3 possibilities for the separation mechanism in reverse phase: 1) partitioning of the solute between the non-polar stationary phase and the polar mobile phase; 2) adsorption of the solute to the non-polar functions of the stationary phase; and 3) partitioning of the solute molecules between the mobile phase and a "new" stationary phase which is the organic modifier (usually methanol or acetonitrile) added to the mobile phase and adsorbed onto the stationary phase [107].

Ionized compounds, such as amino acids, elute very quickly or yield poorly defined peaks with severe tailing on a reverse phase column. There are several ways to suppress the ionization of the molecules to aid in their separation. One such way is to chemically modify the ionic species by bonding another molecule of less polarity to it [104]. Usually this derivatization process not only decreases the ionization of the molecule (polarity), but also usually produces a chromophore which enhances the detection of the molecule [104]. Another method for controlling the ionization of the molecule is by controlling the pH of the mobile phase in order to suppress the ionization of the molecule. This technique is called ion-suppression, and is useful for weak acids and bases in the pH range of 2 to 8. This technique cannot be used with strong acids and bases which remain ionized in that pH range.

When using strong acids or bases, another technique is available for

their separation. With this technique the tailing and poor peak shapes can be circumvented by forming ion pairs with a counter ion dissolved in the mobile phase. This ion-pair chromatography (IPC) technique has several possible modes of retention of a solute. One mode is the actual pairing between the sample and the counter ion, thereby decreasing the polarity as shown below:

$$A^+_{(aq)} + B^-_{(aq)} \longrightarrow AB_{(org)}$$

where A^+ is the aqueous sample; B^- is the counter ion in the mobile phase; and AB is the ion paired species. Another possible mechanism is the conversion of the reverse phase column to a pseudo-ion-exchange column, thereby possessing both characteristics of a reverse phase column and an ion-exchange column. The actual separation mechanism may be a combination of the two processes [107].

In this part of the research, the desmosines are separated and quantitated by several of the described HPLC techniques, including ion-exchange HPLC, ion-suppression HPLC, derivatization with a fluorogenic compound, and finally by ion paired HPLC.

CHAPTER 3

METHODS AND MATERIALS

PURIFICATION OF DESMOSINE AND ISODESMOSINE

Commercially prepared desmosine and isodesmosine (Elastin Products, St. Louis, Mo.) were dissolved in 5.0 mL of 0.2 mol/L sodium citrate buffer, pH 2.20. This solution was then applied to a jacketed column, 85 x 5 cm, packed with Aminex Ms "C" resin. The column temperature was maintained at 45 ^{o}C with a circulating water bath. The flow rate was maintained at 12.0 mL/min. The column was first eluted with approximately 100 mL of 0.2 mol/L sodium citrate buffer, pH 3.25, followed by 1.0 L of 0.38 mol/L sodium citrate buffer, pH 4.00. The desmosine or isodesmosine was then eluted with 0.38 mol/L sodium citrate buffer, pH 5.65.

The eluted desmosine or isodesmosine was then lyophilized and redissolved in a minimum of 0.1 mol/L hydrochloric acid and quantitatively transferred to a 45 x 2 cm column packed with Dowex 50WX8 cation exchange resin, 50-100 mesh, in the hydrogen form. The salts were eluted with 3.0 L of glass distilled, deionized water at a flow rate of 9.0 mL/min and the desmosine or isodesmosine was then eluted with 0.5 mol/L ammonium hydroxide. This solution was then lyophilized.

Following the elution of the desmosine or isodesmosine, the hydrogen form of the cation exchange resin was regenerated.

AMINO ACID ANALYSIS

The purity of the desmosines, as well as the amino acid analysis of the protein and tissue hydrolysates, was accomplished with a Beckman 121 C amino acid analyzer. The analyzer utilized an 11.0 x 0.9 cm jacketed column packed with PA-35, a sperical 7.5% cross-linked sulfonic acid resin, with a mean particle size of 13.0 ± 0.6 mm. The column temperature was maintained at 56 ^{o}C.

The automatic program (Fig. 4) employed for the separation and quantitation of the amino acids was as follows. The samples were diluted with 0.2 mol/L sodium citrate buffer, pH 2.20. Of this dilution 0.5 mL was injected and the column was first eluted with 0.2 mol/L sodium citrate buffer, pH 3.45 for 15 min, followed by a second elution with 0.35 mol/L sodium citrate buffer, pH 4.25 for 60 min. The final eluting buffer was 0.35 mol/L sodium citrate, pH 5.25, for a period of 60 min. All buffers were pumped at approximately 100 psig, at a flow of 68 mL/min.

Following the elution of the amino acids, the column was regenerated with 0.2 mol/L sodium hydroxide, followed by column equilibration with the initial buffer. All buffers contained 0.4 mL of 25% thiodiglycol/L to establish a reducing environment and 1.0 mL of toluene/L to retard bacterial growth.

The eluted amino acids were detected by reaction with ninhydrin. The ninhydrin was prepared according to the Beckman amino acid manual.

SPECTRA DETERMINATION OF DESMOSINE AND ISODESMOSINE

The purified desmosine or isodesmosine (5.43 mg each) was dissolved

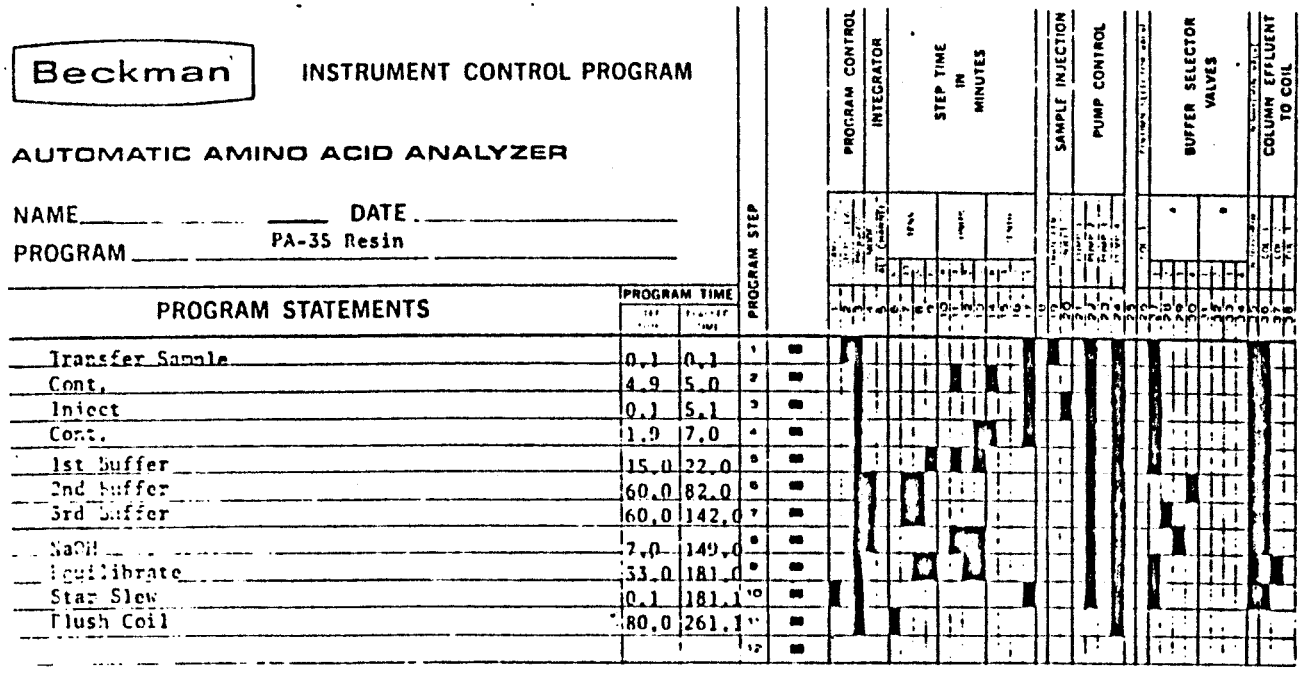


Fig. 4. Automated program employed on the Beckman 121 C amino acid analyzer.

in 10.0 mL of deionized, glass distilled water, hereafter referred to as water. Each solution was scanned against a water blank from 340 to 190 nm with a Perkin-Elmer recording spectrophotometer.

CONJUGATION AND ANTIBODY PRODUCTION

Purification of the Glutaraldehyde Solution:

To 100 mL of commercially prepared 25% glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.), 15 to 25 g of activated charcoal was added and throughly mixed. This mixture was then filtered through a 0.45 μm millipore filter. This procedure of Anderson was performed several times until the filtrate changed from a straw yellow color to a clear solution with a "fruity" fragrence [109]. The spectrum of the purified glutaraldehyde was then recorded from 340 to 200 nm with a Beckman DB-1 recording spectrophotometer and compared to the literature spectrum [109].

Conjugation of the Desmosines to Bovine Serum Albumin:

The purified desmosine or isodesmosine was conjugated to bovine serum albumin via a modified method of several authors [110-114]. In this procedure, 50 mg (0.735 nmol) of bovine serum albumin (BSA) was dissolved in 9.78 mL of 0.1 mol/L sodium acetate buffer, pH 5.0. To this was added 24.0 mg (44.2 nmol) of desmosine or isodesmosine. These amounts were added to establish a desmosine or isodesmosine to BSA ratio of 60 [114]. While slowly stirring, a purified 2% glutaraldehyde solution was added dropwise in various amounts to establish a glutaraldehyde to desmosine or isodesmosine mol ratio of 1 to 4 [114]. The mixture was allowed to react for 3 hours at room temperature, after which the mixture was centrifuged

at 1,000 x g for 20 min to remove any protein-protein macro-conjugates. The remaining supernatant was either dialyzed against water at 4 ^{o}C for 24 hours or placed on a Sephadex G-50 column (45 x 1 cm) and eluted with 0.1 mol/L sodium acetate buffer, pH 5.0. The eluant was monitored at 280 nm. The protein conjugate eluted from the column was then dialyzed against water for 24 hours at 4 ^{o}C to remove the buffer. The dialysate was then lyophilized and stored dessicated at 0 ^{o}C until needed.

All other conjugation procedures were modifications of this method.

Conjugation of the Desmosines to Lysozyme:

Purified desmosine or isodesmosine was conjugated to lysozyme (EC 3.2.1.17, Sigma Chemical Co., St. Louis, Mo.), by the method previously described for BSA with the following modifications. Fifty mg of lysozyme (3.47×10^{-6} mol) was dissolved in 9.78 mL of 0.066 mol/L potassium phosphate (monobasic) buffer, pH 6.24. To this solution was added 113 mg of desmosine or isodesmosine (2.083×10^{-4} mol), followed by the addition of 1.05 mL of a 2% glutaraldehyde solution (2.083×10^{-4} mol), dropwise.

Following the conjugation reaction and centrifugation, the supernatant was dialyzed against water for 24 hours at 4 ^{o}C, lyophilized and stored desiccated at 0 ^{o}C.

Conjugation of the Desmosines to Glucose-6-Phosphate Dehydrogenase:

Purified desmosine or isodesmosine was conjugated to glucose-6-phosphate dehydrogenase (EC 1.1.1.49, Sigma Chemical Co., St. Louis, Mo.), grade IV, by the method previously described for BSA with the following modifications. One mL of a glucose-6-phosphate dehydrogenase solution,

containing 2.05 mg (2.05×10^{-5} mol) protein, was added to 3.97 mL of a 0.1 mol/L Tris buffer, pH 7.6. To this was added 0.67 mL of a desmosine or isodesmosine solution (1 mg/mL; 1.23×10^{-4} mol). While slowly stirring, 0.013 mL of a 2% glutaraldehyde solution was added dropwise.

Following the conjugation reaction and centrifugation, the supernatant was dialyzed against water for 24 hours at 4 ^{o}C, lyophilized, and stored desiccated at 0 ^{o}C.

Determination of Lysozyme Activity:

Lysozyme, grade I, from egg whites, was assayed by a modified procedure of Shugar [115]. The substrate, Micrococcus lysodeikticus (Sigma Chemical Co., St. Louis, Mo.) was prepared by adding approximately 1.5 mg M. lysodeikticus to 10.0 mL of 0.066 mol/L potassium phosphate buffer, pH 6.24. The absorbance of this solution was adjusted to between 0.6 and 0.7 at 450 nm with buffer. Of this adjusted substrate suspension, 0.625 mL was added to a micro silica cuvette. To this was added 0.1-0.2 mL of either buffer or prepared antisera and mixed. Finally, 0.1 mL of a lysozyme solution or lysozyme-desmosine or isodesmosine conjugate solution (1.0 mg lysozyme or lysozyme conjugate dissolved in 10.0 mL of 0.066 mol/L potassium phosphate buffer, pH 6.24) was added and quickly mixed by inversion. Following an initial equilibration period of between 15 and 120 seconds, the absorbance at 450 nm (A_{450}) was recorded every 30 seconds. The A_{450} was recorded for a period of 2 to 4 min following the initial equilibration, and the relative rate, A_{450} change/min (ΔA_{450}) was determined.

This assay was utilized to determine the rate of the reaction for

unconjugated and the conjugated enzyme, as well as for titering the antisera for the quantitation of the desmosines.

Determination of Glucose-6-Phosphate Dehydrogenase Activity:

Glucose-6-phosphate dehydrogenase activity was assayed by a modified procedure of Bergmeyer et al [116]. The activity was determined by the change in absorbance at 340 nm. The assay was as follows: 2.95 mL of a 0.1 mol/L Tris buffer, pH 7.6, containing 6.9 mmol/L magnesium chloride, 1.0 mmol/L glucose-6-phosphate, and 0.39 mmol/L nicotine adenine dinucleotide phosphate (NADP) was added to a 3.0 mL cuvette. At time zero, 0.02 mL of a glucose-6-phosphate dehydrogenase (G-6-PDase) solution (2.05 mg protein/mL buffer) was added to the sample cuvette and mixed. The reference cuvette contained 2.92 mL of 0.1 mol/L Tris buffer, pH 7.6, containing the various cofactors. The absorbance at 340 nm was recorded for a period of 5 min and the relative rate, A_{340} change/min was determined.

This assay was utilized to determine the rate of the reaction for the unconjugated and the conjugated enzyme for the quantitation of the desmosines.

Production of Antibodies to Desmosine and Isodesmosine:

Various amounts of lyophilized BSA-desmosine or BSA-isodesmosine conjugates (2.5 to 5.0 mg) were dissolved in a minimal amount of sterile normal saline. An equal volume of Freund's complete adjuvant was added and the mixture was emulsified by repeatedly aspirating and expelling the mixture from a sterile glass syringe fitted with a 21 gauge needle [117]. A frothy white appearence indicated complete emulsification.

The emulsified mixture was administered by a modified method of Vaitukaitis et al [117]. The fur was shaved from the back and proximal limbs of New Zealand white rabbits, and the emulsion was injected intradermally at 15 to 20 sites, such that each rabbit received a total of 2.0 mL.

Following the initial inoculation, booster injections of from 2.5 to 5.0 mg of emulsified conjugates (Freund's incomplete adjuvant) were administered intramuscularly bi-weekly. After 8 weeks the rabbits were bled [117]. Bleeding was accomplished via the central ear artery, following the shaving of the ear and application of xylene for vasodilitation. The blood was collected into sterile 10 mL glass syringes, and transferred to silicone coated vacutainers. Two to 4 mL of blood was collected from each rabbit per bleeding. After clotting and centrifuging, the sera were frozen in 1.0 mL aliquots. Booster injections were continued throughout the course of bleeding the rabbits.

During the course of these procedures, the rabbits were housed at the animal research facility, Loyola University Medical Center, and fed standard lab chow and allowed water ad lib.

Titering of the Antisera:

The antisera collected from the rabbits were diluted 1:1, 1:10, 1:100, and 1:1000 with either 0.066 mol/L potassium phosphate buffer, pH 6.24, or 0.1 mol/L Tris buffer, pH 7.6 to be used in the lysozyme or G-6-PDase assays, respectively. The various dilutions were then added to their respective assays, as previously described, and the relative rate of the reaction calculated for each.

^{3}H-Isodesmosine Conjugation to BSA:

To 24.0 mg of unlabeled isodesmosine, approximately 0.5 mL of ^{3}H-isodesmosine (0.02 mg; ∿100 μCi), prepared by Amersham (Arlington Heights, IL.) was added. This mixture was then conjugated to BSA as previously described with an isodesmosine to BSA ratio of 60 and a glutaraldehyde to isodesmosine ratio of 1. Following its lyophilization, the conjugate was dissolved in a minimum amount of glass distilled, deionized water. To this was added 8.0 mL of scintillation fluor (4% PPO, 10% BSS, toluene, w/v) and the conjugate was counted to a 0.2% error in a Beckman scintillation counter.

HPLC DETERMINATIONS

All HPLC determinations for desmosine or isodesmosine utilized a Water's M-6000 pump system connected to a Water's U6K injector fitted with a 2.0 mL injection loop. The amino acids were detected with either a Water's M-440 dual wavelength detector (254 and 280 nm) or a Water's M-450 variable wavelength detector at 205 nm, or a Water's M-420 fluorescence detector (334 nm excitation, 450 nm emission). An Omniscribe dual pen recorder was utilized to quantitate the various amino acids as they eluted via the peak height method. All injection were made with a Hamilton 50 μL syringe, designed for the Water's injector.

Ion-Exchange HPLC:

For all ion-exchange determinations, a Whatman SCX/Partisil 10, 25 x 0.4 cm column was utilized. The column consisted of sulfonic acid functional groups bound to inert particles 10 μm in size. For determina-

tions the amino acids were first dissolved in 0.1 mol/L potassium hydroxide (10 mg amino acid/50 mL). Five µL of each were injected onto the column and eluted with 0.2 mol/L ammonium phosphate (dibasic) buffer, with the pH varied from 2.5 to 3.5. The mobile phase was maintained at a constant flow of 0.7 mL/min (300 psig). The amino acids were detected at either 280, 245, or 205 nm as they eluted from the column.

Reverse Phase and Ion-Suppression HPLC:

For all reverse phase and reverse phase-ion suppression HPLC determinations a Water's C-18 µ-Bondapak 30.0 x 4.0 mm, 10 µm particle size, column was utilized. This column consists of octadecyl alkane moieties chemically bonded to silica particles. The amino acids were separated and quantitated by a modified method of Hancock [104]. In this procedure, 5 to 10 µL of the amino acid solutions (10 mg amino acid/50 mL water) were injected onto the column and eluted with a mobile phase, which consisted of a methanol/water/phosphoric acid solution, which varied from 0/99/1 to 40/59/1 (v/v). The pH varied from 2.5 to 6.0. The flow rate was maintained at 1.0 mL/min (1800 to 2500 psig, depending on the methanol percentage of the mobile phase). The amino acids were detected at 205 nm as they eluted from the column.

Derivatization with ortho-Phthaldialdehyde:

The methods of Lindroth and Mopper[105], and Gardner and Miller [106], were employed for the derivatization and detection of the amino acids. The method utilized a pre-column derivatization procedure prior to the injection of the amino acids onto the C-18 column. Again a Water's C-18

column was utilized. The amino acids were derivatized with o-phthaldaldehyde (OPA) reagent, which consisted of 270 mg OPA (Sigma Chemical Co., St. Louis, Mo.) dissolved in 5.0 mL of absolute ethanol. To this was added 200 µL of 2-mercaptoethanol. The OPA reagent was then made to 500 mL with 0.4 mol/L boric acid, pH 9.5.

To derivatize the amino acids, 100 µL of OPA reagent was added to 20 µL of the sample, and allowed to react at room temperature for 2.0 min. Ten µL of the derivatized sample was then injected onto the column and eluted with 0.05 mol/L sodium phosphate (monobasic) buffer, pH 7.3, with the methanol percentage varied between 50 and 95%. The mobile phase was maintained at a constant flow of 1.0 mL/min, and the amino acids were detected as they eluted from the column by fluorescence, at an excitation wavelenth of 340 nm and an emission wavelength of 450 nm.

Ion-Paired HPLC:

This method of separation (PIC ^{R} Supplement, Water's Inc.) utilized the sodium salt of heptane sulfonic acid (Eastman Kodak Co., Rochester, N.Y.) as the counter ion. For separation of the desmosines, this system employed either a Water's C-18 column as previously described or a Bio-Rad ODS-5, 15 cm x 4 mm i.d., C-18 column (5 µm particle size). The amino acids either from standards or from hydrolysates were eluted with a methanol/water solvent, with the methanol percentage varied between 19 and 30%. The solvent contained 0.01 mol/L heptane sulfonic acid, sodium salt, and was adjusted to pH 3.0 with phosphoric acid. The mobile phase was maintained at a constant flow of 1.0 mL/min (1000 to 2500 psig, depending on the methanol percentage in the mobile phase). The eluted

amino acids were detected at either 205, 254, or 280 nm and were quantitated by peak height, as compared to a standard curve.

Tissue Preparation:

Tissue samples from both canine and human aortas were first cleaned by removing any plaque and fat deposits which were visibly present. The tissue was minced and weighed to yield a wet weight of from 2 to 4 g. The minced tissue was then dried at 60 ^{o}C at 0.05 mm Hg pressure overnight to a constant weight. The dried tissue was then defatted by a series of ethanol, ethanol-ether (1:1), and finally ether extractions. The tissue was again dried and weighed to yield a fat free dry weight (FFDW).

The FFDW tissue was then hydrolyzed at 110 ^{o}C for 48 hours with 6 mol/L hydrochloric acid. Following hydrolysis, 20 to 30 mg of activated charcoal was added to the hydrolysate and the mixture was again heated to 60 ^{o}C with agitation for 10 min, followed by filtering through a 3 μm Millipore filter to remove the charcoal.

For amino acid analysis by the Beckman 121 C, the hydrolysate was diluted with 0.2 mol/L sodium citrate buffer, pH 2.2, and analyzed as previously described. For analysis by HPLC, the hydrochloric acid had first to be removed. This was accomplished by a series of washings with water, each followed by evaporation under vacuum. Once the hydrochloric acid was removed, the hydrolysate was diluted to final volume with glass distilled, deionized water.

RECOVERY STUDIES OF THE DESMOSINES USING HPLC

Recovery from Hydrolyzed BSA:

To determine the analytical recovery and to check the linearity of the ion-paired HPLC method, desmosine and isodesmosine were added to BSA, prehydrolysis. In this method, 50 mg of BSA was added to consecutively numbered hydrolysis tubes. To the first tube, no desmosine or isodesmosine was added. To the remaining tubes, desmosine and isodesmosine were added in various amounts to achieve the following concentrations: Tube 2, 0.0224 and 0.0130 mmol/L; tube 3, 0.0448 and 0.0180 mmol/L; tube 4, 0.0597 and 0.0265 mmol/L; and to tube 5, 0.0784 and 0.0397 mmol/L, desmosine and isodesmosine, respectively. The BSA was then hydrolyzed as previously described, followed by the determination of the desmosines.

Recovery from Hydrolyzed Canine Aorta:

The above recovery studies were then repeated substituting prepared canine aorta for the BSA. Once the initial desmosine and isodesmosine concentration of the aorta sample was determined, the remaining tissue was spiked (prehydrolysis) with desmosine and isodesmosine to achieve the following concentrations of desmosine and isodesmosine, respectively: 0.056 and 0.049 mmol/L; 0.060 and 0.056 mmol/L; 0.070 and 0.061 mmol/L; 0.072 and 0.066 mmol/L; and finally 0.080 and 0.078 mmol/L. The spiked aorta samples were then hydrolyzed and the desmosine and isodesmosine concentrations determined, as previously described.

COLLAGEN DETERMINATIONS

Hydroxyproline Determinations:

The collagen content of all the tissue hydrolysates was determined by the method of Blumenkrantz and Asboe-Hanson [40]. In this method, the hydroxyproline content is correlated to the amount of collagen present in the tissue. For this method, 1.0 mL of hydrolysate was mixed with 1.5 mL of a borate-alanine buffer, pH 8.7 and 600 µL of a 0.2 mol/L chloramine T solution. Following a 20 min reaction time, 2.0 mL of a 3.6 mol/L thiosulfate solution was added and throughly mixed. Sodium chloride was then added to the mixture to saturation levels. After a 20-30 minutes, 3.0 mL of toluene was added to the tube , followed by heating at 100 ^{o}C in a water bath. The tubes were then cooled and allowed to set for 15 min followed by a slow speed centrifugation for 10 min.

One mL of the organic layer was then removed and mixed with 0.4 mL of Ehrlich's reagent (Appendix A). After a 30 min reaction time in the dark, at room temperature, the solution was read in a spectrophotometer at 565 nm against a reagent blank. The A_{565} was compared to a standard curve for hydroxyproline.

CHAPTER 4

RESULTS

PURIFICATION OF THE DESMOSINES

From the amino acid analysis of the commercially prepared desmosine and isodesmosine, it was observed that mutiple unknown peaks appeared on the aminograms. Since the desmosines would be used in the conjugation reactions, it was decided that they should be further purified. The desmosines were then purified as described in Chapter 3.

Two criteria were used to judge the purity of the desalted, purified desmosines, the first being a single eluting peak on amino acid analysis, and secondly a comparison of their respective ultra-violet (UV) spectra to those published. Following their purification, desmosine and isodesmosine were both found to elute as single peaks on amino acid analysis, at 70 and 78 min, respectively, devoid of any extraneous peaks first observed on amino acid analysis of the unpurified desmosines (Figs. 5,6). These purified fractions were then subjected to UV analysis, as previously described. Both desmosine and isodesmosine were found to display UV absorption in the 200 to 280 nm range, desmosine displaying its characteristic bi-modal absorption spectrum, having absorbance maxima at 238 and 268 nm, with a slight inflection at 273 nm (Fig. 7), while isodesmosine displaying its characteristic single absorption maximum at 278 nm, with an inflection at 217 nm (Fig. 8). Both these obtained spectra correlated well with those previously published [8,9].

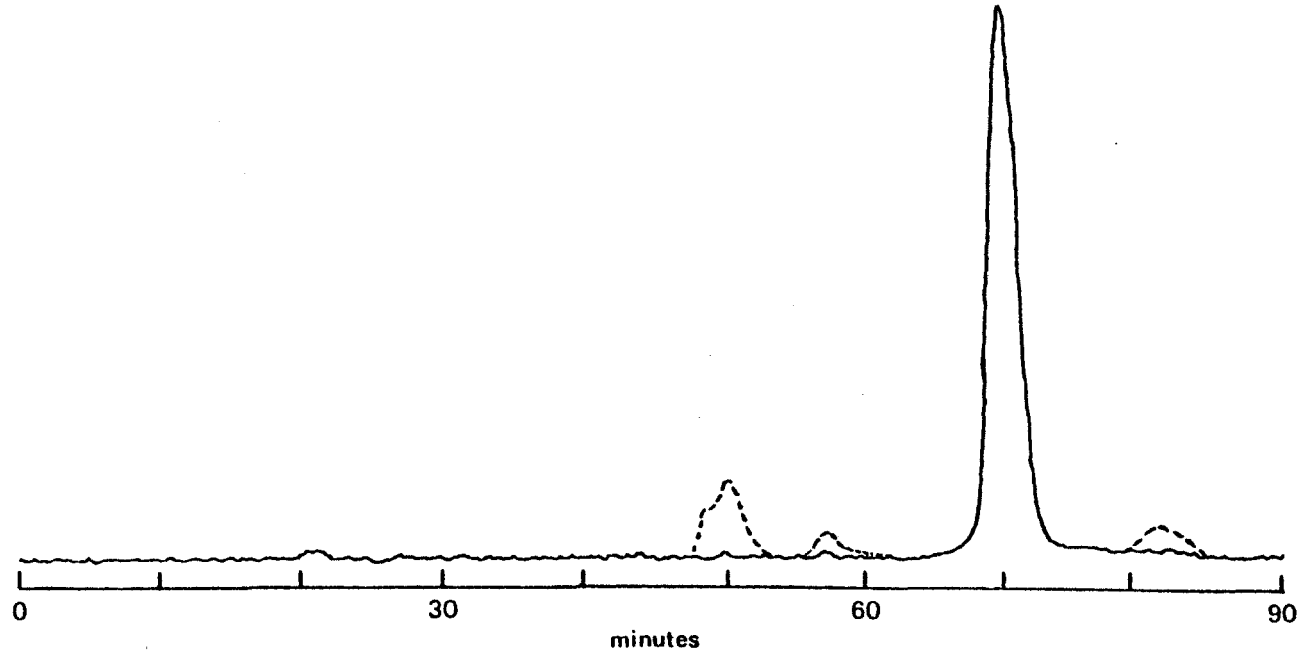


Fig. 5. Aminogram of desmosine. (------) Impurities present in commercially prepared desmosine. (———) Aminogram of desmosine following purification of commercial preparation.

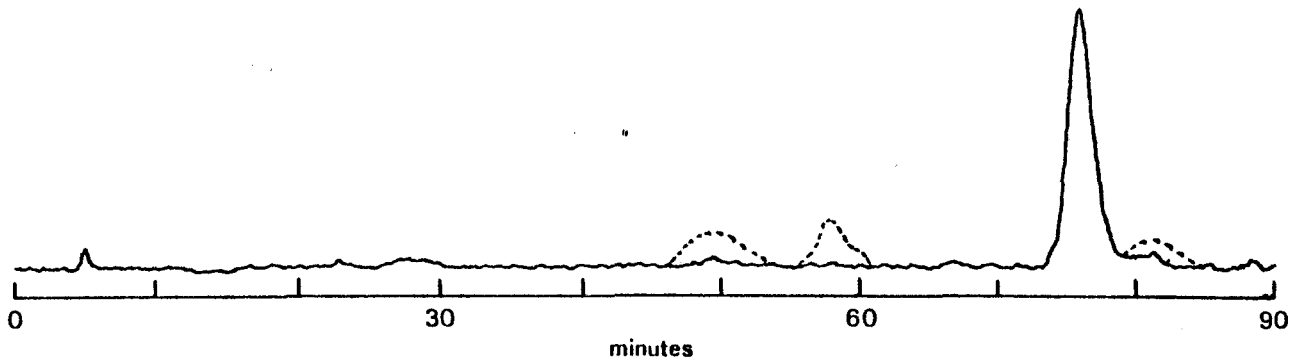


Fig. 6. Aminogram of isodesmosine. (------) Impurities present in commercially prepared isodesmosine. (———) Aminogram of isodesmosine following purification of commercial preparation.

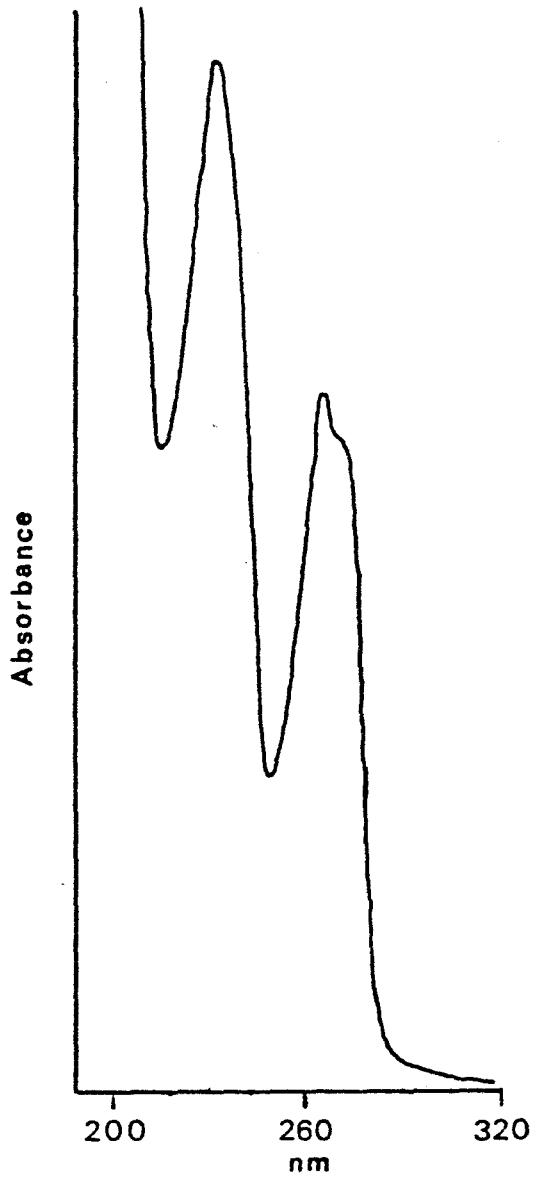


Fig. 7. Ultraviolet spectrum of purified desmosine.

Fig. 8. Ultraviolet spectrum of purified isodesmosine.

Therefore, from the results obtained from amino acid analysis and UV analysis, it was determined that the desmosine and isodesmosine preparations were pure. The purified desmosine and isodesmosine, hereinafter referred to as desmosine and isodesmosine, were used in the conjugation and antibody production phase of the research, as well as for the standards in all automated amino acid analysis and HPLC method development.

CONJUGATION AND ANTIBODY PRODUCTION

Purification of Glutaraldehyde:

In order to elicit an antigenic response from the inoculated rabbits, desmosine or isodesmosine first had to be conjugated to a carrier molecule, BSA fraction V. It was decided that this conjugation of either Des or Ide to BSA should be attempted via a reaction with glutaraldehyde. However, before attempting this conjugation, the commercially prepared 25% glutaraldehyde was purified. This purification involved the stirring of the glutaraldehyde solution with activated charcoal, as described in Chapter 3. According to the literature, an absence of an absorbance peak at 235 nm, as well as the solution being clear and colorless with a distinct "fruity" fragrance, are all indicative of a pure solution [109]. In the commercially prepared solution of glutaraldehyde, a strong absorption peak was noted at 235 nm and the solution had a yellowish tinge. However, on treatment with activated charcoal, the filtrate was observed to be clear and colorless, with a "fruity" fragrance. The purified solution also did not display any absorption peak at 235 nm. Using the before mentioned criteria, it was determined that the glutaraldehyde solution was pure and suitable to be utilized in the conjugation reac-

tion.

Conjugation of the Desmosines to BSA:

Following the purification of the desmosines, and the glutaraldehyde solution, conjugation of either Des or Ide to BSA was attempted, as previously described in the methods. In all conjugation studies the mol ratio of Des or Ide to BSA was maintained at 60:1, with the mol ratio of glutaraldehyde to Des or Ide varied from 1:1 to 10:1.

During the course of the conjugation reaction, it was noted that the mixture changed from a clear, colorless solution to a straw yellow color, and that a white flocculant precipitate formed. Upon completion of the reaction, the solution was placed on a Sephadex G-50 column, and eluted with the appropriate buffer. The eluant was monitored at 280 nm and 2 peaks were observed (Fig. 9). The first peak eluted with the void volume, while the second eluted at approximately 60 mL. The first peak was found to contain the protein portion of the reaction, while the second peak contained the unconjugated Des or Ide. No attempt was made to quantitate the unconjugated Des or Ide. The white flocculant precipitate which formed was thought to be protein-protein macroconjugates, which form as a side reaction to the conjugation process [114].

Following the initial conjugation, the glutaraldehyde (Glu) to Des ratio was varied from 1-10. In these conjugation reactions, the appearence, color and the amount of precipitation was qualitatively noted. Finally, the amount of protein remaining in the supernatant, following the 1,000 x g centrifugation, was quantitated by the Lowry method.

Table 5 shows the results of the various conjugation conditions uti-

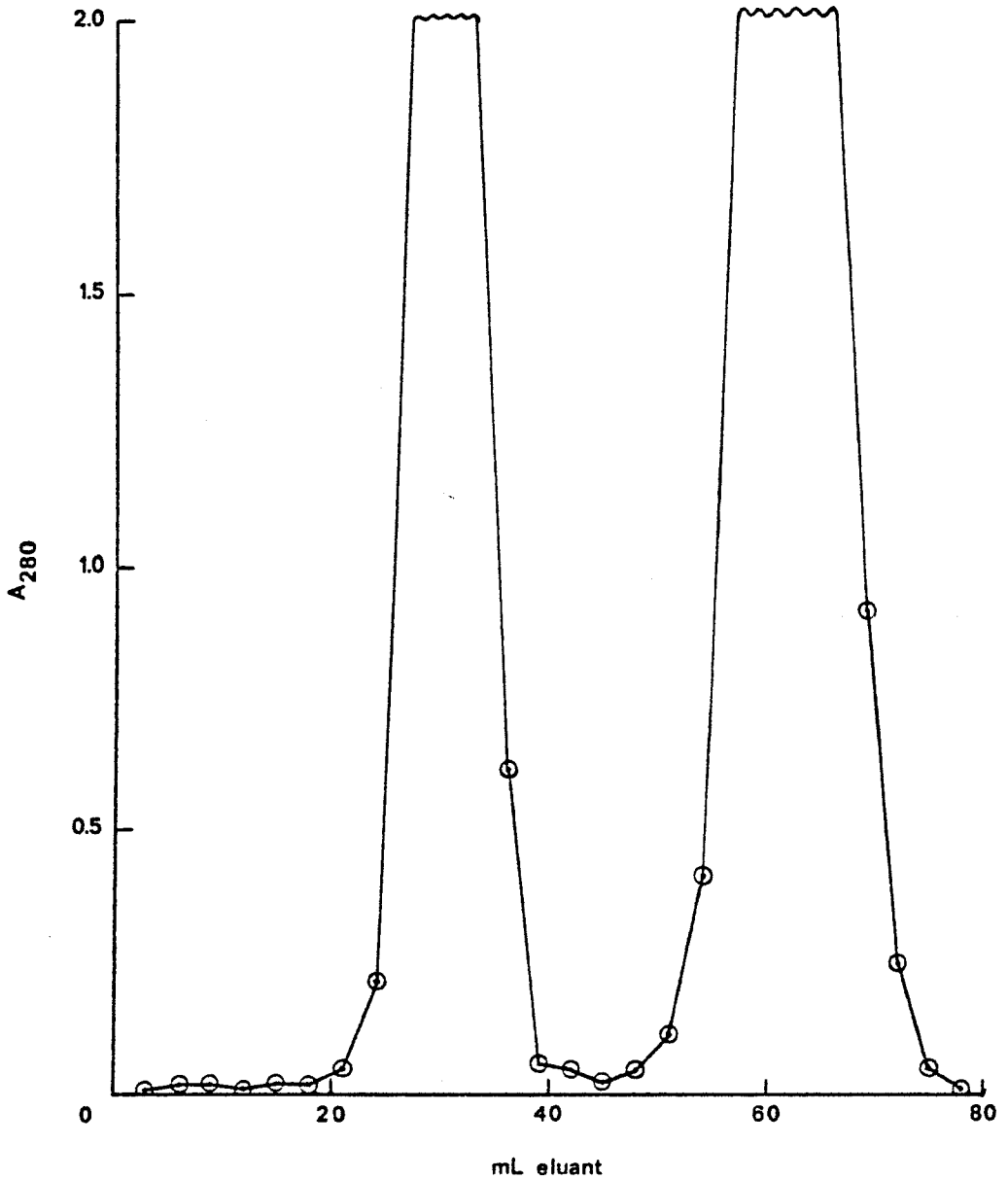


Fig. 9. Elution profile of conjugation reaction mixture applied to a Sephadex G-50 column, and eluted with 0.1 mol/L sodium acetate buffer, pH 5.0. BSA-Desmosine conjugate and unreacted desmosine detected at 280 nm.

TABLE 5

DETERMINATION OF GLUTARALDEHYDE TO DESMOSINE RATIO IN BSA-DESMOSINE CONJUGATION

GLU/DES	APPEARANCE*	AMOUNT PRECIPITATION**	PROTEIN***
0	Clear	None	48 mg
1	Light Yellow	Slight	46 mg
2	Straw Yellow	Moderate	40 mg
3	Yellow	Moderate-Heavy	20 mg
4	Yellow	Heavy	0 mg

Determination of glutaraldehyde to desmosine ratio for conjugation of desmosine to BSA. Ratios are molar and based on a desmosine to BSA ratio of 60 using 50 mg of BSA in the reaction.

* Appearance of reaction mixture following 3 hr. at room temperature.

** Relative amount of precipitate following centrifugation.

*** Protein remaining in supernatant following centrifugation.

ized to determine the optimum glutaraldehyde to Des or Ide ratio. It was noted that as the glutaraldehyde to Des ratio was increased from 1 to 4, the amount of protein precipitation increased, with a concomitant decrease in the amount of protein remaining in the supernatant. It was also observed that during the course of the reaction, the appearance of the mixture changed from a colorless solution to a dark yellow, the color dependent on the glutaraldehyde to Des ratio.

From these findings, it was determined that all conjugation reactions would utilize a glutaraldehyde to Des or Ide ratio of 1 in order to assure a maximum yield of conjugate (protein remaining in the supernatant) with a minimum of precipitation.

Conjugation of the Desmosines to Lysozyme or G-6-PDase:

Des or Ide was conjugated to lysozyme and G-6-PDase as previously described in the methods. The glutaraldehyde to Des or Ide ratio was maintained at 1 (as determined in their conjugation to BSA) in order to minimize any protein-protein macrocomplexes, which would decrease the amount of available enzyme. No visible precipitation was noted upon the completion of the conjugation reaction; however, following the centrifugation, a minute amount of a white precipitate was noted.

In order to determine the loss of lysozyme activity due to the conjugation procedure, unconjugated lysozyme activity was compared to that of the lysozyme-Des or -Ide conjugate. When the two were normalized to mg protein, a substantial decrease was noted in the conjugated enzyme (9.05 ± 0.16 ΔA/min/mg vs. 1.37 ± 0.07 ΔA/min/mg, respectively). This decrease amounts to a loss of approximately 85% of the unconjugated lyso-

zyme activity. This loss, however, was not unexpected, as loss of enzyme activity upon conjugation has been reported [110,111,113]. Even with the 85% loss of activity, it was decided that the remaining activity should be sufficient for the quantitation of the desmosines utilizing lysozyme.

Glucose-6-phosphate dehydrogenase activity was also compared between the unconjugated and conjugated enzyme. In these assays the rates were normalized to units of activity. Upon normalization, it was found that 51% of the unconjugated enzyme activity was lost due to the conjugation process (2.05 ± 0.04 ΔA/min/unit vs. 1.23 ± 0.06 ΔA/min/unit).

Of the two enzyme assays, there was a greater sensitivity, as well as a greater yield of active enzyme, with G-6-PDase. However, both enzymes were utilized to titer the antisera, in order to determine which enzyme was better suited for the quantitation of the desmosines.

Antibody Production:

Bovine serum albumin-desmosine or isodesmosine conjugates were injected into New Zealand white rabbits as previously described. A total of 8 rabbits (4 groups) were inoculated with either 2.5 or 5.0 mg conjugate/injection. Rabbits 1 and 2 recieved 2.5 mg BSA-Des conjugate/injection, while rabbits 3 and 4 received 5.0 mg BSA-Des conjugate/injection. Rabbits 5 and 6, and 7 and 8, were injected with 2.5 and 5.0 mg BSA-Ide conjugate/injection, respectively.

Following the initial inoculations, lesions developed at the sites of the injections. These lesion eventually cleared, and no other gross problems were encountered during the course of the antibody production. The initial bleeding of the rabbits occurred after 8 weeks, and was

continued every 2 weeks thereafter, up to 16 weeks. Booster injections were administered every 2 weeks, with Freund's incomplete adjuvant, and continued throughout the 16 weeks.

The antisera collected from each bleeding were diluted to various titers (1, 1:1, 1:10, 1:100, 1:1000) with buffer used for each enzyme. These various titers were then assayed for antibody activity utilizing both the Des or Ide conjugated lysozyme or G-6-PDase preparations. If antibody activity was present, then a decrease in enzyme activity (rate) from the control activity (sera from untreated rabbits) should be observed. When the various antisera obtained from the rabbits over several bleedings were assayed, no decrease in either enzyme activity from control values were noted (Tables 6-9). The specific data shown is for the final bleeding at 16 weeks. No differences were noted between the rabbits in each group, or the time interval from the initial inoculations. It was noted that the enzyme activity substantially increased when any serum (both control and antiserum) was added to the assay mixture. This increase is thought to be due, in part, to non-specific activation of the enzymes by the proteins or the co-factors present in the serum of the rabbits.

The lack of inhibition of either enzyme assayed, at the various titers of the antisera, could be accounted for by several reasons. One, that the assays were not sufficiently sensitive enough to detect the decrease in the activity (rate) of the enzymes, or two, that antibodies to either Des or Ide were not produced. Since both enzymes employed in this research have been extensively utilized in this type of assay, it was decided to investigate the possibility that antibodies were not being

TABLE 6

TITERING OF DESMOSINE ANTISERA
FROM RABBIT 1

TITER OF ANTISERA	LYSOZYME (ΔA/MIN/MG PROTEIN)	G-6-PDASE (ΔA/MIN/UNIT)
0*	1.36 ± 0.07	1.22 ± 0.05
1	1.66 ± 0.03	1.52 ± 0.04
1:1	1.69 ± 0.04	1.51 ± 0.03
1:10	1.67 ± 0.03	1.49 ± 0.06
1:100	1.65 ± 0.02	1.53 ± 0.02
1:1000	1.68 ± 0.03	1.50 ± 0.03
CONTROL**	1.63 ± 0.05	1.53 ± 0.04

RATE OF REACTION FOR LYSOZYME- AND G-6-PDASE-DESMOSINE CONJUGATES AT VARIOUS TITERS OF ANTISERA FROM RABBIT 1.
ADMINISTERED 2.5 MG BSA-DESMOSINE CONJUGATE/INJECTION.
* ASSAYED WITH APPROPRIATE BUFFER, REPLACING ANTISERA.
** ASSAYED WITH SERA FROM UNTREATED RABBIT.

TABLE 7

TITERING OF DESMOSINE ANTISERA
FROM RABBIT 3

TITER OF ANTISERA	LYSOZYME (ΔA/MIN/MG PROTEIN)	G-6-PDASE (ΔA/MIN/UNIT)
0*	1.36 ± 0.07	1.22 ± 0.05
1	1.69 ± 0.05	1.54 ± 0.05
1:1	1.65 ± 0.03	1.58 ± 0.04
1:10	1.67 ± 0.03	1.58 ± 0.06
1:100	1.68 ± 0.04	1.56 ± 0.02
1:1000	1.65 ± 0.03	1.52 ± 0.03
CONTROL**	1.66 ± 0.06	1.52 ± 0.04

RATE OF REACTION FOR LYSOZYME- AND G-6-PDASE- DESMOSINE CONJUGATES AT VARIOUS TITERS OF ANTISERA FROM RABBIT 1.
ADMINISTERED 5.0 MG BSA-DESMOSINE CONJUGATE/INJECTION
* ASSAYED WITH APPROPRIATE BUFFER, REPLACING ANTISERA.
** ASSAYED WITH SERA FROM UNTREATED RABBIT.

TABLE 8

TITERING OF ISODESMOSINE ANTISERA
FROM RABBIT 5

TITER OF ANTISERA	LYSOZYME (ΔA/MIN/MG PROTEIN)	G-6-PDASE (ΔA/MIN/UNIT)
0*	1.36 ± 0.07	1.22 ± 0.05
1	1.64 ± 0.03	1.45 ± 0.04
1:1	1.67 ± 0.04	1.43 ± 0.07
1:10	1.60 ± 0.05	1.48 ± 0.06
1:100	1.65 ± 0.03	1.45 ± 0.03
1:1000	1.63 ± 0.04	1.44 ± 0.04
CONTROL**	1.64 ± 0.02	1.46 ± 0.03

RATE OF REACTION FOR LYSOZYME- AND G-6-PDASE-ISODESMOSINE CONJUGATE AT VARIOUS TITERS OF ANTISERA FROM RABBIT 5. ADMINISTERED 2.5 MG BSA-ISODESMOSINE CONJUGATE/INJECTION.

* ASSAYED WITH APPROPRIATE BUFFER, REPLACING ANTISERA.

** ASSAYED WITH SERA FROM UNTREATED RABBIT.

TABLE 9

TITERING OF ISODESMOSINE ANTISERA
FROM RABBIT 7

TITER OF ANTISERA	LYSOZYME (ΔA/MIN/MG PROTEIN)	G-6-PDASE (ΔA/MIN/UNIT)
0*	1.36 ± 0.07	1.22 ± 0.05
1	1.56 ± 0.05	1.40 ± 0.03
1:1	1.50 ± 0.04	1.38 ± 0.04
1:10	1.55 ± 0.06	1.42 ± 0.02
1:100	1.52 ± 0.05	1.40 ± 0.05
1:1000	1.50 ± 0.03	1.41 ± 0.03
CONTROL**	1.53 ± 0.04	1.40 ± 0.06

RATE OF REACTION FOR LYSOZYME- AND G-6-PDASE-ISODESMOSINE CONJUGATES AT VARIOUS TITERS OF ANTISERA FROM RABBIT 7. ADMINISTERED 5.0 MG BSA-ISODESMOSINE CONJUGATE/INJECTION

* ASSAYED WITH APPROPRIATE BUFFER, REPLACING ANTISERA.

** ASSAYED WITH SERA FROM UNTREATED RABBIT.

produced to either Des or Ide.

Immunodiffusion Studies:

In order to determine if in fact antibodies were being produced to the desmosines, immunodiffusion studies were undertaken. In this technique, six wells are punched in a 1% agarose gel film, in a hexagonal shape, with a seventh well punched in the center of the hexagon. The antisera is placed in the center well, while the antigens are placed in the surrounding wells. As the solutions diffuse through the agarose, they eventually interface with each other. At the interface between the antisera and its specific antigen, a precipitin band will be observed, due to the antigen-antibody complex which is formed.

In this study, prepared immunodiffusion plates (Travenol, 1% agarose, containing 0.1% sodium azide to retard bacterial growth) were utilized. The various titers of each antisera were placed into the center well, while the outer wells contained solutions of the various possible antigens. To one well, a 10% solution of BSA was added, while to another well, either BSA-Des or BSA-Ide was added, depending on the antisera in the center well. To the third well, the corresponding G-6-PDase-Des or -Ide conjugate was added. Finally the corresponding lysozyme-Des or -Ide conjugate was added. The plates were allowed to incubate at 5 ^{o}C for periods up to 1 week, with daily observation.

It was observed that precipitin bands formed at all titers investigated (1:1, 1:10, and 1:100, antisera in 0.9% sterile saline) to the 10% BSA solution, as well as the BSA-conjugates, within 1 days incubation. No precipitin bands were observed between the antisera and the corres-

ponding enzyme conjugates by the end of the fifth day.

In order to determine if the two buffers utilized in the enzyme assays had an effect on the antigen-antibody complex formation, the experiment was repeated using the corresponding enzyme buffers in lieu of the 0.9% sterile saline solution used to prepare the various titers of the antisera. The results were essentially the same, precipitin bands were formed between all titers of the antisera to the BSA solution or the BSA-conjugates. Again, however, no precipitin bands were observed between the antisera and its respective enzyme conjugate.

In both of the immunodiffusion studies, untreated rabbit sera was run as a control, and in all cases, no precipitin bands were formed between any of the solutions during the 5 day incubation preiod.

From these results it was concluded that antibodies were being produced to BSA, however, no evidence existed that antibodies to enzyme conjugates were being produced. These results implied that antibodies to Des or Ide were not being produced by the rabbits.

Conjugation Studies:

The results from the previous study, i.e., the production of antibodies to BSA, while no apparent antibody production to Des or Ide, could be explained by the failure to conjugate the desmosines to the BSA molecule. This occurence would also explain the large amount of unreacted Des or Ide eluted from the Sephadex G-50 column following conjugation. Therefore, in order to determine if this situation existed, Des was conjugated to BSA in the manner previously described, maintaining the Des to BSA ratio of 60, and varying the glutaraldehyde to Des ratio from 0 to 4.

Following the conjugation reaction, the conjugate was separated into its two fractions via gel filtration, both fractions being collected. The first fraction (presumptive conjugate) plus any precipitate formed during the conjugation reaction was collected and hydrolyzed as previously described. The hydrolysates were then analyzed for their amino acid composition, as was the second fraction eluted from the column. Table 10 displays the results from these studies, and as can be observed, no Des was detected in the first fraction at any of the Glu to Des ratios investigated, and that the amount of Des recovered from the second fraction did not significantly differ from the control reaction (Glu to Des = 0).

From these results it was concluded that Des or Ide, in amounts capable of being detected by automated amino acid analysis, were not being conjugated to the BSA molecule at the glutaraldehyde to Des or Ide ratio utilized in the initial preparation of the conjugates.

^{3}H-Isodesmosine Conjugation Study:

In the event that the amino acid analyzer was not sensitive enough to detect the amount of Des or Ide being conjugated to the BSA molecule, a radiolabeled tracer (^{3}H-isodesmosine) was utilized in a conjugation reaction. The labeled isodesmosine was reacted with the BSA as described in the methods, and following the conjugation, the amount of ^{3}H-label incorporated into the BSA molecule was determined by liquid scintillation counting.

At a glutaraldehyde to Ide ratio of 1, the ratio employed in the initial conjugation reactions, no activity above background could be detected in the first fraction off the column, while the second fraction

TABLE 10

DESMOSINE CONJUGATED TO BSA AT
VARIOUS GLUTARALDEHYDE TO DESMOSINE RATIOS

GLU/DES	MOLE DES/MOLE BSA	DES RECOVERED ***
CONTROL*	0	0
0**	0	0.043 MMOLE
1	0	0.043 MMOLE
2	0	0.042 MMOLE
3	0	0.043 MMOLE

DETERMINATION OF THE AMOUNT DESMOSINE CONJUGATED TO BSA AT VARIOUS GLUTARALDEHYDE TO DESMOSINE RATIOS BY METHOD PREVIOUSLY DESCRIBED. DESMOSINE DETERMINED BY AMINO ACID ANALYSIS.

* FIFTY MG BSA HYDROLYZED AND ANALYZED.

** AS IN CONJUGATION REACTION WITHOUT ADDITION OF GLUTARALDEHYDE.

*** DETERMINED FROM DESMOSINE FRACTION FROM SEPHADEX G-50 COLUMN.

collected contained significant activity.

From this qualitative study, when combined with the other conjugation studies, it was determined that at the glutaraldehyde to Des or Ide ratio utilized in this project, the two derived amino acids were not being conjugated to BSA, and consequently not producing antibodies.

HPLC STUDIES

Due to the inability to produce antibodies for use in an EIA for the desmosines, alternative methods of quantitation were investigated. These methods utilized high performance liquid chromatography, specifically, ion-exchange, reverse phase, reverse phase with ion suppression, and ion-pairing for the separation and quantitation of the desmosines.

Ion-Exchange HPLC:

The first technique investigated for the separation and quantitation of the desmosines was ion-exchange HPLC, due to its similarity to automated amino acid analysis (AAA). However, in contrast to AAA, which uses several buffers to elute the amino acids, this HPLC system uses only one buffer to elute all the amino acids (isocratic).

Initial attempts to separate the desmosines from the other amino acids used a system which consisted of 0.2 mol/L ammonium phosphate buffer, pH 3.5, with the eluted amino acids detected at either 205 or 254 nm. A representitive chromatogram of an amino acid standard containing Trp, Phe, and Des is shown in Fig. 10. With this system, the 3 amino acids separated from one another, Trp eluting first, followed by Phe and Des. However, when a mixture of Des and Ide was injected, the two amino

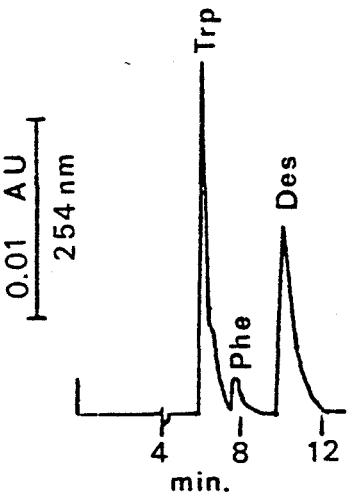


Fig. 10. Cation exchange HPLC separation of amino acid standard containing Trp, Phe, and Des. Standards eluted with 0.2 mol/L ammonium phosphate buffer, pH 3.5, and detected at 254 nm.

TABLE 11

K' VALUES OF VARIOUS AMINO ACIDS AT pH 3.5 AND 2.5 USING ION-EXCHANGE HPLC

AMINO ACID	pH 3.5 K'	pH 2.5 K'
Tyr	**	0.25
Ala	**	0.28
Trp	0.52	0.68
Leu	**	1.03
Ile	**	1.03
Phe	0.90	1.21
Des	1.50	1.97
Ide	1.50	1.97

K' determined for various amino acids using ion-exchange HPLC analysis with a mobile phase of 0.2 mol/L ammonium phosphate buffer, pH 3.5 and 2.5, with a flow rate of 0.7 ml/min (300 psig). Amino acids detected at 205 and 254 nm.

* Ten mg of amino acid dissolved in 50 ml of 0.01 mol/L KOH. Injection volume of 5 µL.

** Not determined.

acids failed to separate. This result was further verified by injecting single amino acid standards of Trp, Phe, Des, and Ide, and determining their respective k' values. The k' values for Trp, Phe, and Des; or Trp, Phe, and Ide were sufficiently different such that they could be quantitated (Table 11). However, the k' values for Des and Ide were identical, making it impossible to accurately quantitate them in a mixture.

In an attempt to separate the desmosines, the pH of the mobile phase was lowered to 2.5, and again a series of amino acid standards were injected. With this modified system, the k' values of the amino acids increased from those obtained initially, and other amino acids were now resolvable (Tyr, Ala, Leu, or Ile). However, Des and Ide were still unresolvable, as was Leu and Ile (Table 11).

From these results, it was concluded that although Des and Ide could be separated from various other amino acids, it was not possible to separate the two utilizing either of the two isocratic elution schemes discribed. These results were also confirmed by Farris and co-workers, when they failed to separate Des form Ide utilizing a similar ion-exchange system [118].

Reverse Phase HPLC:

Since the desmosines were found to be unresolvable with either of the isocratic, ion-exchange systems described, another separation technique was investigated. Reverse phase HPLC encompasses 3 different methods of separation, all of which rely on the interaction between the organic characteristics of the analytes and the octadecyl (C-18) column.

The first method investigated utilized a system which would separate

the desmosines without any enhancement of their organic nature. This system consisted of a methanol/phosphoric acid/water (30/1/69, v/v) buffer, pH 5.85. The eluted amino acids were detected at either 205 or 254 nm. A representitive chromatogram of a standard containing Des, Phe, and Trp is shown in Fig. 11. With this system the 3 amino acids were found to elute in reverse order from the ion-exchange system (Des, Phe, and Trp), and with sufficiently different retention times to facilitate their quantitation. However, it was noted that Des eluted with the void volume of the column, and that when a mixture of Des and Ide was injected, they both eluted with the void volume. These results were indicative of no interaction between the desmosines and the C-18 column (Table 12).

Since the desmosines were eluting unresolved with the void volume of the column, it was attempted to increase their interaction with the column by lowering the methanol percentage of the mobile phase to 10%. This modification should allow for more hydrophobic interactions to occur between the desmosines and the column. It was found that while this modification of the mobile phase did increase the k' values of the other various amino acids, though not substantially, Des and Ide still eluted with the void volume (Table 12).

Reverse Phase-Ion Suppression HPLC Studies:

With the failure to resolve the desmosines with a lowering of the methanol percentage in the mobile phase, it was decided to decrease their polarity by partially suppressing their ionic nature. With this ion suppression, the desmosines should interact with the C-18 moieties of the column more effectively. Therefore, the pH of the mobile phase was

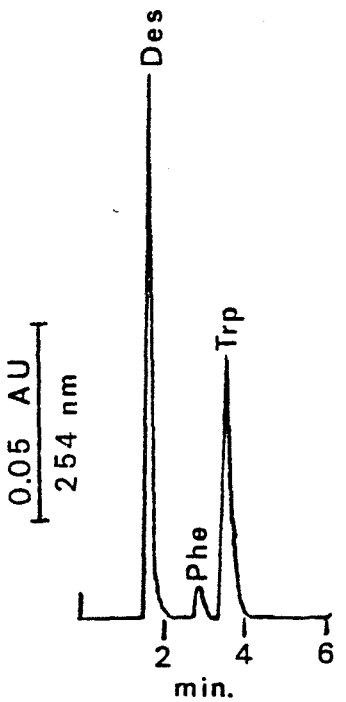


Fig. 11. Reverse phase HPLC separation of amino acid standard containing Trp, Phe, and Des. Standards eluted with a mobile phase consisting of methanol/water/phosphoric acid (30/69/1, v/v/v) and detected at 254 nm.

TABLE 12

K' VALUES OF VARIOUS AMINO ACIDS
ELUTED WITH 30 AND 10 % METHANOL
USING REVERSE PHASE HPLC

AMINO ACID*	30% K'	10% K'
DES	0.00	0.00
IDE	0.00	0.00
PHE	0.66	0.72
TRP	1.11	1.20

K' DETERMINED FOR VARIOUS AMINO ACIDS USING REVERSE PHASE HPLC ANALYSIS WITH A MOBILE PHASE OF METHANOL/PHOSPHORIC ACID/WATER (30/1/69 AND 10/1/89, v/v), pH 5.35, WITH A FLOW RATE OF 1 ML/MIN. AMINO ACIDS DETECTED AT 254 NM.

* TEN MG AMINO ACID DISSOLVED IN 50 ML OF WATER. INJECTION VOLUME OF 5 μL.

to 2.5, a pH at which several of the carboxyl functions on the desmosines would be protonated, thereby decreasing their polarity. To further enhance their interaction with the column, the methanol was entirely removed from the mobile phase.

Amino acid standards of Des, Ide, Phe, and Trp were then injected and eluted with this modified mobile phase of water/phosphoric acid (99/1, v/v), pH 2.5 (Table 13). It was found that, though the k' values of Phe and Trp did substantually increase, Des and Ide still eluted with the void volume of the column.

From the results of the reverse phase and ion-suppression studies, it was concluded that although the desmosines were found to separate from other various amino acids using these particular systems, the desmosines themselves were still unresolvable.

Reverse Phase with OPA Derivatization:

From the previous studies it was determined that the polarity of the desmosines would have to be further reduced in order to achieve any type of separation between the two. One method which should substantially decrease their polarity would be to chemically modify the molecule. This modification could be accomplished by derivatizing the desmosines with a fluorogenic compound, such as OPA. This derivatization would not only enhance their interaction with the C-18 moieties, but also aid in their detection by being fluorescent.

The OPA derivatized amino acids were initially eluted with a mobile phase consisting of 0.05 mol/L sodium phosphate buffer/methanol (50/50, v/v), pH 7.3, and detected by fluorescence emission at 405 nm. A repre-

TABLE 13

K' VALUES OF VARIOUS AMINO ACIDS
USING ION SUPPRESSION HPLC

AMINO ACID*	K'
DES	0.00
IDE	0.00
PHE	1.16
TRP	2.02

K' DETERMINED FOR THE VARIOUS AMINO ACIDS USING REVERSE PHASE-ION SUPPRESSION HPLC ANALYSIS, WITH A MOBILE PHASE OF WATER/PHOSPHORIC ACID (99/1, V/V), PH 2.5, AT A FLOW RATE OF 1.0 ML/MIN. THE AMINO ACIDS WERE DETECTED AT 254 NM.

* TEN MG AMINO ACID DISSOLVED IN 50 ML OF WATER. INJECTION VOLUME OF 5 μL.

sentative chromatogram of an amino acid standard is shown in Fig. 12.

It was found that several of the derivatized amino acids were resolvable from one another. However, upon injection of derivatized Des or Ide no peaks were detected. It was initially thought that since the desmosines contained 4 amino groups available for derivatization with OPA, they could be retained by the column longer than 30 min. Therefore, it was decided to increase the methanol percentage of the mobile phase to 70% in order to decrease their retention time.

Fig. 13 is a representative chromatogram of the same amino acid standard as previuosly injected, this time eluted with the modified mobile phase containing 70% methanol. With this modification it was found that all the derivatized amino acids eluted within 10 min, decreasing their retention times by approximately one third. Again, however, Des and Ide were not detected. Finally, the methanol percentage was increased to 90%, and the same amino acid standards injected. With this increase in the methanol percentage, it was found that all the amino acids eluted within 1 min, yet Des and Ide were still undetected.

After reviewing the results from the derivatization method, it was discovered that Des and Ide were eluting close to the void volume of the column, and appeared as multiple peaks on the chromatogram. Multiple peaks could result from the 4 amino groups having different reactivities with the OPA. Similar results have been reported for dibasic amino acids with OPA derivatization by Lindroth and Mopper [105].

As the desmosines were found to elute in several fractions with the OPA derivatization method, and as the desmosines were unresolvable using the other reverse phase methods described, it was decided to attempt

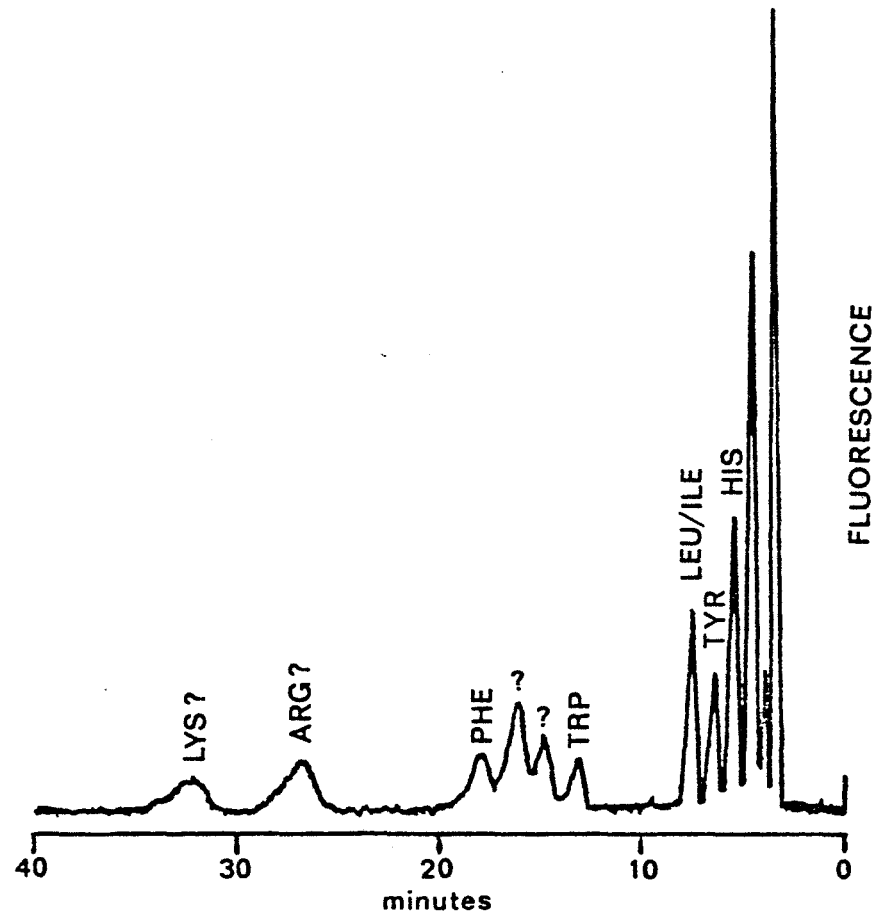


Fig. 12. Reverse phase HPLC separation of amino acid standard containing His, Tyr, Leu, Ile, Phe, Arg, Lys, Ide, and Des pre-derivatized with OPA. Standard eluted with 0.05 mol/L sodium phosphate buffer/methanol (50/50, v/v), pH 7.3, and detected by fluorescence (340 nm excitation; 450 nm emission).

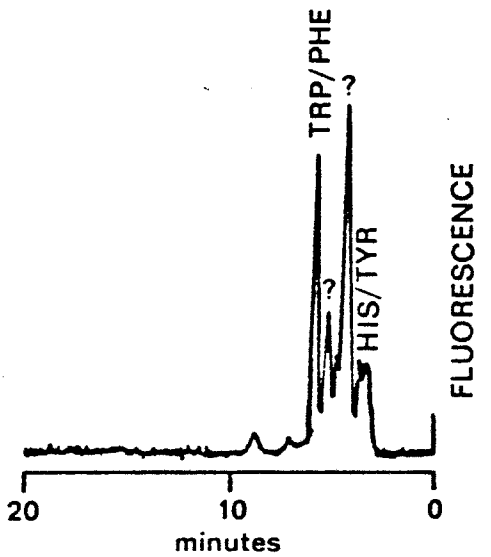


Fig. 13. Reverse phase HPLC separation of amino acid standard containing His, Tyr, Leu, Ile, Phe, Arg, Lys, Ide and Des pre-derivatized with OPA. Standard eluted with 0.05 mol/L sodium phosphate buffer/methanol (30/70, v/v), pH 7.3, and detected by fluorescence (340 nm excitation; 450 nm emission).

their separation by ion-paired HPLC.

Ion-Paired HPLC:

Ion-paired HPLC utilizes a counter ion to one of the ionic species present on the molecules to be separated [119]. It was decided to use a counter ion to protonated amino groups which could be generated on the desmosines. This would require the mobile phase to be acidic, an environment suitable for the C-18 column. The counter ion chosen for this method was the sodium salt of heptane sulfonic acid, due to its widespread use in ion-paired systems, and its general availability [119].

The concentration of the counter ion in most ion-paired systems vary from 0.005 mol/L to 0.02 mol/L. For this method of separation, knowing that hydrolysates were to be used, it was decided initially to use a concentration of 0.01 mol/L.

Thus the ion-paired system utilized a methanol/water mobile phase, containing 0.01 mol/L sodium heptane sulfonate at an acid pH. Initially, a pH of 3 was used because at this pH several of the amino groups of the desmosines were protonated. Also, at this pH, minimal deterioration of the column occurs (optimal pH range for C-18 columns is between 2.5 and 7.0) [12,119]. The eluted amino acids were detected at 205 nm, therefore, the pH of the mobile phase was adjusted with phosphoric acid [104].

With this system, it was found that many amino acids could be separated from one another, including the desmosines. It was also found that by varying the methanol percentage of the mobile phase, the retention times of the amino acids could be varied. With this knowledge, it was decided to use a methanol percentage which would yield minimum

retention times for the desmosines, yet allow their separation. Thus in these preliminary studies, the methanol percentage of the mobile phase was varied from 10 to 30%.

Initial attempts of the separation of the desmosines proved fruitful, in that it was found that the desmosines could be resolved using this ion-paired system. Finally, it was determined that by using a system consisting of methanol/water (26/74, v/v), pH 3.0, containing 0.01 mol/L heptane sulfonic acid, baseline resolution of the two desmosines could be achieved. With this method, Ide and Des were found to elute at 57 and 62 min, respectively, having k' values of 50.80 and 57.33.

Once it was determined that the desmosines could be resolved, other amino acids were investigated to determine if they would interfere. These amino acids were then injected and their k' values determined (Table 14). It was found that from this series of 25 amino acids, all of which could possibly be present in the hydrolysates to be analyzed, none eluted close to either Ide or Des, the closest being Trp with a k' value of 22.00. Tryptophan, however, should be destroyed in the acid hydrolysis procedure, thereby separating the desmosines from the other amino acids by approximately 30 min.

Once it was determined that the method would reproducibly separate the desmosines from the other amino acids, the various chromatographic constants (k', α, R_s, and N) of the method were investigated (Table 15). As previously described in Chapter 2, the capacity factor, k', is a measure of the retention of a particular solute by the system. Though usually, in the interest of time, the k' values are kept below 10, in the case of the desmosines, the k' values were approximately 5 times

TABLE 14

K' VALUES FOR VARIOUS AMINO ACIDS USING ION-PAIRED HPLC

AMINO ACID	K'
Hydroxyproline	0.56
Proline	0.76
Cysteine	0.81
Glycine	1.10
Alanine	1.12
Glutamic Acid	1.25
Glutamine	1.30
Serine	1.30
Threonine	1.35
Aspartic Acid	1.42
Asparagine	1.44
Valine	3.40
Ornithine	5.96
Hydroxylysine	6.36
Lysine	6.92
Tyrosine	7.25
Histidine	7.47
Isoleucine	7.60
Leucine	8.80
Arginine	8.85
Norleucine	9.00
Phenylalanine	9.80
Tryptophan	22.00
Isodesmosine	50.80
Desmosine	57.33

k' values of various amino acid standards, calculated as described in Chapter 2. Amino acids eluted with methanol/water (26/74, v/v), pH 3.0, containing 0.01 mol/L heptane sulfonic acid, and detected at 205 nm.

TABLE 15

CHROMATOGRAPHIC CONSTANTS FOR DESMOSINE AND ISODESMOSINE SEPARATION USING ION-PAIRED HPLC

K'		α	R_S	N	
IDE	DES			IDE (HETP)*	DES (HETP)
50.80	57.32	1.13	4.80	29,026 (0.001)	25,600 (0.001)

VARIOUS CHROMATOGRAPHIC CONSTANTS DETERMINED FROM DES AND IDE STANDARDS, ELUTED WITH A MOBILE PHASE OF METHANOL/WATER (26/74, V/V), PH 3.0, WITH A FLOW RATE OF 1.0 ML/MIN.

AMINO ACID DETECTION AT 205 NM.

CONSTANTS CALCULATED AS DESCRIBED IN CHAPTER 2.

* HEIGHT EQUIVALENT TO THEORETICAL PLATES.

that value. In an attempt to lower the k' values for the desmosines either by increasing the methanol percentage in the mobile phase, or by adjusting the pH, the resolution of the two amino acids decreased. Therefore, it was decided not to adjust the k' values.

Although the k' values of Des and Ide were higher than usual, the separation factor, α, was found to be in an acceptable range. This factor, as described in Chapter 2, is actually a measure of the relative retention of Des and Ide, and is related to the chemical parameters of the method. By definition, this factor should be greater than 1.00, and since the factor utilizing this method of separation was greater than 1.3, modifications were not necessary [119].

The efficiency (N) of the C-18 column to separate the desmosines was found to be excellent, both having theoretical plate values exceeding 25,000. Though these values are somewhat higher than those usually encountered in an HPLC method, this can be explained by the long retention times of the desmosines and their relatively narrow peak width on elution [119].

Another measure of the efficiency of the column to separate the analytes is the height equivalent to theoretical plates (HETP), sometimes abbreviated H [119]. This value is simply the column length in cm divided by the efficiency factor, N. For this constant, the better the efficiency, the lower the H value, just opposite to the definition for the N factor.

Finally, the ability of this method to resolve the desmosines was determined. The factor, R_s, should be greater than 1 for quantitation, and greater than 1.5 for baseline resolution [119]. As described in

Chapter 2, this factor takes into account all the other calculated constants determined for that specific system. Thus, R_s is a measure of the selectivity of the column for the analytes (as determined by the a factor); secondly, it takes into account the efficiency of the column for the analytes (as determined by the N factor); and thirdly, it takes into account the capacity of the system for the analytes (as determined by k_1').

Utilizing this method for the separation of the desmosines, the R_s value was in excess of 4, indicating, for the amount of time on the column, the separation of the two eluted amino acids approached the baseline. Thus, the desmosines should be able to be quantitated by this method with no interference from the other amino acids, or themselves.

Method Parameters:

As previously stated, α is a term which is dependent on the chemical interactions of the analytes with the system. Therefore, it was decided to investigate several of the chemical parameters of the method, and determine their effects on the separation of the desmosines. The first parameter investigated was the effect of the methanol percentage of the mobile phase on the separation of the desmosines. In the initial development of the method, the methanol percentage was varied to achieve maximum separation of the desmosines. Qualitatively, as the methanol percentage increased, the retention times of the desmosines decreased, as did their resolution. Thus as the methanol increased, N, α, and R_s decreased. It was found that above a methanol percentage of 30, the efficiency (N) decreased drastically as did the separation factor, α, which

decreased to 1. The R_s was also found to decrease from 4.8 with 26% methanol to less than 1 with a percentage above 36%. In all cases, the methanol percentage yielding maximal chromatographic characteristics was determined to be between 25 and 28%.

It was also found that as the column aged, the retention times of the desmosines decreased. In order to offset this decrease, the concentration of the methanol was lowered. The decrease in the retention time by the column is believed due to deterioration of the column with usage [119]. Finally, it was noted that as the methanol percentage increased, the column head pressure also increased.

The next chemical parameter investigated was the effect of the mobile phase pH on the separation of the desmosines. From these studies, it was determined that as the pH increased above 3.0, the k' values of the amino acids decreased (Fig. 14). It was noted that the desmosines could effectively be separated up to a pH of approximately 3.2; however, several of the other amino acids could not be resolved at this pH. It was also found that if the pH was decreased below 3.0, no substantial effects were observed. Therefore, it was decided to maintain the pH at 3.0.

From these studies it was determined that the pH decreased the value of N, α, and R_s constants, similar to increasing the methanol concentration. These interactions are believed due to the amount of ionization of the amine groups on the amino acids with the sulfonic acid residues of the counter ion [119].

Finally, the effect of the counter ion concentration was investigated. With ion-paired systems, the solvent strength can be varied by

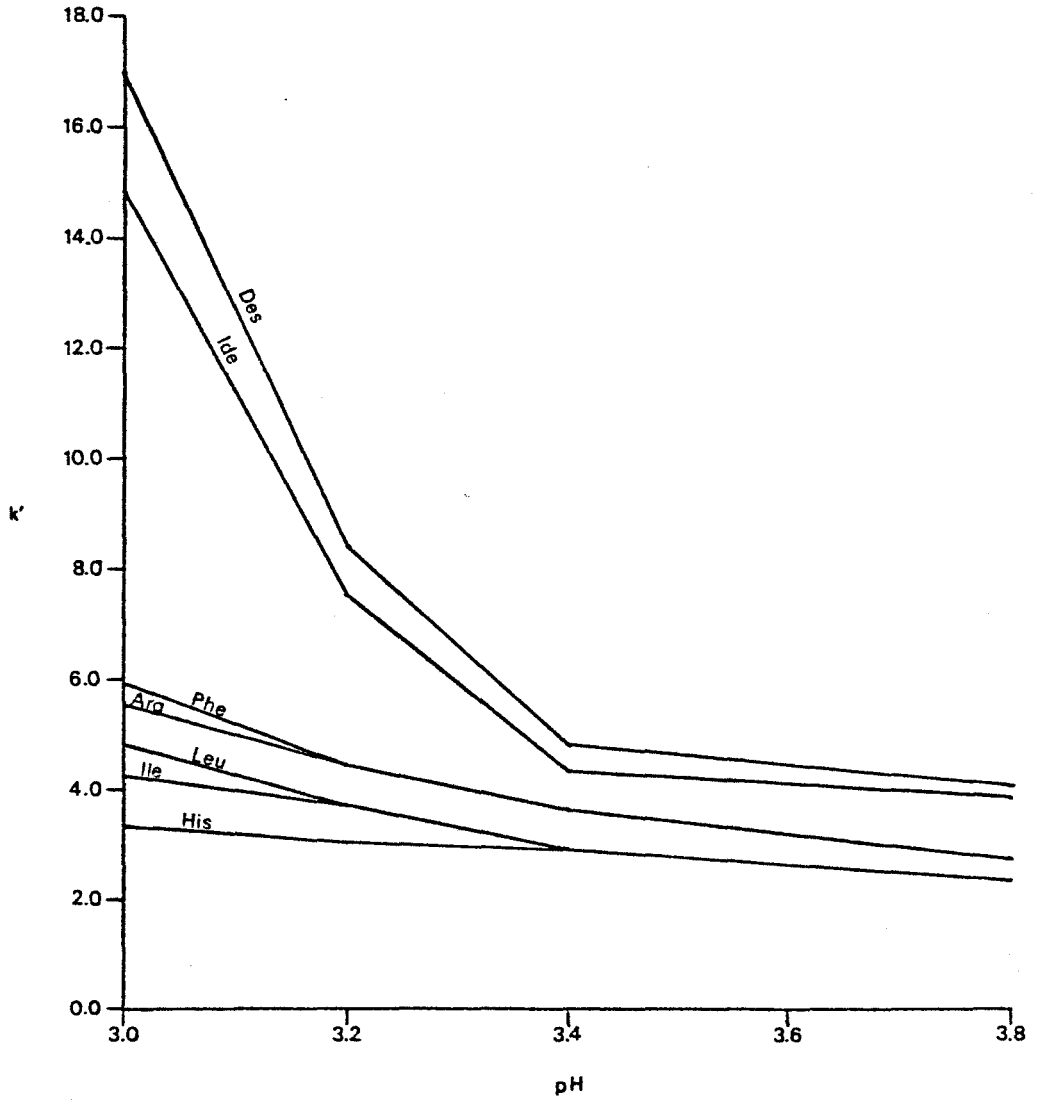


Fig. 14. Effect of ion-paired mobile phase pH on k' values of several amino acid standards. Mobile phase consisted of methanol/water (26/74, v/v), containing 0.01 mol/L heptane sulfonic acid, with the pH varied from 3.0 to 3.8.

changing the counter ion concentration [119]. Thus, by increasing the concentration of the counter ion in the mobile phase, the k' values of the analytes can be increased, if the analyte is not fully ionized [119]. Therefore, a change would be expected in α. If however, the analyte is fully ionized, little or no change should be observed in α.

When the heptane sulfonic acid concentration was increased to 0.02 mol/L, a very small increase was noticed in the k' values of the desmosines. However, no change was observed in the α factor. If the concentration was decreased, both Des and Ide k' values were found to decrease, substantually decreasing α. Therefore, for this method it was decided to maintain the heptane sulfonic acid concentration at 0.01 mol/L.

Quantitation of the Desmosines:

Once it was determined that the method would reproducibly separate the desmosines from one another and other amino acids, their quantitation was attempted. In order to access their ability to be quantitated, a standard curve was constructed, as described in Chapter 3. Essentially, various concentrations of Des and Ide standards were injected separately and together, such that any deviations, with their simultaneous quantitation, would be detected.

Desmosine and Ide were found to display a linear response to the various concentrations injected, both singly and together, when detected at 205 nm (Figs. 15, 16; circles). Upon analysis of the curve, Des and Ide yielded almost identical equations of the lines ($y = 68.28x + 0.04$ for Des, and $y = 64.55x - 0.07$ for Ide). Not only did both amino acids display similar slopes and y-intercepts, they were found to display

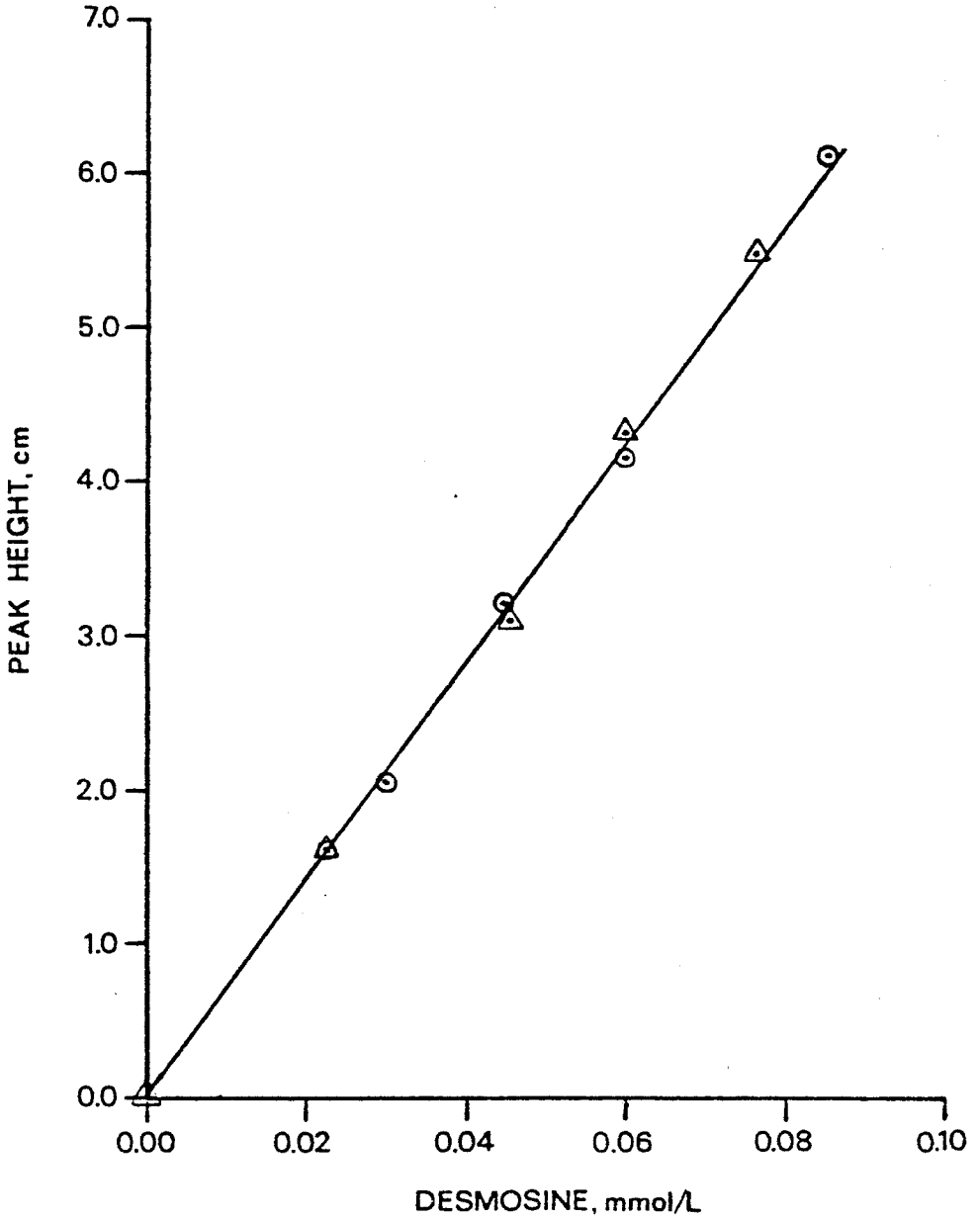


Fig. 15. Standard curve (⊙) and recovery from hydrolyzed BSA (△) of desmosine using the ion-paired HPLC method.
(⊙) Known concentrations of purified desmosine standards injected and peak heights determined.
(△) Known concentrations of purified desmosine standards added to BSA prior to hydrolysis. Recovery peak heights compared to standard curve peak heights.

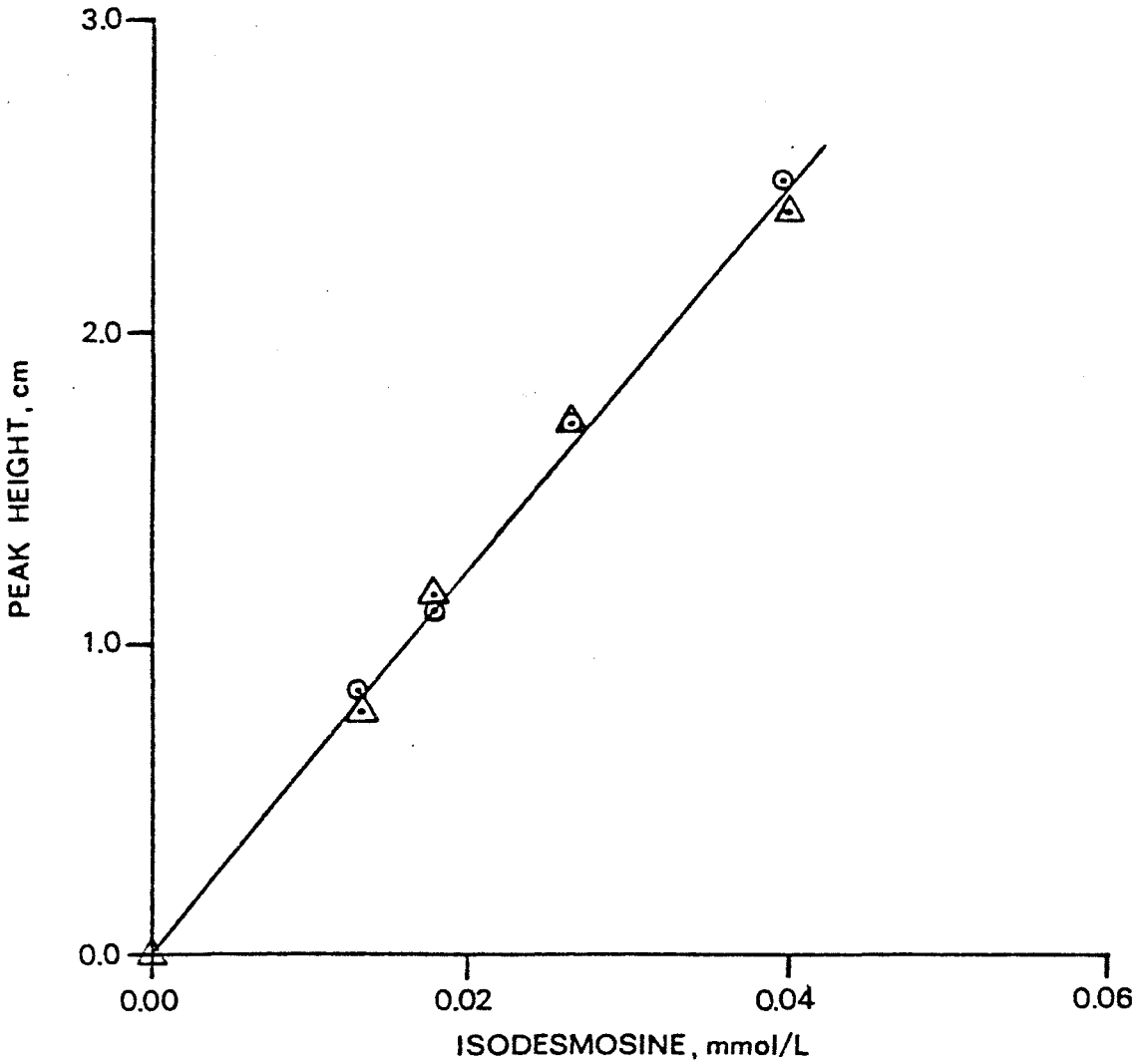


Fig. 16. Standard curve (⊙) and recovery from hydrolyzed BSA (△) of isodesmosine using the ion-paired HPLC method.
(⊙) Known concentrations of purified isodesmosine standard injected and peak heights determined.
(△) Known concentrations of purified isodesmosine standards added to BSA prior to hydrolysis. Recovery peak heights compared to standard curve peak heights.

excellent correlation between their respective concentrations and peak heights (r greater than 0.99 for both).

Once it was determined that the desmosines could be quantitated at 205 nm, various other wavelengths were used in their detection. Again, the peak height was used as the measure of quantitation. Initailly, the wavelength was decreased in order to increase the sensitivity of the detection. However, it was found that as the wavelength decreased the baseline absorbance increased, such that the desmosines were undetectable. This effect was due to the methanol in the mobile phase, as methanol has been shown to have an absorbance cutoff at 205 nm.

When the wavelength was increased, the sensitivity for the detection of the desmosines decreased. While this decrease was not substantial (approximately 10% at 215 nm), it was considered significant. Therefore, the desmosines were detected at 205 nm, as previously described.

From these studies it was concluded that not only would this system separate the desmosines, but would simultaneously quantitate Des and Ide.

Once it was determined that the desmosines could be quantitated, the reproducibility of the method was investigated. For this precision study,standards of Des and Ide (0.0851 and 0.0569 mmol/L, respectively) were injected according to two schedules. For the intra-day precision, Des and Ide were injected 10 times daily, while for the inter-day precision, Des and Ide were injected once daily for 10 days. In all cases, the peak heights were determined and used in the calculations.

It was found that the standard deviations (S.D.) and the coefficients of variation (C.V.) were less than 0.2 cm and 3%, respectively, for both the intra- and inter-day precision studies (Table 16). It was

TABLE 16

INTRA- AND INTER-DAY PRECISION STUDIES
FOR DESMOSINE AND ISODESMOSINE DETERMINATIONS
BY ION-PAIRED HPLC
N=10

	INTRA-DAY		INTER-DAY	
	DES	IDE	DES	IDE
PEAK HEIGHT (cm)	7.99	3.98	7.96	3.79
S.D. (cm)	0.07	0.06	0.14	0.09
C.V. (%)	0.93	1.58	1.69	2.39

Desmosine and isodesmosine standards (0.0851 and 0.0569 mmol/L injected 10 times per day for intra-day precision, and once daily for 10 days for the inter-day precision determinations.

also noted that the S.D. and the C.V. of the intra-day determinations were approximately one-half the inter-day determinations. This result is to be expected, as the within day precision is almost always greater than the between day precision [120].

A C.V. of less than 5% is usually acceptable for analytical determinations [120]. Therefore, with this method displaying a low C.V. for both the desmosines (less than 3%), it was concluded that it was well within the acceptable limit of reproducibility for an analytical method.

Recovery Studies:

The final analytical parameter investigated was the percent recovery of Des and Ide from various samples, to demonstrate the accuracy of the method. The first study utilized BSA, a protein which contains no desmosines. To this protein, Des and Ide were added, prehydrolysis, and the samples prepared as described in Chapter 3.

Following the analysis of the hydrolysates, the Des and Ide concentrations were determined, and compared to their standard curves. Both Des and Ide were well within the peak heights for their respective spikes (Figs. 15, 16; triangles). The slope and y-intercept for both the Des and Ide spikes ($y = 71.48x - 0.02$ and $y = 60.56x + 0.02$, respectively), correlated well with the slopes and y-intercepts of the standard curves for Des and Ide ($r = 0.99$). The range of recovery for the Des spikes were between 98 and 102%, with an average recovery from the spiked BSA of 99.6%. The average recovery of Ide from spiked BSA was 98.5%, with a recovery range from 96 to 102%.

Also, from this initial recovery study, the lower limit of detection

for either Des or Ide was approximately 100 pmol. Below this level, the desmosine peak was indistinguishable from the baseline noise of the detector.

Following the determination of the recoveries from a sample which contained no desmosines, a Des and Ide spike was added to a sample known to contain both the amino acids. For these studies, a canine aorta sample was obtained and prepared as described in Chapter 3. Again, the Des and Ide was added prior to the hydrolysis procedure. In this study, one sample of the aorta remained unspiked, while the remaining 5 samples were spiked with both Des and Ide.

Upon analysis and determinations of the Des and Ide concentrations of the unspiked and spiked samples, the recoveries were calculated, and the observed values (ion-paired HPLC determinations) vs. the predicted values (calculated from the amount of the spike) were plotted. The recoveries for both Des and Ide were found to be excellent. The recovery range for Des was between 95 and 100%, with an average recovery of 97.2%. The recovery range for Ide was determined to be between 96 and 102%, with an average recovery of 99%.

When the observed concentrations were compared to the predicted concentrations, both Des and Ide were found to display a linear relationship (Figs. 17, 18). Upon statistical analysis of the recoveries, both Des and Ide displayed similar slopes and y-intercepts ($y = 0.99x + 0.002$ for Des, and $y = 0.95x + 0.003$ for Ide), and were found to correlate well with their respective predicted values (r greater than 0.97 for both amino acids).

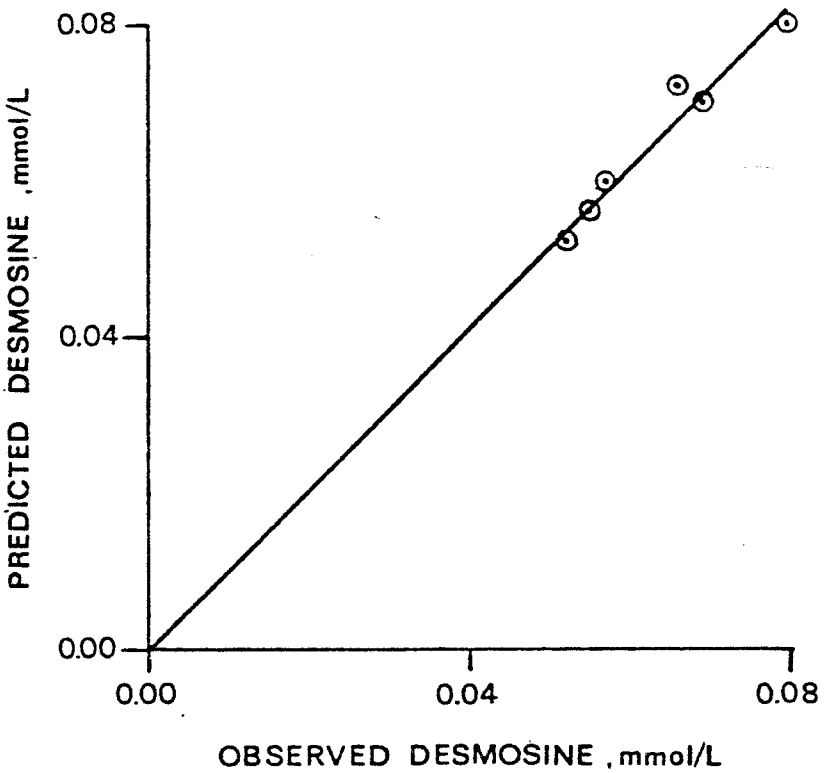


Fig. 17. Recovery of purified desmosine from hydrolyzed canine aorta as described in Chapter 3. Recovered (observed) concentrations plotted against the predicted concentrations.

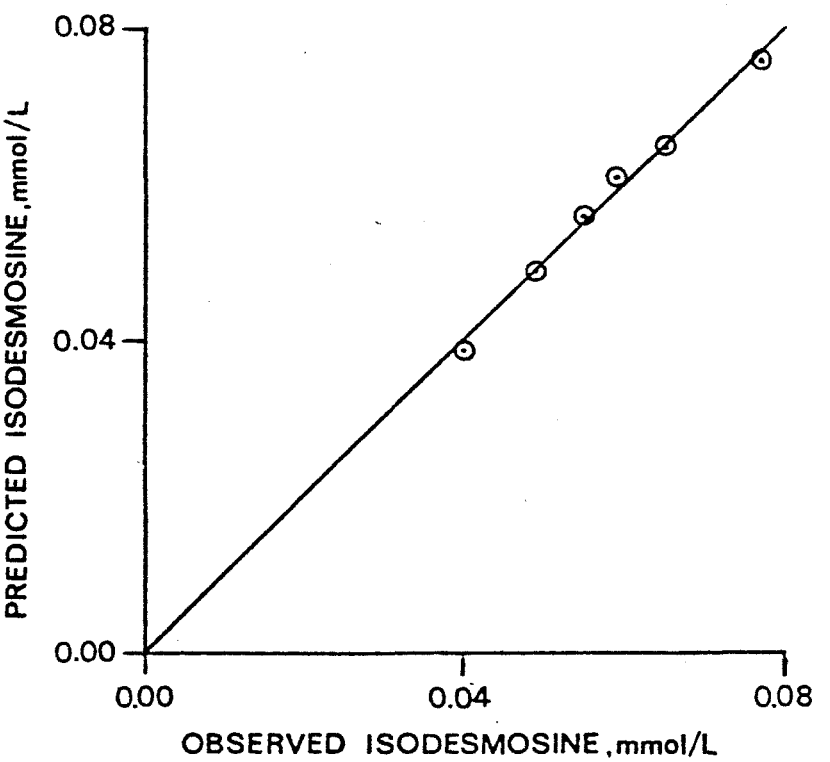


Fig. 18. Recovery of purified isodesmosine from hydrolyzed canine aorta as described in Chapter 3. Recovered (observed) concentrations plotted against the predicted concentrations.

Canine Aorta Studies:

From all the previous analytical studies, the method was determined to be reproducible, accurate and precise for the separation and quantitation of the desmosines [119,120]. This having been established, it was decided to attempt to quantitate the desmosines, using tissue samples. The tissue to be investigated was canine aorta (aortic arch region). This tissue was chosen because the concentration of both the desmosines, as well as the elastin content, is well documented [101,121-123]. In this study, the Des and Ide concentrations of the aorta samples, determined by ion-paired HPLC, were compared to those concentrations obtained by automated amino acid analysis (AAA). Finally , the elastin content of the canine aortas was estimated from the Des and Ide concentrations of the samples, and compared to the elastin content as determined by gravimetic procedure.

Thirteen canine aortas were obtained and prepared as described in Chapter 3. Of the prepared, diluted hydrolysates, 10 µL of each was injected, and the desmosines quantitated as described by the ion-paired method. The desmosines were found to elute in a manner similar to the Ide and Des standards, with retention times of 52 and 57 min, respectively (Fig. 19). The other amino acids present in the hydrolysates were also found to elute similar to their respective standards. In all the chromatograms of the aorta samples, one prominent peak was observed to elute between Phe and Ide, with a k' value different from any investigated amino acid (31.50). This peak was thought to be lysinonorleucine, a minor cross-link found in elastin (see Chapter 1).

The concentrations of Des and Ide were then calculated from their

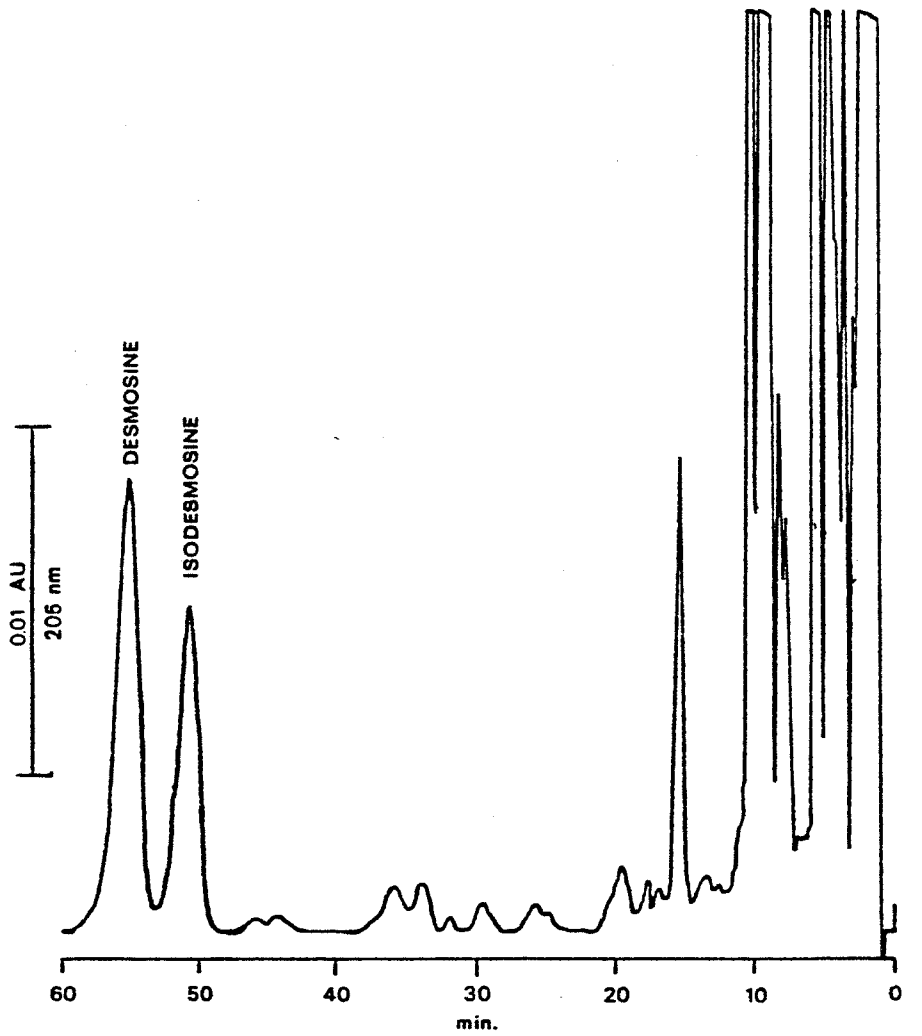


Fig. 19. Representative ion-paired HPLC chromatogram of hydrolyzed canine aorta. Hydrolysate eluted with methanol/water (26/74, v/v) mobile phase, containing 0.01 mol/L heptane sulfonic acid, pH 3.0. Detection at 205 nm.

respective standard curves, for each of the 13 canine tissue samples (Table 17). In a similar fashion, the Des and Ide concentrations of each of the 13 samples were determined by AAA, as previously described in Chapter 3 (Table 17).

Once the Des and Ide concentrations were determined by both methods, the results were compared. Both Des and Ide were found to correlate well with the AAA, both having a linear relationship, with slopes of approximately one with small intercepts, and with correlation coefficients of greater than 0.97 (Figs. 20, 21). These results indicated that no significant difference existed between the two methods of determination for Des and Ide ($p<0.001$).

From this study it was concluded that Des and Ide concentrations could be determined from tissue samples using the ion-paired HPLC method, with no significant difference between the results obtained by ion-paired HPLC, and those obtained from the accepted method of AAA [124-126].

Estimation of Elastin and Collagen Content of the Aorta:

The elastin content of the aorta was estimated from the Des and Ide concentrations [39,121,122]. For this estimation, it was initially necessary to determine the percentage of the total desmosines (Des plus Ide) in elastin. In order to determine this percentage, the amino acid analyses of purified elastins from human and canine aorta and lung were obtained from several investigators [101,121-123]. Lung elastin was included for several reasons: one, it has been shown that the amino acid content of purified elastin from aorta and lung are very similar; and secondly, the majority of recent publications on the purification and amino acid

TABLE 17

DESMOSINE AND ISODESMOSINE CONCENTRATIONS
AS DETERMINED BY HPLC AND AUTOMATED
AMINO ACID ANALYSIS

DOG	AUTOMATED AMINO ACID ANALYSIS		HPLC	
	DES (MMOL/L)	IDE	DES (MMOL/L)	IDE
1	0.406	0.305	0.441	0.295
3	0.204	0.165	0.257	0.184
5	0.123	0.097	0.117	0.103
6	0.474	0.367	0.446	0.360
7	0.310	0.256	0.280	0.285
8	0.318	0.254	0.301	0.293
9	0.348	0.295	0.341	0.306
10	0.482	0.346	0.510	0.361
11	0.267	0.256	0.289	0.271
12	0.452	0.360	0.458	0.344
13	0.338	0.290	0.360	0.312
14	0.328	0.276	0.363	0.297
15	0.516	0.436	0.565	0.473

DESMOSINE AND ISODESMOSINE CONCENTRATIONS OF 13 CANINE AORTA SAMPLES PREPARED AS DESCRIBED IN CHAPTER 3.

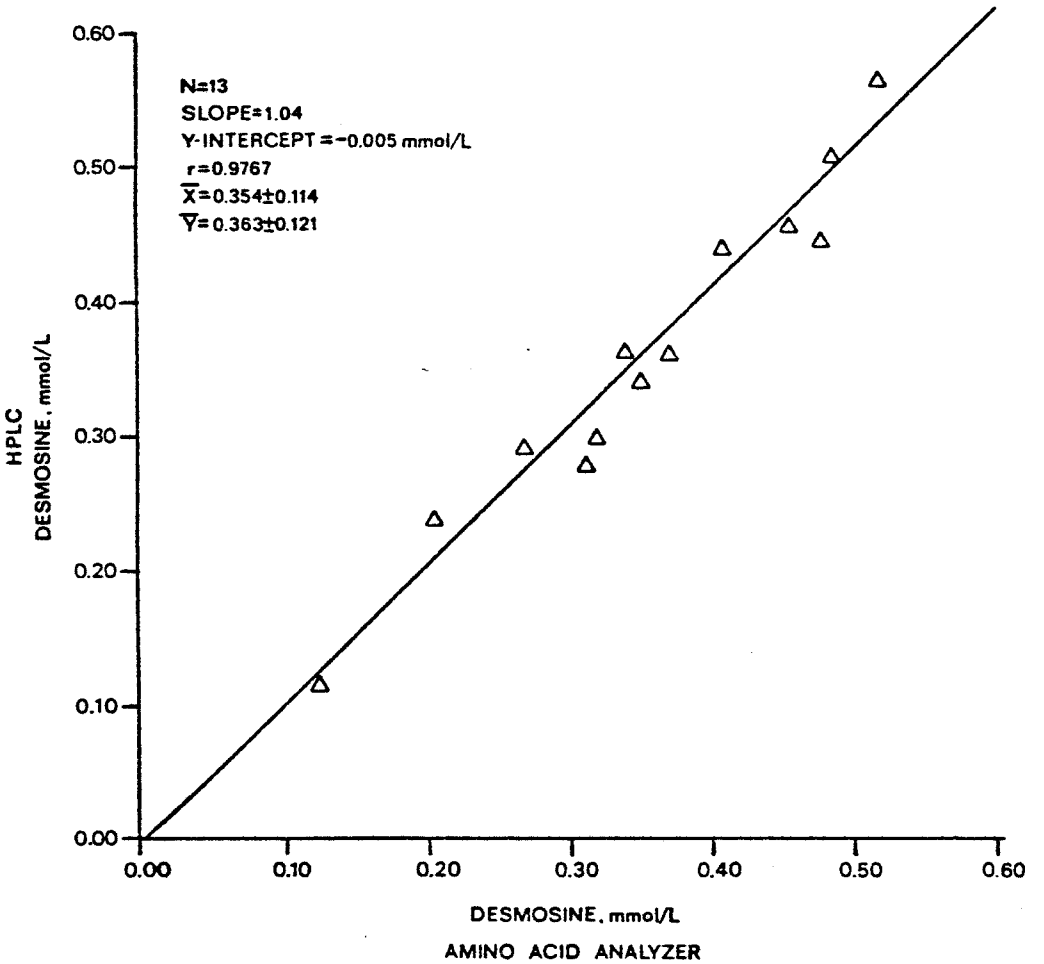


Fig. 20. Regression analysis of desmosine concentrations from 13 hydrolyzed canine aortas as determined by AAA and ion-paired HPLC.

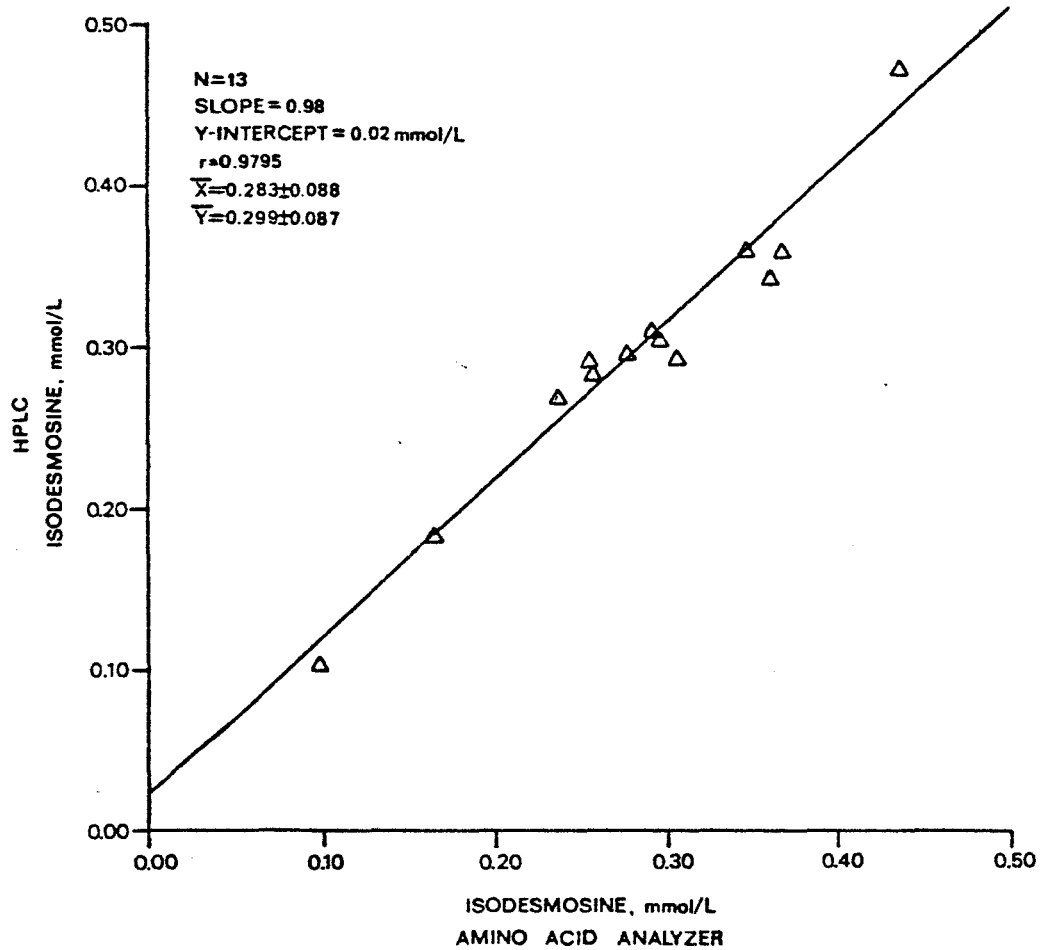


Fig. 21. Regression analysis of isodesmosine concentrations of 13 hydrolyzed canine aortas as determined by AAA and ion-paired HPLC.

content of elastin has utilized lung tissue [101,121-123].

Upon examination of the amino acid content of these elastin preparations, it was found that there are approximately 2-4 total desmosine residues per 1000 residues. Thus in order to determine the percent total desmosines in elastin, the molecular weight (MW) contribution of the total desmosines was divided by the total MW of the 1000 residues of elastin and then multiplyed by 100.

From these determinations, it was found that approximately 1.3% of elastin is total desmosines, or for every mg elastin, 13 μg is contributed by the desmosines. To further substantiate the use of the purified lung elastin in the percentage determination, when the percent desmosines were calculated in the lung preparation only (not incorporating any data from the aorta), the percent was again found to be 1.3.

Thus for the elastin calculations, the Des and Ide concentrations were first converted from mmol/L to nmol total desmosines/mg aorta FFDW. The nmol total desmosines were converted to μg total desmosines by the MW of the desmosines, yielding a μg total desmosines/mg aorta FFDW. The elastin content was finally arrived at by using the conversion factor of 13 μg total desmosines per mg elastin.

These calculations were performed on the Des and Ide results, obtained by ion-paired HPLC and AAA, from the 13 canine aorta samples (Table 18). Upon statistical analysis, both methods were found to be in good agreement with one another, displaying a linear relationship (Fig. 22), with a slope of approximately one ($y = 0.91x + 53$), and an r value of 0.9326, indicating excellent correlation between the two methods ($p<0.001$). Also, no significant difference was found between the mean

TABLE 18

ELASTIN AND COLLAGEN CONTENT OF CANINE AORTA

DOG	% ELASTIN		% COLLAGEN		% CTP**	
	AAA	HPLC	UNCORR.*	CORR.*	UNCORR.*	CORR.*
1	38.9	40.3	28.6	22.5	68.0	62.8
2	30.9	37.1	46.9	41.3	84.0	78.4
5	25.5	25.5	52.0	48.1	77.5	73.6
6	43.8	42.0	30.5	24.1	72.5	66.1
7	38.5	38.5	27.4	20.7	65.9	59.2
8	41.0	42.5	24.9	18.4	67.4	60.9
9	44.8	45.0	21.2	14.3	66.2	59.3
10	41.2	43.5	27.2	25.0	70.7	64.0
11	31.3	34.9	30.4	25.0	65.3	59.9
12	45.2	44.6	28.8	22.0	73.4	64.6
13	42.5	45.4	26.9	20.0	72.3	65.4
14	37.8	41.2	23.6	17.3	64.8	58.5
15	46.2	50.9	29.0	21.3	79.9	72.2

ELASTIN AND COLLAGEN CONTENT (UNCORRECTED AND CORRECTED) CALCULATED AS DESCRIBED IN CHAPTER 4.

* UNCORR = UNCORRECTED; CORR. = CORRECTED

** % CTP = % CONNECTIVE TISSUE PROTEIN, AS DETERMINED BY PERCENT ELASTIN PLUS COLLAGEN.

ELASTIN CONTENT DETERMINED FROM DES AND IDE QUANTITATION BY ION-PAIRED HPLC

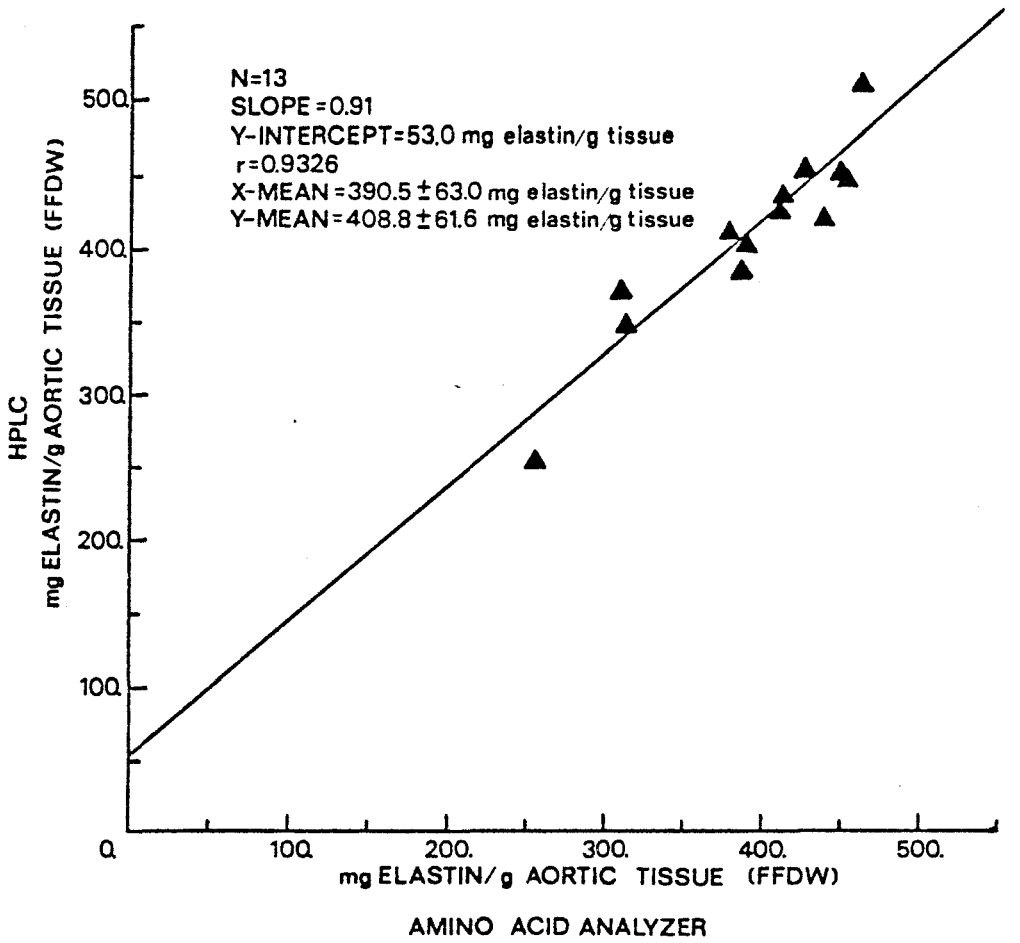


Fig. 22. Regression analysis of elastin content of 13 canine aortas, calculated from Des and Ide concentrations as determined by AAA and ion-paired HPLC.

elastin content obtained from AAA (390.5 ± 63 mg elastin/g aorta FFDW) and the mean elastin content as determined by ion-paired HPLC (408.8 ± 62 mg elastin/g aorta FFDW).

Once the elastin content had been calculated from the Des and Ide concentrations, it was decided to compare these results to the elastin content of the same canine aorta samples as determined by a gravimetric procedure. This method of elastin determination relied on the insolubility of the protein through several extractions including; a 1% sodium chloride extraction, followed by several autoclavings at 121 ^{o}C and 18 psig, until the resulting supernatant was devoid of protein, and finally, the remaining residue was extracted with hot (100 ^{o}C) 0.1 mol/L sodium hydroxide to remove any remaining non-elastin proteins. The residue remaining following the alkali extraction was considered elastin [27,29].

This procedure was performed on 5 canine aorta samples, and the elastin content as determined by both methods (gravimetric and calculated) were compared. The average elastin content obtained by the calculation method (408.7 ± 61 mg elastin/g aorta FFDW) was found to be somewhat higher than the elastin content as determined by the gravimetric procedure (325.1 ± 27.5 mg elastin/g aorta FFDW), though no significant difference between the two methods was noted.

From this study it was determined that no significant difference existed between the gravimatric and calculation procedures for the estimation of the elastin content of the canine aorta. This finding, however, may be biased by one result which yielded the same elastin content by both procedures. If this result was not included, a significant increase was observed in the elastin content when determined by HPLC.

Aorta Collagen Determinations:

Though elastin has been shown to be a major component of the aorta, another major connective tissue protein, collagen, is also present. Therefore, in order to determine the total connective tissue protein content of the aorta, the collagen content of the aorta samples was determined. Collagen content is frequently estimated by the hydroxyproline (Hyp) content of the tissue, in a manner similar to the use of Des and Ide for the estimation of the elastin content [40]. Therefore, in order to determine the collagen content, the percent Hyp in collagen was determined. This determination was based on the amino acid analyses of various purified collagen preparations [127-129]. It was found that there were between 88 and 95 Hyp residues per 1000 residues of collagen. From this, the percent Hyp in collagen was calclated, by the previously described methods, to be 11.1%. Therefore, for every mg collagen, 111 µg is contributed by Hyp.

Using this Hyp percentage, the collagen content of the 13 canine aorta samples were determined as described in Chapter 3 (Table 18). It was found that the collagen content of the aortic samples varied from 20 to 50%, with an average collagen content of 305.7 ± 110.6 mg collagen per g aorta FFDW. The total connective tissue protein content of the aorta samples were found to vary from 60 to 80% of the total weight, with an average of 700.0 mg total connective tissue protein/g aorta FFDW. During the course of this study it was noted that generally, as the elastin content increased, the collagen content decreased.

As previously mentioned, the collagen content is derived from the quantitation of Hyp. However, other proteins, specifically elastin, have

been shown to contain Hyp [101,121-123]. Since it has been shown that the aorta contains a significant amount of elastin (approximately 40%), the Hyp from elastin is being included in the total amount of Hyp in the tissue. Thus, non-collagen Hyp would tend to elevate the collagen levels in tissues which are rich in elastin.

In order to determine if this apparent increase in the collagen content would be significant, the Hyp component of elastin was calculated by determining the percent Hyp in purified preparations of elastin. It was found that elastin contained approximately 1.7% by weight Hyp [101, 121-123]. This non-collagen Hyp was then subtracted from the total Hyp and the collagen content was recalculated, to obtain a "corrected" collagen content of the canine aortas (Table 18). Upon analysis of the "uncorrected" and "corrected" collagen content, it was found that the difference between the uncorrected and corrected values was significant ($p<0.01$), and that the corrected values were approximately 10% less than the uncorrected values. It was also noted that the largest apparent decrease in the collagen content occurred in samples which contained high percentages of elastin.

As previously stated, it was found that as the elastin content of the aorta increased, the collagen content was found to decrease. When the various elastin and collagen percentages of the 13 canine aortas were plotted, an inverse relationship was, in fact, found to exist (Fig. 23). This relationship may be biased by the inclusion of two samples which were found to be high in collagen and low in elastin (Dogs 2 and 5). If these two samples were excluded from the population no relationship could be inferred. However, no justification existed for their exclusion,

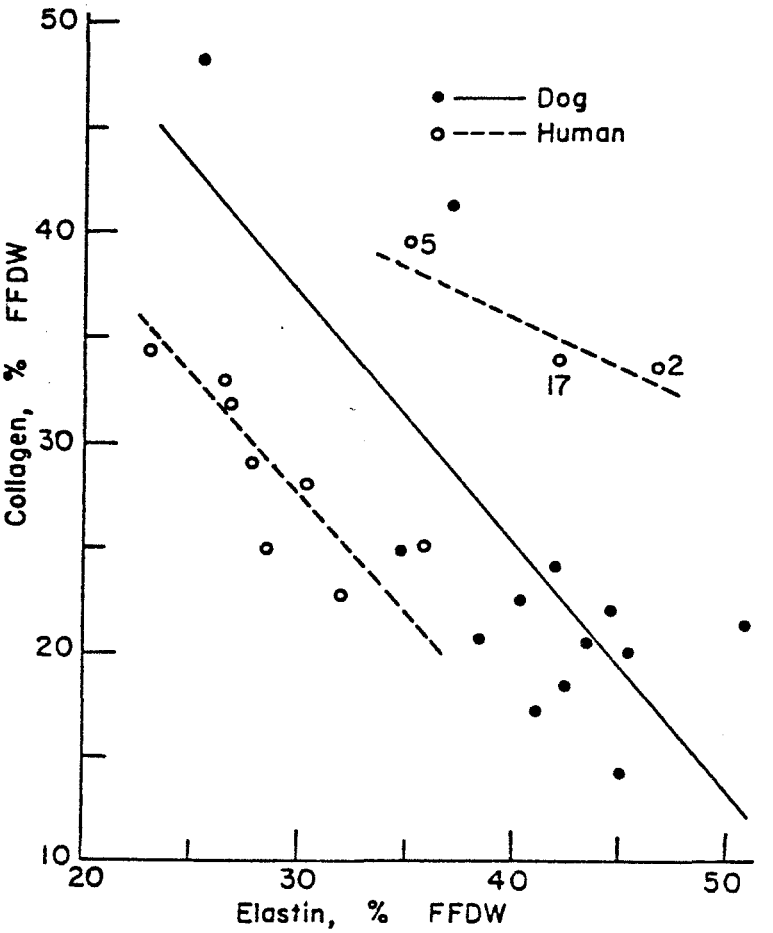


Fig. 23. Relationship between elastin and collagen (corrected) content for canine and human aortic tissues. Elastin calculated from Des and Ide concentrations as determined by ion-paired HPLC. Collagen content calculated from corrected Hyp concentrations.

therefore, they were included in the population.

Effect of Age on the Elastin and Collagen Content of Human Aortas:

Having determined the validity of both the ion-paired HPLC method for the quantitation of the desmosines, and their subsequent use for the estimation of the elastin content, an investigation of the effect of age on both the elastin and collagen content of human aortic arch tissues was undertaken. For this study, samples of human aorta (aortic arch region) were obtained from eleven post-mortem examinations at Loyola University Medical Center. Only tissue that on gross examination by a pathologist appeared free of pathological processes were used in this study. The age and sex of each subject from whom the tissues were obtained were noted (Table 19). The all Caucasian population included 5 females and 6 males, ranging in ages from 2 to 90 years.

Following the tissue preparation (as described in Chapter 3), the Des and Ide concentrations were determined by both the ion-paired HPLC method, and by AAA (Table 20). It was noted that the ion-paired HPLC chromatograms obtained from the human aorta hydrolysates were very similar to the chromatograms obtained from the canine hydrolysates, with Ide and Des eluting at 59 and 66 min, respectively (Fig. 24).

Following the quantitation of the desmosines in the human aorta hydrolysates, the percent elastin in each was estimated as previously described in the canine aorta studies (Table 21). From these calculations, it was found that the results obtained from the ion-paired HPLC method again correlated well ($r = 0.96$) with the results obtained via the AAA, yielding a linear relationship between the two methods ($y = 1.00x +$

TABLE 19

DEMOGRAPHICS OF HUMAN SAMPLES

SAMPLE	LAB # *	SEX	AGE (YRS)
1	81A-21	M	2
2	81A-20	F	5
3	81A-22	M	17
4	81A-30	F	34
5	81A-31	M	58
6	81A-19	F	66
7	81A-33	M	66
8	81A-32	F	67
9	81A-18	M	70
10	81A-34	M	70
11	81A-17	F	90

AGE AND SEX INFORMATION OF SAMPLES OBTAINED FROM HUMAN SUBJECTS.

* LABORATORY ASSIGNED NUMBER.

TABLE 20

DESMOSINE AND ISODESMOSINE CONCENTRATIONS OF HUMAN AORTIC ARCH TISSUES AS DETERMINED BY HPLC AND AUTOMATED AMINO ACID ANALYSIS

SAMPLE	AUTOMATED AMINO ACID ANALYSIS		HPLC	
	DES (MMOL/L)	IDE	DES (MMOL/L)	IDE
1	0.174	0.155	0.186	0.149
2	0.114	0.119	0.139	0.113
3	0.177	0.130	0.176	0.126
4	0.123	0.103	0.118	0.088
5	0.106	0.071	0.110	0.083
6	0.131	0.088	0.132	0.098
7	0.114	0.094	0.116	0.086
8	0.102	0.062	0.109	0.083
9	0.131	0.076	0.147	0.101
10	0.117	0.095	0.116	0.094
11	0.098	0.078	0.094	0.072

DESMOSINE AND ISODESMOSINE CONCENTRATIONS OF HUMAN AORTIC ADCH SAMPLES PREPARED AS DESCRIBE IN CHAPTER 3. ALL SAMPLES RUN IN DUPLICATE.

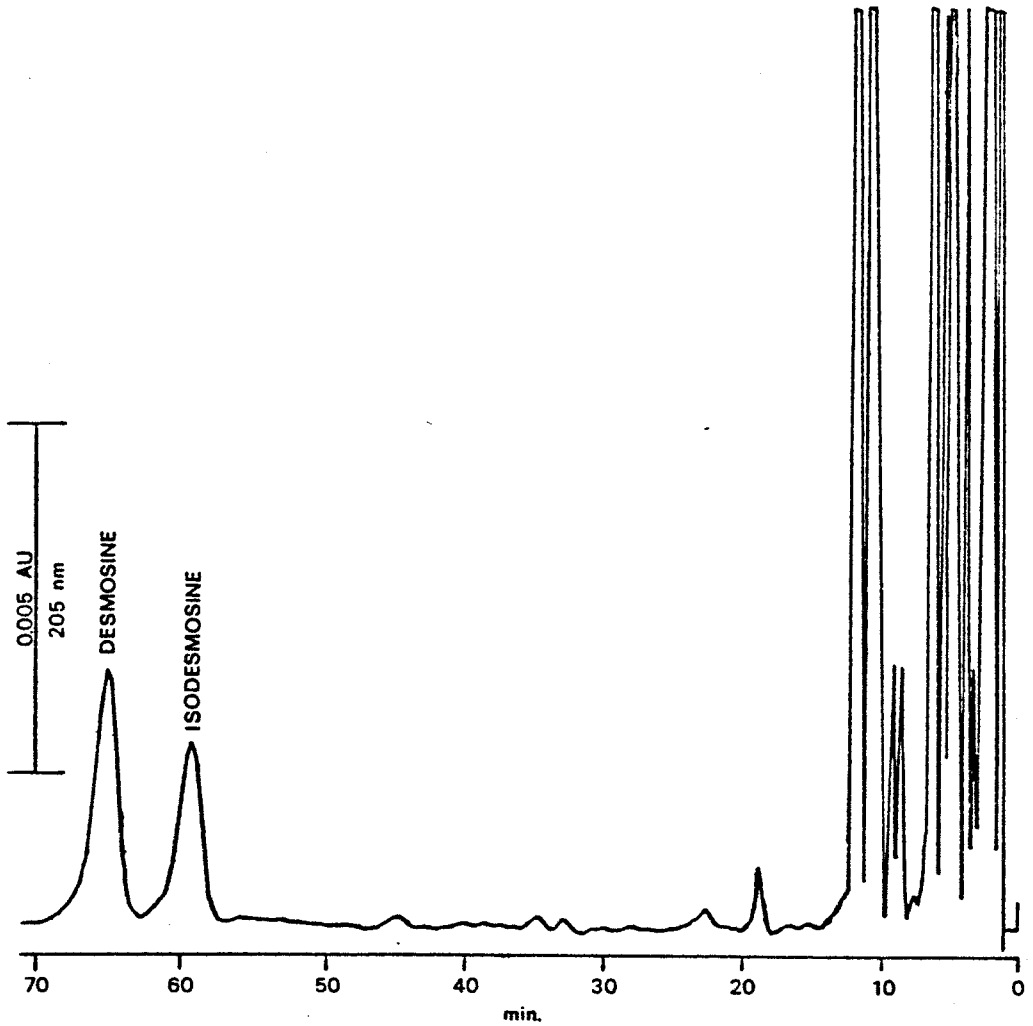


Fig. 24. Representative ion-paired HPLC chromatogram of hydrolyzed human aorta. Hydrolysate eluted with methanol/water (26/74, v/v) mobile phase containing 0.01 mol/L heptane sulfonic acid, pH 3.0. Detection at 205 nm.

TABLE 21

ELASTIN AND COLLAGEN CONTENT OF HUMAN AORTIC ARCH TISSUE

SAMPLE	% ELASTIN		% COLLAGEN		% CTP**	
	AAA	HPLC	UNCORR.*	CORR.*	UNCORR.*	CORR.*
1	45.8	46.6	40.6	33.5	87.2	80.1
2	31.2	35.1	44.7	39.5	79.8	74.6
3	42.7	42.1	40.4	33.9	82.5	76.0
4	31.5	28.6	31.2	25.1	59.8	53.7
5	27.0	26.8	36.0	31.9	62.8	58.7
6	24.6	26.9	37.0	32.9	63.9	59.8
7	30.5	32.0	26.7	22.7	58.7	54.7
8	28.4	27.8	33.1	28.9	60.9	56.7
9	33.0	35.8	30.5	25.0	66.3	60.8
10	29.5	30.4	32.7	28.0	63.1	58.4
11	24.5	23.1	37.9	34.4	61.0	57.5

ELASTIN AND COLLAGEN (UNCORRECTED AND CORRECTED) CONTENT CALCULATED AS DESCRIBED IN CHAPTER 4.

* UNCORR. = UNCORRECTED; CORR. = CORRECTED

** % CTP = % CONNECTIVE TISSUE PROTEIN, AS DETERMINED BY PERCENT ELASTIN PLUS COLLAGEN. ELASTIN CONTENT DETERMINED FROM DES AND IDE QUANTITATION BY ION-PAIRED HPLC.

0.6). Therefore, the elastin content, as determined by the ion-paired HPLC quantitation of the desmosines was utilized in all further analyses of the human aorta samples.

Upon statistical analysis of the human aorta samples, it was found that they segregated into two groups. The first group (age 2-17) had a mean elastin content of 412.6 ± 58.1 mg/g aorta FFDW, while the second group (age 34-90) was found to have a mean elastin content of 288.7 ± 38.1 mg/g aorta FFDW. These two means were determined to be statistically different ($p<0.01$). From these results it was concluded that with age, the elastin content of the aorta (aortic arch region) decreased significantly.

Following the determination of the elastin content of the human aorta samples, the collagen content of these same samples were determined, as previously described in the canine aorta study. Again, both the uncorrected and corrected collagen content of each sample was calculated (Table 21).

Upon statistical analysis, it was found that the uncorrected collagen content of the aorta samples were segregated into 2 age groups, identical to the age groups found in the elastin study. The first group (age 2-17) had a mean collagen content of 419.0 ± 24.5 mg collagen/g aorta FFDW, while the second age group (34-90) was found to have a mean collagen content of 331.2 ± 37.0 mg collagen/g aorta FFDW. These two means were found to be statistically different ($p=0.05$). However, when the correction for the non-collagen Hyp content of the aorta samples were made, and the corrected collagen content determined, it was found that while the younger age group had a somewhat higher mean collagen content

than the older group (356.0 ± 34.4 mg collagen/g aorta FFDW vs. 286.9 ± 45.5 mg collagen/g aorta FFDW), and that there was a decrease in the collagen content from young to old, the significance observed in the uncorrected collagen diminished. This decreasing tendency in the collagen content of aortic samples with age has been observed by other investigators [134,135].

From these results it was concluded that the significant age difference observed in the uncorrected collagen content between of two groups may be due to the Hyp content of the elastin. Thus, when the non-collagen Hyp is subtracted from the total Hyp content of the tissues, the age difference previously observed in the uncorrected collagen content diminished, although a decreasing trend in the corrected collagen content with age is still observed. These results stress the necessity to correct for the non-collagen Hyp content in tissues which are rich in elastin, such as the aorta.

Following the collagen determinations, the percent connective tissue protein in the human aortas was calculated as previously described (Table 21). Again, a significant difference was observed between the two age groups, whether the uncorrected or corrected collagen content was used in the calculations. Using the corrected collagen content of the aortas, the mean percent connective tissue protein for the 2-17 year group was determined to be 76.9 ± 2.85%, while the mean percentage determined in the 34-90 year group was 57.5 ± 2.4%. Clearly, these results show a significant decrease (approximately 1/3) in the total connective tissue component of the aorta with age ($p<0.001$). This result is to be expected since this research has shown the elastin, and possibly, the

collagen content of the aorta decrease with age.

Hydroxyproline Determination by Ion-Paired HPLC:

During the development of the ion-paired HPLC method for the quantitation of the desmosines, it was observed that standards of Hyp and Pro displayed k' values different from the other amino acids investigated (Table 14). From this finding, it was thought that it may be possible to simultaneously quantitate the Hyp and the desmosines in tissue hydrolysates, thereby yielding an estimate of both the elastin and collagen content from one analysis. However, it was found that that the mobile phase which maximized the separation of the desmosines failed to sufficiently resolve Hyp and Pro in the tissue hydrolysates.

In an attempt to separate Hyp from Pro and the other amino acids in the hydrolysate, the methanol percentage of the mobile phase was decreased to 19%. At this methanol percentage, Hyp was observed to completly separate from Pro (Fig. 25). The elution profile of the desmosines at this decreased methanol percentage, however, was such that they were unable to be quantitated, the peaks being very broad and unsymmetrical, due to the extended length of time on the column.

It may still be possible to quantitate Hyp with the desmosines if a gradient elution technique is applied to the existing ion-paired HPLC method. With this system, the methanol percentage of the mobile phase would be gradually increased from 19% to 26% during the course of the analysis. Therefore, the Hyp, eluted at the lower methanol percentage, would separate from the other amino acids, and the desmosines, eluted at the higher methanol percentage, could still be quantitated.

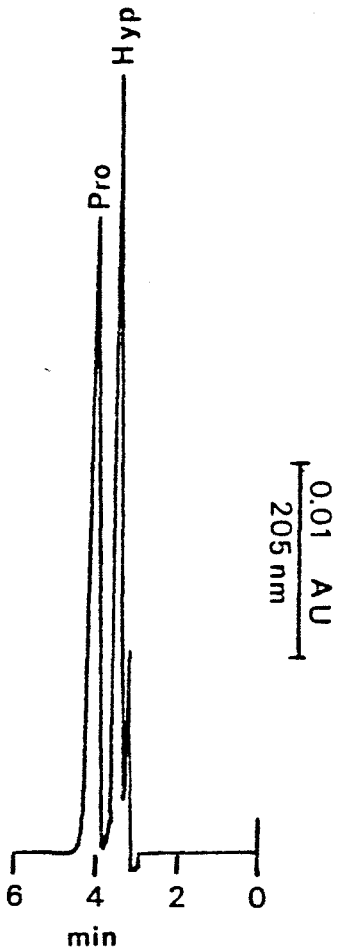


Fig. 25. Chromatogram of Hyp and Pro separation by ion-paired HPLC. Amino acid standards eluted with methanol/water (19/81, v/v), pH 3.0, containing 0.01 mol/L heptane sulfonic acid. Amino acids detected at 205 nm.

CHAPTER 5

DISCUSSION

Elastin is one of the major connective tissue proteins of the body, and is unique in that it displays both elasticity and tensile strength. This protein has been implicated in several pathological conditions, including atherosclerosis, emphysema, and a rare hereditary disease, pseudoxanthoma elasticum. Thus, it would be advantageous to determine the amount and composition of elastin in both "normal" and "pathological" processes. However, this information is lacking in that the major source of data pertaining to the quantitation of elastin is derived from gravimetric determinations of the residue remaining following several extraction procedures.

These extraction results have been shown to be non-reproducible when compared to both with-in and between method evaluations [14,24-26]. This variability of results has been partially explained by the glycoprotein content of the remaining residue, which is often defined as elastin. This glycoprotein content has been shown to vary considerably with the source and age of the tissue, and may be due to the method utilized to extract the various other proteins [46,86]. Also, these extraction methods are tedious, require several days for completion, and prone to methodological errors.

Recently, a method for the estimation of elastin, which relies on the cross-linking amino acids Des and Ide, has been proposed [39]. This method utilizes the quantitation of the two derived amino acids as an

index to the amount of elastin present in the tissue, just as the quantitation of Hyp is frequently used as an index to the amount of collagen present [49]. This inference is possible because Des and Ide have only been found in elastin and the non-elastin protein in egg shell membranes.

This method of quantitation has several advantages over the extraction procedures. For one, after the tissue is dried and defatted, it is simply hydrolyzed in one step, which negates any errors associated with the possible loss of elastin by extraction procedures. Secondly, by its nature, the procedure is less tedious, and eliminates several methodological errors introduced by the extraction procedures.

With this method, the desmosines are routinely quantitated by automated amino acid analysis. While this method of quantitation has been the accepted technique, there are disadvantages associated with the amino acid analyzers. For one, relatively few laboratories have a completely automated amino acid analyzer at their disposal, the instrument being somewhat cost prohibitive. Secondly, amino acid analysis is a time consuming procedure, the average analysis requiring 3 to 6 hours. Finally, the limit of detectability for the desmosines with an amino acid analyzer, utilizing ninhydrin from detection, is at best 5 nmols, thereby limiting its use to tissues rich in elastin (aorta, lung) unless concentration procedures are utilized.

The results described in Chapter 4 show that an alternative method of quantitation of the desmosines is possible. This ion-paired HPLC method for the separation and quantitation of Des and Ide was shown not only to correlate well with the accepted method of quantitation, but to rival the amino acid analyzer in terms of general availability (not as

cost prohibitive), analysis time (less than 1 hour for complete analysis with no column regeneration), and sensitivity (lower limit of detection being approximately 100 pmol).

This research has also shown that the estimation of the elastin content of canine aortic tissues, when calculated from the concentration of the desmosines, as opposed to determination by gravimetric procedures, were higher in 4 of the 5 samples studied. Yet due to one high gravimetric result, the difference observed between the two methods was not significant. This finding may be biased by the limited number of samples investigated. The higher elastin content obtained by the HPLC method in the 4 samples mey be explained by sample degradation due to the harshness of the extraction procedures utilized in the gravimetric method [14, 24-26]. The elastin results obtained by the quantitation of the desmosines, when compared to those previously reported, were found to be nearly identical [101].

When the elastin content of the canine aortas were combined with their respective collagen contents, it was observed that there was an inverse relationship between the two connective tisue proteins, and that the elastin plus collagen content of the canine aortas accounted for approximately 2/3 of their total FFDW. If, however, the non-collagen Hyp content of the aortas (elastin contains approximately 1.7% Hyp) was subtracted from the total Hyp content, and the collagen determined using these corrected concentrations, a significant decrease of approximately 10% was observed in the canine aortas. This is the first report of the overestimation of the collagen content in tissues rich in elastin.

hydrolysates (aortic arch region), it was observed that the elastin content varied according to the age of the individual. It was found that in the 11 human aorta samples studied, the highest elastin content occurred in the first two decades of life, and decreased thereafter. These findings have been substantiated by others, though the absolute decrease varied between investigators, depending on the method utilized for the estimation of the elastin content [130-132].

The average elastin content of the human aortas was found to be somewhat less than those previously published [101]. This difference may be accounted for by the age dependence associated with the elastin content, and the sample population being skewed towards the aged.

The collagen content of the human aortas were again shown to be inversly proportional to the elastin content, and this relationship was observed to fall into the 2 age groups described for the elastin content ($p=0.05$). Also, a substantial decrease was noted in the collagen content of the human aortas when the non-collagen Hyp from elastin was subtracted from the total Hyp content.

There have been conflicting reports on the age dependence of the collagen content of human aortas, varying from a decrease with age to reports of increases observed in the collagen content with age [133-135]. These conflicting results may, in part, be explained by the non-collagen Hyp contribution of elastin. However, no definitive statement can be made in regard to the significance of the dependence of the collagen content with age from the results described in Chapter 4. These findings, however, further emphasize the necessity for correcting for the Hyp content of elastin, in tissues rich in this protein.

Finally, the connective tissue protein content of the human aortas was determined to be approximately 2/3 the total FFDW, using the corrected collagen content. This value is approximately 10% higher than those of a previous report [18]. However, the method of elastin and collagen content utilized in that report involved extremely harsh extraction procedures, which could lead to sample degradation.

As previously described in Chapter 4, an unknown peak appeared on the chromatograms of all the canine and human aorta hydrolysates. This peak was speculated to be lysinonorleucine, a minor cross-link found in elastin. With further observations, the relative peak height was found to increase with the age of the human aorta samples. If this peak is in fact lysinonorleucine, perhaps an inverse relationship may exist between it and the the desmosines. Thus with age, as the concentration of the desmosines decrease, the concentration of lysinonorleucine would increase. This finding has been substantiated by other investigators [130].

This finding leads to a problem associated with the measurement of elastin by the quantitation of the desmosines, or any other amino acid. This problem is the question of possible changes in the absolute number of cross-links (Des, Ide, or lysinonorleucine) per unit elastin with time. There is some evidence that the Des and Ide cross-links in early fetal elastin is very low, and then increases in late fetal elastin [136]. Also, there is some evidence of a decrease in the number of Des and Ide cross-links per unit elastin in aged individuals [130]. If, in fact, this occurs, an argument can be made for a concomitant increase in the lysinonorleucine cross-links, with a decrease in the number of Des and Ide cross-links. Assuming that the Des and Ide cross-links are required for

the elasticity and tensile strength displayed by the elastin molecule, a decrease in their absolute number, with a concomitant increase in the non-functional lysinonorleucine cross-links, may explain the "brittleness" often associated with aging of the aorta.

Yet, with these drawbacks, it may well be that the quantitation of the desmosines for their use as an index to the elastin content is as good as index as the others thus far suggested for postnatal measurements. This would especially be true in dealing with tissues from a given species, of similar age.

Recently, 3 other methods have been developed for the quantitation of the desmosines for use in the estimation of elastin [137-139]. The first of these methods relies on the Hyp content of the tissue to determine both the elastin and collagen content [137]. While, this method would offer simultaneous quantitation of the two connective tissue proteins, there are certain drawbacks associated with it. For one, since Hyp is contained in both elastin and collagen, each would be susceptible to contamination from one another, thus tending to increase their amounts. Secondly, these methods rely on extraction procedures, whose problems have been previously described. Both of these problems can be eliminated if Des and Ide are utilized in place of the Hyp, since Des and Ide are specific markers for elastin, and their quantitation requires no extraction procedures.

Another method recently developed employs an RIA for the quantitation of desmosine [138]. Undoubtedly, this method would be more sensitive than any method previously described. However, this method is very dependent on antibody production to desmosine, which has been shown to be

a difficult procedure, by both the developers of the RIA method and in this research (Chapter 4). Also, the time of the assay is somewhat limiting, in that it requires a long equilibration procedure.

Finally, Faris and co-workers have reported using a HPLC system similar to the ion-exchange method investigated during the course of this study, for the quantitation of the desmosines [139]. Though their system fails to resolve Des from Ide, they claim to quantitate the total desmosine concentration by detection at 275 nm. While this method may be useful as a preparative procedure, the absolute quantitation of the desmosines would be suspect for several reasons. For one, their method assumes that the molar absorbance coefficients of Des and Ide are identical at 275 nm, however, their data fails to confirm this. Secondly, their method of quantitation assumes the Des to Ide ratio to be constant. This, however, was not shown in their report, in fact, the Des to Ide ratio has been shown to vary from tissue to tissue [101,121-123]. It was for these reasons that the ion-exchange method was abandoned during the early HPLC investigations in this research.

Therefore, the ion-paired HPLC method for the quantitation of the desmosines, and their subsequent use for the estimation of the elastin content, is clearly as good as, if not superior, to the methods previously described. Also, with further refinements (gradient elution), simultaneous quantitation of Hyp, Des, and Ide may be possible with the ion-paired HPLC method. Thus the quantitation of these 3 amino acids from one analysis would yield an estimation of both the elastin and collagen content, negating the use of a separate assay for Hyp.

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APPENDIX A

APPENDIX A

PREPARATION OF EHRLICH'S REAGENT:

Solution A:

To 25 mL of cold absolute ethanol, 3.42 mL of concentrated sulfuric acid was slowly added. The mixture was kept cooled until needed.

Solution B:

To 25 mL of cold absolute ethanol, 15 g of para-dimethylaminobenzaldehyde was added and dissolved.

Reagent:

Solution A was slowly added to solution B (1:1) in an ice bath. The reagent was stable for 4 weeks if stored in a cold dark room.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

14 April 1982
Date

Albert A. Dietz
Director