BIOCHEMICAL STUDIES OF ELASTIC TISSUE

By

Lawrence B. Sandberg, M.D.

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

June 1966

APPROVED:

. . . .
(Professor in Charge of Thesis)

. . . .
(Chairman, Graduate Council)

TABLE OF CONTENTS

	Page
Preface	1
Historical introduction	2
Aims and objectives of the experimental sections	11
Experimental sections	
Section 1: Studies on the composition of bovine ligamentum nuchae	13
Introduction	13
Materials and methods	14
Determination of ligament ages and weights	14
Determination of elastin content	14
Determination of total collagen content	16
Reagents	17
Procedure	17
Determination of moisture content	18
Evaluation of DNA content	18
Reagents	18
Procedure	18
Ligament histology	20
Results and discussion	20
Ligament weights	20
Water and DNA content	24
Collagen and elastin content	24
Collagen, elastin and water content : changes with age	27
Histologic correlations	32

	Page
Summary	39
Section II: Ultrastructural studies of elastogenesis	40
Introduction	40
Materials and Methods	41
Observations and discussion	41
Pre-elastin filaments	42
Mature elastin	42
Collagen	43
Polysaccharide-protein complexes	43
Summary	53
Section III: Swelling studies on bovine ligamentum nuchae	54
Introduction	54
Materials and methods	57
Materials	57
Solutions	57
Swelling procedure	59
Determination of pH	59
Determination of solubilization	60
Calculation of swelling	60
Results and discussion	61
Swelling and solubilization at acid pH	61
Without salt	61
With 2M NaCl	61
Swelling and solubilization at neutral and mildly alkaline pH	61
Without salt	61

Page

With 2M NaCl 68

Swelling and solubilization at very alkaline pH 68

Without salt 68

With 2M NaCl 69

Summary 70

Section IV: A study of the chemical composition of pure elastins 71

Introduction 71

Materials and methods 72

Preparation of elastins 72

Hydrolysis of elastins in preparation for amino acid analysis 72

Amino acid analysis 73

Modified Woesner II method for determination of hydroxyproline 78

Troll and Lindsley method of proline determination 79

Reagents 79

Procedure 79

Determination of total nitrogen content of elastins and hydrolysates of elastin 81

Amide nitrogen and ash determinations of elastins 82

Sodium hydroxide treatment of purified elastins 82

Results and discussion 85

Automated determination of total nitrogen 85

Method of amino acid analysis 89

Results of amino acid analysis 92

Page

Amide nitrogen determinations 97

The effects of sodium hydroxide treatment on purified elastins 97

Summary 105

Section V: Metabolic studies of lysine incorporation into growing elastin 106

Introduction 106

Materials and methods 109

Study 1: long-pulse study 109

Study 2: short-pulse study 110

Results and discussion 111

Summary 122

Section VI: Studies on the antigenic properties of elastin 123

Introduction 123

Materials and methods 124

Preparation of α and β elastin 124

Development of anti-α elastin antibodies 124

Precipitin tests 125

Passive cutaneous anaphylaxis test 125

The hemagglutination reaction for detection of antigen-antibody reactions using tanned formalinized cells 125

Immunodiffusion and immunoelectrophoresis studies 128

Results and discussion 128

Summary 137

General discussion 138

Page

Summary and conclusions 143

Acknowledgments 145

References 146

LIST OF TABLES

Table		Page
I	Composition of bovine ligamentum nuchae	58
II	Amino acid composition of bovine ligamentum nuchae elastins	93
III	Amino acid composition of bovine ligamentum nuchae elastins from various ages, autoclaved and autoclaved & NaOH treated	94
IV	Results of chemical studies on purified elastins from bovine ligamentum nuchae	98
V	Amino acid composition of bovine ligamentum nuchae elastins from various ages in moles/100gm elastin	101
VI	A comparison of activities in aortic elastin of long and short-pulse lysine uptake studies in the growing chick	117
VII	Long and short-pulse lysine uptake studies expressed in μmoles and DPM/μmol	118
VIII	Effects of hot alkali treatment on recoveries of radioactivity from C^{14} lysine labeled chick aortic elastin (long-pulse study)	121
IX	Precipitating antibody response of rabbit after repeated injections of 2 percent alpha-elastin with equal parts of Freund's Adjuvant	130
X	Hemagglutinations observed when alpha elastin was reacted with anti-alpha elastin:reading made after 12 hr incubation at room temperature	132
XI	Hemagglutinations observed in a 20 percent ethanol treated extract of fetal bovine ligamentum nuchae when reaction with anti-alpha elastin.	136

LIST OF FIGURES

Figure		Page
I	Weight changes of whole bovine fetal ligamentum nuchae with age	23
II	Changes of water and DNA content in bovine fetal, calf, and adult ligamentum nuchae with age	26
III	Collagen and elastin content of bovine fetal, calf and adult ligamentum nuchae as a function of age	29
IV	Collagen, elastin, and other solid matter content of bovine fetal, calf and adult ligamentum nuchae as a function of age	31
V	Verhoeff-Van Giesen's stain of ligamentum nuchae from four bovine ages	34
VI	Masson's Trichrome stain for collagen fibers and cellular detail from four bovine ages	36
VII	Electron micrograph of a portion of a fibroblast in the ligamentum nuchae of a 180-day-old fetal calf	45
VIII	Electron micrograph of elastin fibers sectioned longitudinally	47
IX	Electron micrograph of a cross-section of developing elastin fiber	49
X	Electron micrograph of a cross-section of developing elastin fibers and an adjacent fibroblast	51
XI	Swelling of dried ox hide in HCl-NaCl and NaOH-NaCl systems at 20° for 3 days	56
XII	Swelling and solubilization of fetal bovine ligamentum nuchae in non-saline and in 2M-NaCl solutions at various pH values at 20° for 3 days	63
XIII	Swelling and solubilization of 6 month calf bovine ligamentum nuchae in non-saline and in 2M-NaCl solutions at various pH values at 20° for 3 days	65

Figure		Page
XIV	Swelling and solubilization of adult bovine ligamentum nuchae in non-saline and in 2M-NaCl solutions at various pH values at 20° for 3 days	67
XV	Method of deoxygenation of the nitrogen supply, and storage of the ninhydrin reagent for the Technicon amino acid analysis system	75
XVI	Platter arrangement for automated determination of nitrogen on the Technicon analyzer using the Na phenate-hypochlorite reaction	84
XVII	Curves from the standards of three consecutive series of nitrogen determinations on the Technicon analyzer using the Na phenate-hypochlorite reaction	88
XVIII	A typical amino acid analysis represented by nine year old cow ligamentum nuchae elastin	91
XIX	A graphic representation of the totals of polar and non polar amino acids of elastins before and after treatment with 0.1N hot NaOH	103
XX	A summary of the known degradative products of lysine which occur in rat liver, and also the steps which are believed to occur in the conversion of lysine to desmosine and X_4 in elastin	108
XXI	A portion of the amino acid analysis chromatogram from a lysine uptake study in rat aortic elastin	113
XXII	A interfacial test using various dilutions of calf serum against anti-calf serum	127
XXIII	Photograph of hemagglutinations observed when alpha elastin was reacted with anti-alpha elastin	134

"Comprehension of functions is dependent
on knowledge of structure and composition."

A. N. Richards and W. T. Gies (1902)

PREFACE

Elastin is one of the few substances found in vertebrate tissues in considerable quantities about which relatively little knowledge has been gained regarding its biochemical makeup. A good deal of the descriptive material on elastin involves histologic observations coupled with relatively crude and often ill-conceived physical chemical studies with an attempt to interpret the molecular structure on this basis. Much of the difficulty probably stems from the resistance of this tissue to most of the conventional protein solvents. The pathway of least resistance has been followed by taking advantage of this *inertness* in preparation of "elastin" for physical-chemical studies. Because of the drastic measures used in this preparation, the final product is no doubt greatly altered from its native form. Thus inertia has tended to propagate itself by the study of a substance relatively sterile in its ability to yield data of biochemical usefulness.

An attempt is made in the *Historical Introduction* of this treatise to pick up the thread of the "true biochemical nature of elastin" from its earliest inception in the 19th century up to and including work currently in progress in this field. The *Experimental Sections* will then deal with a series of experiments designed to further illuminate and define this problem. A *General Discussion* section will deal with the conclusions that can be drawn from these studies as well as some "honest speculation" regarding the biosynthesis and molecular structure of this fibrous protein.

HISTORICAL INTRODUCTION

Although some earlier observations on the elasticity of blood vessels had been made, it was not until histology became a definite science that elastin was described as a separate and distinct substance. Jacob Henle (35), in 1841 first recognized the specific elements now called elastic fibers in the lamellae of blood vessels, and he recognized that this was different from the white fibrous collagenous tissues found in other portions of the body. Although von Ebner (106) was the first investigator to attempt specific staining of elastin, it was not until the orcein stain of Tänzer (100) was introduced in 1891 that a specific stain for this fibrous protein was available.

Clinicians in the late 19th century began to describe histopathologic changes in connective tissues. Mall made an extensive study of the properties characterizing elastic, collagenous, and reticular fibers (54). He also studied their formation (55). His work is probably the most outstanding work on connective tissue of the late 19th century. Unna in his treatise on *Histopathology of Diseases of the skin,* in 1896, (105) introduced such terms as "elacin," "collacin," "collastin," and "elastica" in describing degenerative products found in diseased connective tissues. These terms implied there was a chemical change of elastin to collagen and vice versa, or to some intermediate substance with ageing and disease states. Unna later chose to abandon these terms (41), but the damage was already done, and one yet finds modern authors employing these archaic usages. Confusion has thus been compounded by the entertainment of a theory

which is biochemically unsound, though still adhered to by some investigators. One is reminded of Stahl's "phlogiston theory," of the 16th century, which threw back the progress of chemistry for more than a century by the confusion of what the poet calls "the sublime and irrefutable passion of belief" with the purposes of scientific investigation (24).

Despite the confusion, a remarkably clear review and treatise on the chemical nature of elastin was published by Richards and Gies in 1902 (83). This has laid the groundwork for the present day definition of elastin. Their observations were exceedingly precise and comprehensive, considering the relatively crude analytical methods in use in their day.

Fifteen to twenty pounds of ligamentum nuchae from cattle and horses were worked up at a time to carry out elemental analysis, ashing studies, digestibility and heat of combustion experiments. The presence of serum-like proteins in ligamentum nuchae and aortic tissues was recognized to the extent that albumins and globulins were identified by their precipitation patterns with ammonium sulfate. They pointed out the danger of prolonged hot alkali and acid treatment in preparation of the "pure fiber." They also recognized the presence of collagen, which was easily removed by boiling water, to be identical with the collagen of tendon and bone. They identified the presence of a glycoprotein, which was in close association with the elastin fiber and much more difficult to remove. They pointed out that practically all of the nitrogen was present as mono-amino acids, very little free ammonia being liberated on mild acid hydrolysis. Analysis for the basic polar amino acids was carried out, all

yielding low results. From their heat of combustion data, they were able to conclude that the carbon to oxygen ratio was very high compared to other proteins and thus implying that the protein was relatively non-polar. In partial enzymatic hydrolysates, they demonstrated coacervation phenomenon of the solubilized peptides and even observed how this could be altered with the addition of alkali or base. In essence, they dealt with practically all of the special physical-chemical properties of elastin that were to be "rediscovered" in a slightly more refined fashion over the ensueing sixty years. Truly, this is a monumental piece of work.

The following forty years brought forth relatively little new data regarding the biochemistry of any of the connective tissues. In an excellent review, George M. Hass, in 1939, (34) covered the then known facts about elastic tissue. Most of his observations as well as those of other workers cited are based on the tinctorial properties of this fibrous protein. A good deal of his interpretation of physical-chemical properties is based on these empirical staining procedures about which even he states the following: "It may be emphasized here that a change in the physical properties or in the chemical structure of the fiber does not necessarily imply that there is a comparable change in the uniformity or intensity of staining. Neither does a change in staining reaction signify in all instances a parallel change in the functional ability or in the structural integrity of the fiber. Therein lies the weakness of many dogmatic interpretations of histologic appearances." In short, he could say little more about elastin than was known in the early 1900s.

The only refinements were histologic identification and description of embryogenesis.

With biochemical attention being focused increasingly on the dynamic aspects of intermediary metabolism, the relatively inert substances such as collagen and elastin had thus been pushed to the background. The few significant chemical observations on elastin were made in regards to its action during the tanning of leather. Leather chemists found that if allowed to remain as an intact fiber in the hide, elastin interferred considerably with the flexibility of the finished product. Roddy and O'Flaherty in 1938 (86), followed elastin histologically through the tanning process observing its swelling and shrinkage with changes in pH and ionicity of the tanning liquors. They simultaneously observed the same effects on pure elastin and collagen fibers prepared respectively from ligamentum nuchae and tendon. They noted that liming caused the greater differential in swelling of elastin as compared to collagen, and during this process the fibers appeared to break up into fibrils. However, no actual tanning of the elastin was observed during the entire chrome tanning procedure. Dehydrated elastin was found to be very brittle and wood-like.

Other workers earlier had noted the rubber-like properties of wet elastin. Lloyd and Garrod (50) (51) combined these observations with their own to make some of the first sensible modern biochemical observations on elastin, summarized as follows: The long side chains of the non-polar amino acids (valine, leucine, isoleucine) tend to keep the "backbones" of the elastin molecules apart so they can fold

randomly during retraction. The high proportion of non-polar v.s. polar side chains (approximately 20:1) would account for the rubber-like properties of elastin, since the number of possible cross bonds would be too few to hold the molecule in an extended form. The inertness of these fibers to chrome tanning liquors as observed by Roddy and O'Flaherty confirms the absence of polar side chains, since it had been shown that the first stage of chrome tanning is a coupling of the collagen fiber and the basic chrome tanning complex through the carboxyl groups of the collagen. Elastin and lightly vulcanized rubber have similarities in that they both fail to show any sign of molecular orientation by X-ray diffraction methods. However, one important difference exists. The rubber molecule consists only of carbon and hydrogen, and is thus self lubricating under forces of shear. The backbone of the elastin molecule contains both oxygen and nitrogen atoms, with their potentialities for forming hydrogen bonds. The elastin molecule, therefore, is not self-lubricating, but requires the presence of water or some other polar solvent for its rubber-like properties to become apparent. The nature of the molecular cross-links were not known at the time of these investigations, but their presence was appreciated, and it was apparent they must be of an unusual nature, since the protein was so resistant to solubilization. A better chemical definition of elastin was necessary however, before accurate evaluation of the cross-links could be made.

With the advent of rapid methods of amino acid analyses in the early 1950s, the exact chemical definitions of a great many proteins

became more precise to the extent that amino acid composition of a substance became a measure of purity. This was particularly true in regards to collagen and elastin, since there was then no recognition of a molecular species of either of these proteins. Although Stein and Miller (99) carried out the first complete amino acid analysis of elastin in 1938, it was not until the introduction of the rapid methods of amino acid analysis that elastins were examined extensively. Lansing, in 1951 (44), laid down some of the criteria for so called "pure elastin" from aortic tissue by showing that elastin from this organ prepared in the conventional manner of boiling in water contained considerable contaminating proteins. These contaminants were only removed by the treatment, first introduced by Lowry, Gilligan, and Katersky (52), of fibers with hot 0.1N NaOH for 45 minutes. Partridge and co-workers (73) (74) (28) later showed that the amino acid composition of elastin from a given species was relatively constant regardless of source, providing adequate measures were taken to remove contaminating proteins. Elastin from ligamentum nuchae was the easiest to prepare, requiring only saline extraction, followed by autoclaving; elastin from aortic tissue and elastic cartilage was more difficult, requiring the treatment with hot alkali. Thus, the criteria of low hydroxyproline, absence of tryptophan, and high valine content have become accepted as standards in evaluating the purity of an elastin preparation (46) (73).

Several points should be emphasized here, which might not be appreciated fully by the biochemist having limited experience with connective tissue. First of all, though these criteria may seem very

crude, which in fact they are, they represent a tremendous step forward in our understanding of elastin metabolism, since at least there is universal agreement on the chemical definition of this protein fiber. Secondly, having arrived at a definition of the insoluble elastin fiber, we must immediately recognize that this is not necessarily the chemical composition of the "in situ" fiber. There is ample evidence, as will be pointed out later, that considerable loss of polar amino acids occurs in the rigorous purification process of autoclaving and hot alkali treatment.

The demonstration of the metabolic inertia of elastin by several groups of workers should be also regarded as a characteristic. Lansing (45) and Slack (96), both showed that administration of C^{14} labeled glycine resulted in quite permanent fixation of this substance into elastic tissues.

The chemical and metabolic inertness of elastin have made the study of its metabolism a very unusual and difficult problem. In 1963, however, a breakthrough occurred which has revolutionized this entire problem and perhaps will prove to be a key point in the elucidation of a soluble elastin precursor. Thomas, Elsden, and Partridge, at Cambridge, England demonstrated the presence of two heterocyclic nitrogen containing compounds, which apparently are peculiar to elastin. They named the compounds Desmosine and Isodesmosine after the Greek word δεσμοσ, meaning to bond or link. They pointed out that these compounds could very probably be formed by the condensation of four modified lysine chains, which were brought into close proximity of each other by random folding of one or more

elastin peptide chains.

From this point on, studies on elastin metabolism have been moving at a progressively accelerating pace. Miller, Martin, and Piez (61), demonstrated in both organ cultures and live animals that there is an actual diminution of lysine with age and a concomitant increase in the desmosine and isodesmosine content. Transfer of radioactive label from lysine to the desmosines was also clearly shown in vitro. Studies on disease states in which there is impaired elastin formation (i.e. copper deficiency, and lathyrism) by the same workers (62), and also by Carnes and co-workers (109) and O'Dell and co-workers (67), demonstrated a failure for the desmosine cross-links to form, accompanied by an abnormally high lysine content. Franzblau, Sinex, and Faris (22), very recently demonstrated the presence of another peculiar substance in elastin hydrolysates, which has been identified as N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine or lysinonorleucine or X_4. This, they feel, could possibly represent one of the intermediates in the conversion of lysine to desmosine and isodesmosine.

To summarize, the 120 years that have elapsed since the recognition of elastin by the father of modern histology, Jacob Henle, are marked by relatively few important advances in illucidation of the metabolism of this fibrous protein. The peculiar tinctorial properties as demonstrated by Mall and Unna, and the crude, but illuminating chemical studies of Richards and Gies, represent the highlights of the early work. More recently, the relatively obscure leather chemists gave us the first insight into the physical chemistry

of this substance by their observations of behaviour during the tanning process. With the advent of radioisotope tracer studies and accurate and rapid methods of amino acid analysis, their speculations were confirmed. Also metabolic and chemical guide lines were established by such workers as Lansing and Partridge. Finally, the discovery of the peculiar cross-linking agents and their alterations with age have made clear that this tissue has a very unique metabolism, that it is not as inert a substance as it was once thought to be, and has sparked a very keen interest towards a more complete understanding of it in the light of modern biochemical concepts.

AIMS AND OBJECTIVES OF THE EXPERIMENTAL SECTIONS

The general aims of the *Experimental Sections* may be summarized in these three short questions:

1. How is elastin synthesized?
2. Is there a soluble precursor of elastin?
3. What is the role of the cross-linking agents and their precursors in the formation of elastin?

For sake of clarity the materials presented are divided into the *6 Experimental Sections* listed below. The bibliographic references at the end of the sections refer to publications containing the experimental data from that section.

1. *Studies on the Composition of Bovine Ligamentum Nuchae* deals with the chemical nature of fresh ligament in terms of connective tissue components, i.e. elastin, collagen, and cells, and changes in these which occur with age.
2. *Ultrastructural Studies of Elastogenesis* is concerned with the study of a fetal tissue in which elastin, collagen, and cells are quite distinct, as opposed to more adult tissue, so that inferences can be made as to the inter-relationships of these components (20).
3. *The Swelling Properties of Ligamentum Nuchae* has as its objective the definition of the best conditions under which proteins can be solubilized from fresh ligamentum nuchae (39).
4. *A Study of the Chemical Composition of Pure Elastins* may appear to be devious from the main stream of thought but it

was felt essential in order to define, in the accepted chemical sense, the elastins being dealt with in the various ages of fetuses, calves, and cattle. It was also a necessary groundwork for the metabolic study following (12).

5. *Metabolic Studies of Lysine Incorporation into Growing Elastin* is a study of the uptake of radiolysine into aortic elastin of the young chick. The purpose of this study was to identify, if possible, the presence of an early metabolic derivative of lysine which might serve as a marker for identification of soluble elastin (13).

6. *Studies on the Antigenic Properties of Elastin* is an attempt to use immunologic methods as a means of identifying elastin which has been solubilized by various limited hydrolytic methods. The study also applies the same methods to identification of elastin-like components in soluble proteins from ligamentum nuchae (40) (89).

SECTION I

STUDIES ON THE COMPOSITION OF BOVINE LIGAMENTUM NUCHAE

Introduction

A study of the synthesis of elastin should most reasonably be carried out on a tissue having a high content of this fibrous protein. The two most logical tissues to examine would be aorta and ligamentum nuchae. The latter was chosen for this study because its elastin content is the highest of any tissue (6) and also because it is least contaminated by other substances (28). Bovine material was used for this study because it is readily available from commercial abattoirs.

Several problems are inherent in aorta to preclude its use in this study. Several investigators (11), (29), (33), have shown that there is a marked decrease in the elastin content of the aorta from the thoracic to the abdominal region in many animals and man. Associated with this is a concomitant increase in the collagen content. Cleary has also carried out retraction studies on various segments of aortae by measuring the length of a defined segment in situ and then re-measuring the same length after removal to determine the amount of shortening (11). He has shown there is a variation in the retraction of an arterial segment depending on the location of this segment on the aortic trunk. He has further shown that retraction is due to the elastin present but not in a quantitative sense, i.e. retraction is greatest where elastin content is lowest. Thus it appears that there may be a qualitative difference in elastin from various portions of the aorta. Therefore, standard-

ization of results would be extremely difficult.

The objective of this first study was to better define ligamentum nuchae in terms of simple biochemical parameters focusing on the changes which might occur with aging. It was hoped the following questions would be answered:

1. What chemical constituents are present in the ligament other than elastin?
2. Do these constituents change with the age of the animal?
3. If there is a soluble precursor of elastin, when would be the optimal age to look for it?

Materials and Methods

Determination of Ligament Ages and Weights

Fresh ligament was obtained from the abattoir within one-half hour of the death of the cow. If of fetal origin, the age was estimated by measurement of the crown-rump length, and referral was made to standard tables for interpretation of the age of gestation (4) (see thesis pg. 21). If of calf or adult cow origin, the U.S.D.A. Inspectors present were extremely helpful and skilled in the determination of age, judging primarily from dentition and tooth-wear of the animal. The ligaments were carefully dissected from the animals. Care was taken to keep the ligament intact. They were weighed after removal of any adhering muscle and adipose tissue. For commercial reasons, it was only possible to obtain whole fetal ligaments.

Determination of Elastin Content

The procedure used was a slight modification of that outlined by Partridge, Davis, and Adair (74). It was oriented towards the

preparation of a very pure elastin with quantitative recovery for gravimetric assay, as well as the use of the supernatant solutions for evaluation of gelatin content. Preparation of elastin was begun by mincing the ligament, freezing it with liquid nitrogen, and then crushing it with a stainless steel mortar and pestle. The crushed ligament was then weighed and suspended in five to ten volumes of normal saline (see pg. 70 for a later alteration of this extraction solution) and homogenized in a Virtis Homogenizer at high speed. Usually one or two minutes of homogenization were required to give a finely-divided, thick, creamy suspension. Care was taken to carry out the homogenization procedures at cold room temperature (4°) with prior chilling of the apparatus and solutions. If more prolonged homogenization was required to produce a smooth suspension, a cooling period was allowed for every two minutes of operation of the Virtis machine. The sample was then transferred to a centrifuge bottle and placed on a gently-moving, wrist-action shaker for 24 hours in the cold. Several drops of caprylic alcohol were added to the suspension to prevent growth of micro-organisms. At the end of this time the suspension was centrifuged at 10,000 × G for 30 minutes and the supernatant poured off and saved. The elastin residue packed into a rubbery, solid mass. This was easily removed from the centrifuge bottle to repeat the homogenization procedure and extraction.

When supernatants reacted negatively to 5% TCA, which usually required 5 or 6 extractions of adult ligament and up to 12 extractions of fetal ligament, two additional extractions were carried out using cold water. All supernatants were pooled, although it was obvious from

the TCA reactions that the majority of soluble material was removed with the first three saline extractions, even in fetal tissue. The residue was then transferred to a glass bottle, capped with a guaze and cotton plug, and autoclaved at 30 p.s.i. for six hours. This caused a gelatinization of the collagen. The gelatin was poured off after centrifugation at 10,000 × G for 30 minutes. The residue was washed twice with boiling distilled water and twice with cold distilled water. The cold water washings were tested for the presence of protein with the biuret reaction. If the test was positive, the autoclaving and washing procedure was repeated. Repetition of the autoclaving procedure was usually necessary. The washings and gelatin supernatants were pooled with the previous saline washings and saved. The residue was then dehydrated by washing several times with 50% ethanol-ether followed by pure anhydrous ether. It was then allowed to air dry for several days at 37° and weighed. A portion was dried in a 100° oven for 24 hours to determine moisture content. The remainder of air dried elastin was finally put through a Wiley Mill with a 60 mesh screen which produced a cream-colored, free-flowing powder.

Determination of Total Collagen Content

A portion of the pooled supernatants obtained during the preparation of pure elastin was hydrolyzed with 5 ml. HCl in sealed pyrex tubes for 16 to 24 hours at 110°. The hydrolysates were taken to dryness three times on a steam bath, then dissolved in a small amount of water, and made to 10.0 ml. in a volumetric flask. The hydrolysates were filtered before making the final dilution for

determination of hydroxyproline by the Woesner I method (110). The reagents and procedure for this method, with modification, are described below.

Reagents: A standard hydroxyproline solution was prepared containing 100 μgm. of hydroxyproline per ml. This was obtained by dissolving 50 mg. of vacuum-dried L-hydroxyproline in 500 ml. of distilled water containing 20 ml. of concentrated HCl as a preservative. Appropriate dilutions ranging from 0.5 to 4.0 μgm. hydroxyproline per ml. were made from this standard. The standard stock solution could be stored indefinitely. The dilute standards were prepared fresh daily. The buffer, chloramine T, methyl cellosolve, perchloric acid, and p-DAB (para-dinitro-amino-benzaldehyde) were prepared exactly as described by Woesner.

p-DAB (Eastman) was made fresh shortly before its use. This chemical is highly unstable. A grey-green color of the powder or solution indicates deterioration, unspoiled p-DAB having a yellow color in powder form and a deep amber color in solution.

Procedure: 2.0 ml. of the unknown solutions as well as 2.0 ml. of the dilute standards and 2 water blanks were placed in 18 × 150 mm. tubes. Forty determinations were carried out at a time. This included 15 unknowns and 4 standards, all in duplicate, as well as the 2 water blanks. One ml. of the chloramine T was first added to each tube. A strict sequence of tubes was adhered to and total pipetting time for each step was limited to five minutes. The tubes were shaken by the use of a Vortex Jr. mixer and allowed to stand at room temperature for twenty minutes. One ml. of perchloric acid was then added in the

same sequence as above to destroy the oxidative powers of the chloramine T. The tubes were shaken again. It was observed that total oxidation time could vary ±5 minutes, but consistency for the whole run was a necessity. Two to three, but not more than 5 minutes after the addition of perchloric acid, one ml. of p-DAB solution was added with thorough shaking. The tubes were placed in a 60° water bath for 20 minutes, then cooled in tap water for 5 minutes. The absorbancy of the solutions was measured with a spectrophotometer at 557 mu. The color which developed appeared to be stable for approximately one hour. Reproducible accuracy of this test was ±2%.

The collagen content of the pooled supernatants was then calculated by assuming the hydroxyproline content of collagen to be 14.4% which is based on the work of Jackson, Leach, and Jacobs (38).

Determination of Moisture Content

Moisture content was determined gravimetrically after drying 0.5 gm. portions of the fresh ligament at 100° for 24 hours. Results were obtained in triplicate and generally agreed within ±5%.

Evaluation of DNA Content

DNA determinations were done using a modification of the method of Schneider (93). The reagents and procedure for this method are described below.

Reagents: Diphenylamine reagent was prepared by first recrystallizing this compound twice from 70% ethanol. One gm. of this was dissolved in a solution consisting of 100 ml. of glacial acetic acid and 2.75 ml. of concentrated sulfuric acid. Standard DNA solutions were prepared by dissolving 10 mg. of purified and

polymerized calf thymus DNA (Sigma) in 10 to 20 ml. 5% TCA. This required heating at 90°, after which the solution was made up to 50 ml. by the addition of more 5% TCA. Standard dilutions were made by diluting this stock 2:10, 4:10, 6:10, and 8:10. Standards and stock were found to be stable for only 24 hours.

Procedure: Fresh samples of ligamentum nuchae were finely minced, frozen, and crushed as in the determination of elastin and collagen. The samples were then weighed and the fat extracted, first with one washing of absolute ethanol and then with two washings of ethanol-ether 3:1. During the fat extraction procedure an attempt was made to break up the samples into a finely divided state with a stirring rod. Supernatants were discarded and the samples allowed to evaporate almost to dryness. The residues were extracted twice with 7% TCA in the cold (4°), allowing 20 minutes for each extraction. If the residues were too dry at the time of addition of the TCA solution, it was difficult to re-wet them and thus the reason for evaporation of ethanol-ether washed tissues "almost to dryness." Supernatants from these cold TCA washings were discarded. Each sample was then extracted twice with approximately 5 ml. of 5% TCA at 90° for 15 minutes (a total of 30 minutes). These extracts contained the DNA. They were pooled and made up to a convenient volume (e.g. 20 ml.). For color development, 2.0 ml. portions of the 5% hot TCA extracts were pipetted into glass stoppered tubes. A blank and standards in duplicate were also included. Four ml. of the diphenylamine reagent was added to each of the tubes, which were then placed in a boiling water bath for 10 minutes. The tubes

were then cooled rapidly and the contents transferred to spectrophotometer cuvettes for immediate reading. Samples were read against the blank first at 595 mu. and then at 650 mu. The absorbancies obtained at 650 mu. were subtracted from those obtained at 595 mu. for each sample to give a final value.[1]

The quality of reagents was found to be of great importance for this test, particularly in the case of acetic acid and diphenylamine. If the absorbancies of the blanks were too high, a fresh lot of acetic acid was obtained and the test repeated. Sensitivity of the test was 20-200 mgm. DNA/ml.

Ligament Histology

Representative sections of ligament from various ages of fetuses, calves, and cattle were fixed in neutral calcium-free formalin and sectioned in the usual fashion at 5μ. Staining for elastin fibers was accomplished by the technique of Verhoeff with counter-staining of collagen fibers with Van Gieson's picrofuchsin mixture, as described by Mallory (56). This combination will be referred to as VVG stain. Differentiation of collagen from cytoplasm was obtained by the use of Masson's Trichrome stain (58) and will be referred to as such.

Results and Discussion

Ligament Weights

Figure I, representing the weight changes of ligamentum nuchae with fetal age, shows a sharp increase in the weight of the ligaments

[1]Webb and Levy have shown (108) this method of reading at two wave-lengths helps exclude the absorbancy due to substances other than DNA.

begins between 150 and 200 days of fetal life. If reference is made to the growth chart from Bogart (4), reproduced below, it is apparent that 200 days marks the beginning of a period of accelerated growth of the fetus.

Age-length Relationships of Fetal Calves

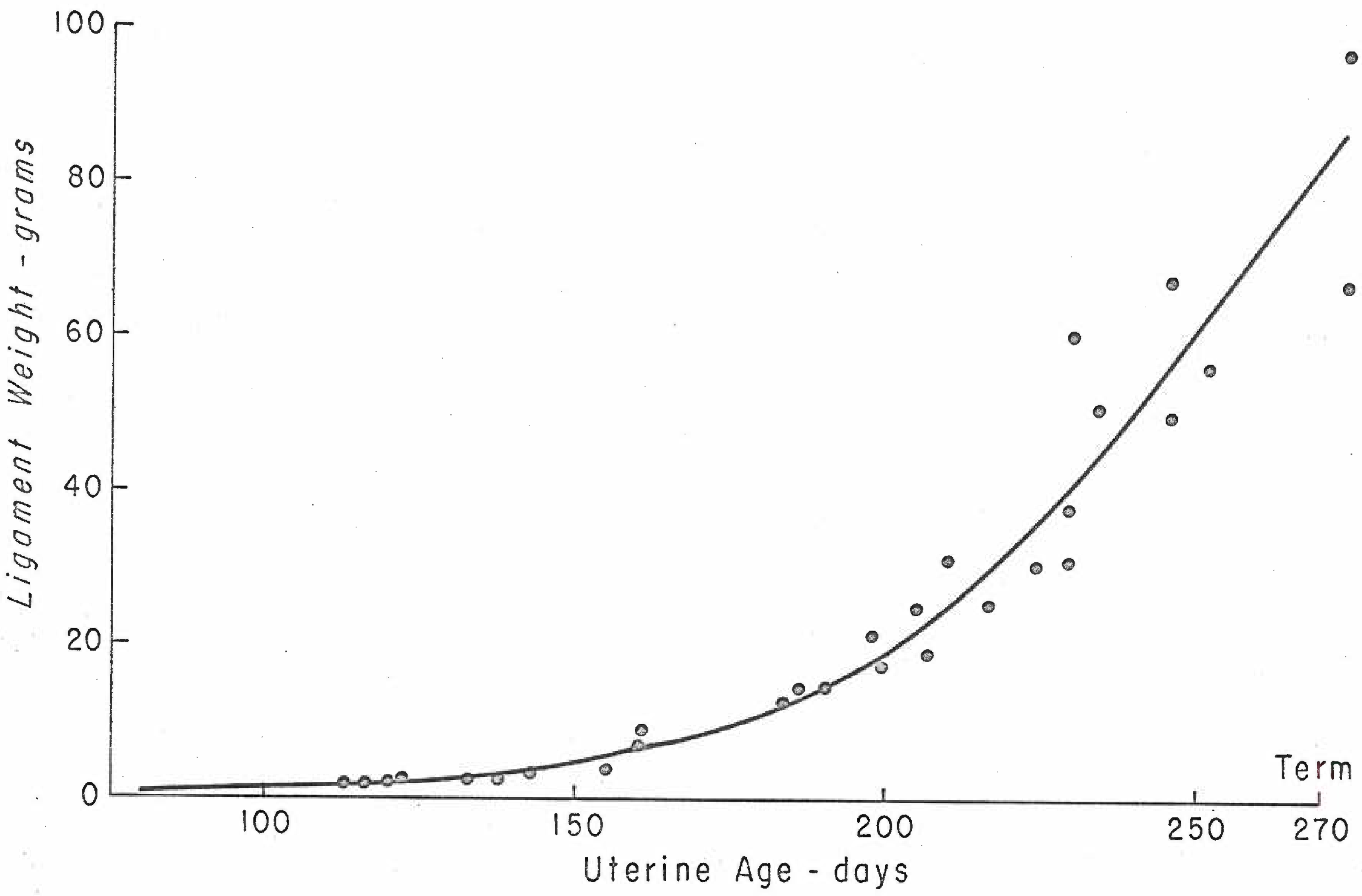
Age (days)	Forehead to Rump Length (cm)
45	3.08
50	3.85
60	6.60
70	9.40
90	16.40
100	18.80
120	27.10
140	32.60
160	43.70
185	54.00
200	58.50
230	73.00
260	87.00

Thus, it would appear that due to some growth stimulus all tissues in the fetal calf are undergoing a period of rapid increase beginning at about 200 days gestation. This period extends up to the time of birth.[2]

[2]Normal gestation for cattle is 270 days.

FIGURE I

Weight changes of whole bovine fetal ligamentum nuchae with age.


WET WEIGHT OF BOVINE FOETAL LIGAMENT
Ligament Weight - grams
100
80
60
40
20
0
100
150
200
250
270
Term
Uterine Age - days

Water and DNA Content

The water and DNA content of the ligament are plotted simultaneously as a function of age on Figure II. This graph points up qualities of the growth of the tissue which require special emphasis. Measurement of DNA content was carried out as an index of cellularity. This, of course, is at best a crude estimation, but it is generally accepted as an adequate method of quantitating cellularity (109). It will be noted (Fig. II), there is a progressive drop in DNA concentration past the time of birth. By the third month of extrauterine life, this level has become steady at less than one μgm. per mg. wet tissue. Thus, in spite of the great increase in mass of the ligament beyond 200 days gestation, the DNA content continues to fall. This would indicate the increase in mass is due to extracellular components.

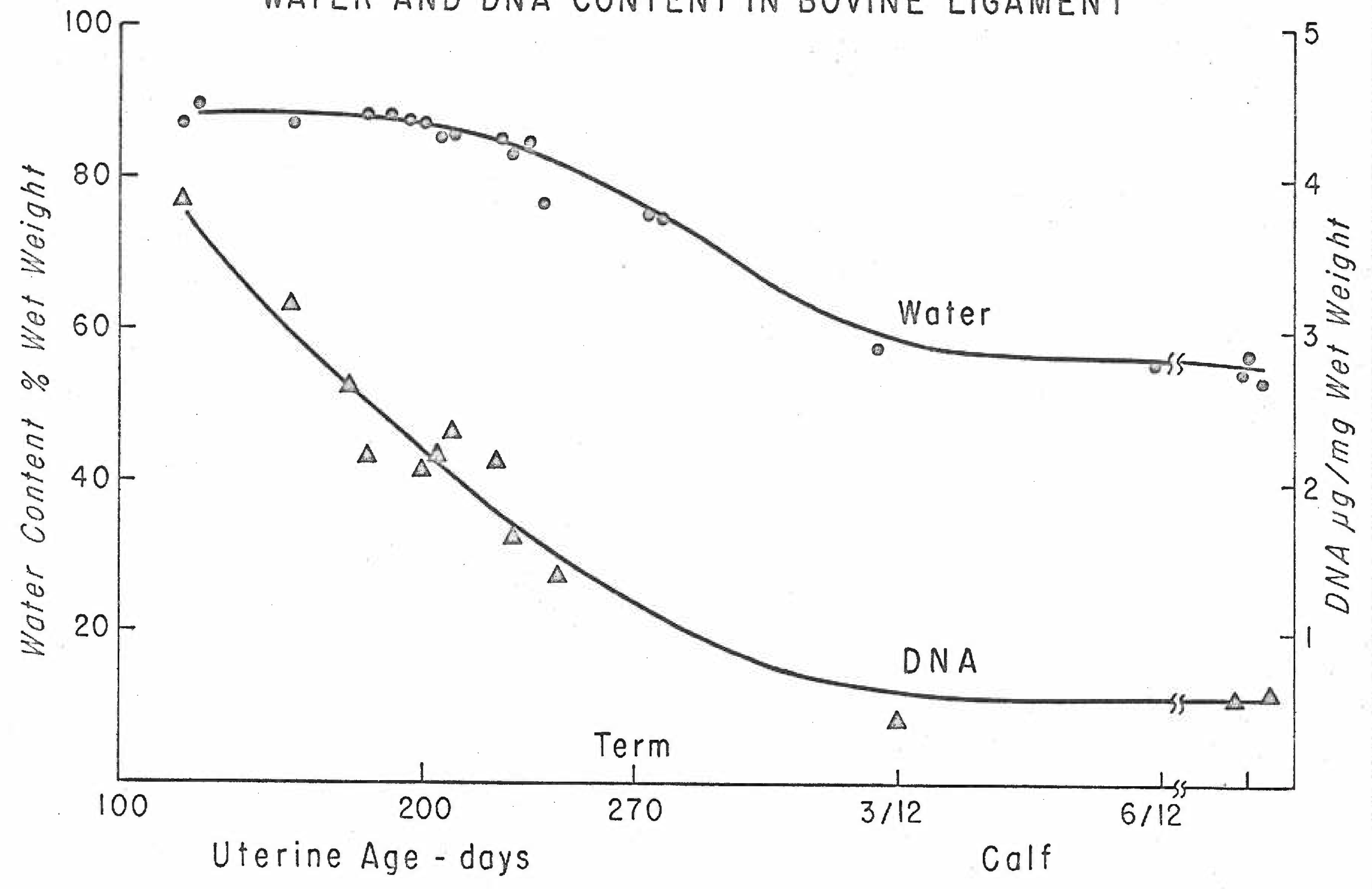
Collagen and Elastin Content

The standard methods for determination of elastin content of a tissue such as the aorta or ligamentum nuchae involve gravimetric evaluation, after all other substances have been dissolved away (46), (52). This is necessary, because there is no specific marker as yet identified by which one can distinguish elastin from other scleroproteins. Collagen, on the other hand, is the only known protein with a high concentration of hydroxyproline. With the advent of the Neuman and Logan colorimetric method for hydroxyproline estimation in 1950 (66), this method and modifications of it have been the basis of most collagen estimations since.

The graphs depicting collagen and elastin content changes as

FIGURE II

Changes of water and DNA content in bovine fetal, calf, and adult ligamentum nuchae with age.


WATER AND DNA CONTENT IN BOVINE LIGAMENT
Water Content % Wet Weight
100
80
60
40
20
DNA μg/mg Wet Weight
5
4
3
2
1
Water
DNA
Term
100
200
270
3/12
6/12
Uterine Age - days
Calf

a function of age are shown in Figure III. These curves would seem to indicate the increase in the mass of the ligament is due to the increase in its elastin content, since the elastin curve takes an upward swing just before 200 days. The totals of collagen and elastin show a steady increase up to adulthood. Because this chart (Fig. III) is based on fat-free, dry weight, it would seem that some of this increase would be due to the decrease in cellular components as demonstrated by the fall in DNA content (Fig. II). Also, there may be other extracellular components such as serum-like proteins or polysaccharide-protein complexes which are decreasing in concentration as the tissue ages.

Collagen, Elastin and Water Content : Changes with Age

The decrease in water content of the ligament as age progresses (Fig. II) is probably not remarkable, because this is the usual pattern of all fetal tissues. However, when the water content graph (Fig. II) is combined with the elastin-collagen graph (Fig. III) to give an approximate representation of the amount of these substances present in the wet tissues (Fig. IV), one gets a much better idea of what is actually taking place in this tissue during its development. The term "approximate representation" is used, because there is some lipid material which has been removed during the process of fat extraction prior to autoclaving. This lipid content of ligamentum nuchae has been found to be variable, in the range of 0.1 to 4% of dry weight. Fat free weight has been found to be a better and more consistent basis for the determination of total collagen and elastin content, due to this variability of lipid concentration. Thus, in

FIGURE III

Collagen and elastin content of bovine fetal, calf and adult ligamentum nuchae as a function of age. Values expressed as % of fat free dry weight.

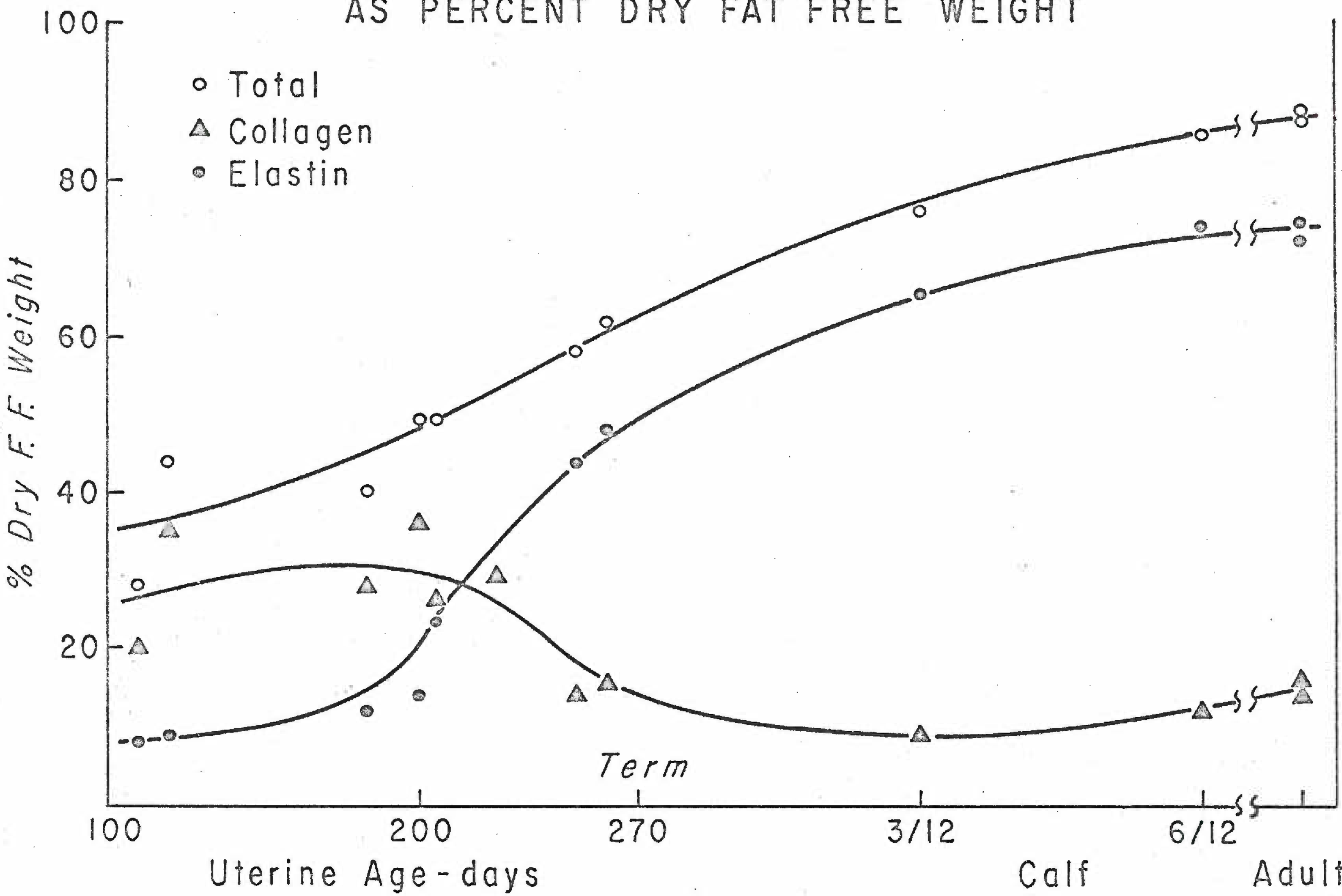
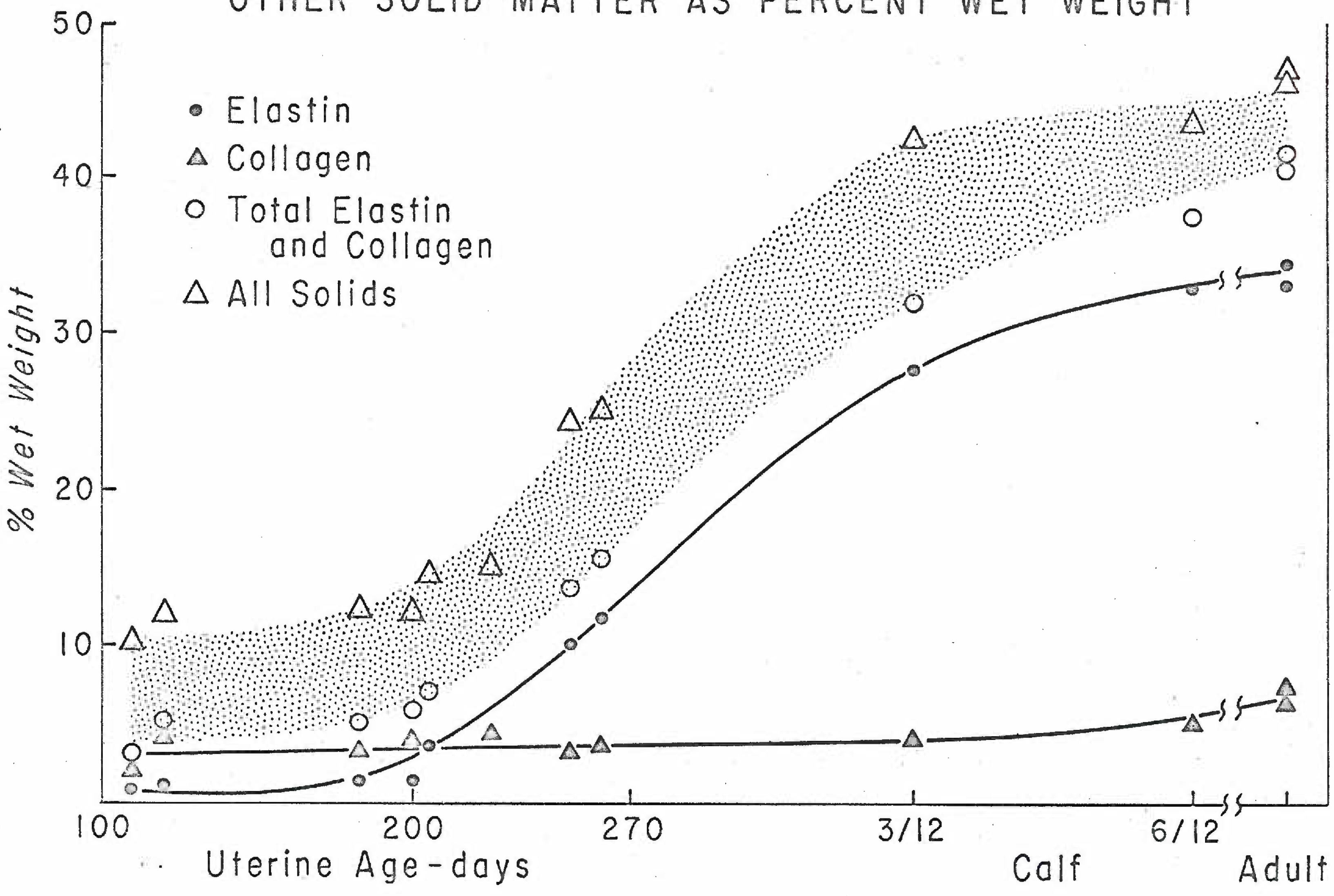

BOVINE LIGAMENT ELASTIN AND COLLAGEN CONTENT
AS PERCENT DRY FAT FREE WEIGHT
Total
Collagen
Elastin
% Dry F. F. Weight
100
80
60
40
20
100
200
270
3/12
6/12
Term
Uterine Age-days
Calf
Adult

FIGURE IV

Collagen, elastin, and other solid matter content of bovine fetal, calf and adult ligamentum nuchae as a function of age. Values expressed as % of wet weight.


BOVINE LIGAMENT ELASTIN, COLLAGEN AND
OTHER SOLID MATTER AS PERCENT WET WEIGHT
Elastin
Collagen
Total Elastin
and Collagen
All Solids
% Wet Weight
50
40
30
20
10
100
200
270
3/12
6/12
Uterine Age-days
Calf
Adult

calculation of the points for Figure IV, it was assumed fat free dry weight of the ligament was the same as dry weight which is not too erroneous, because the experimental error of the methods is greater than the maximal lipid content.

The elastin concentration curve in Figure IV makes a dramatic, sharp upward bend at just 200 days of gestation, as if some hidden force were suddenly released to carry out elastogenesis. The slope of this elastin curve continues unabated until approximately three months of life (post-partum) when a gradual leveling off begins. It is interesting to note the collagen concentration when plotted in this manner is quite constant, at approximately 5%, as is the concentration of substances other than collagen and insoluble elastin represented by the stippled area in Figure IV. More will be said about this stippled area later.

Histologic Correlations

Histologic sections are represented by Figures V and VI. Figure V represents 4 ages of ligamentum nuchae stained by the VVG method. Figure VI represents the same 4 ages of ligament stained with Masson's trichrome. The VVG stain imparts a purple-black coloration to the elastin, the remaining collagen shows as a faint pink. This stain is not adequate for identification of cellular detail. The collagen content of the photographs (Fig. V.) can not be appreciated due to the black and white nature of the reproductions, however the general degree of "blackness" is a rough, but fairly dramatic visual demonstration of the amount of elastin present. The Verhoeff's stain used here is a modification of the orcein stain

FIGURE V

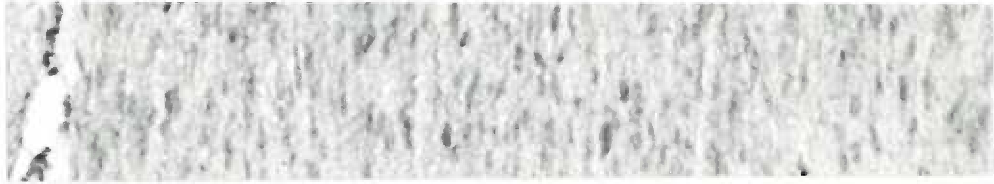
Verhoeff-Van Gieson's stain of ligamentum nuchae from four bovine ages in consecutive order.

(a) 6 month fetus

(b) Term fetus

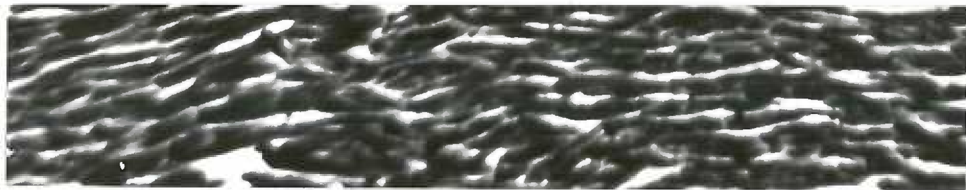
(c) 3 month calf

(d) 1-2 year adult



(a)

(b)



(c)

(d)

FIGURE VI

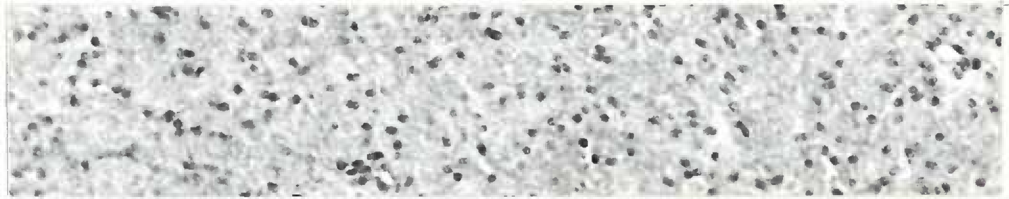
Masson's Trichrome stain for collagen fibers and cellular detail from four bovine ages in consecutive order.

(a) 6 month fetus

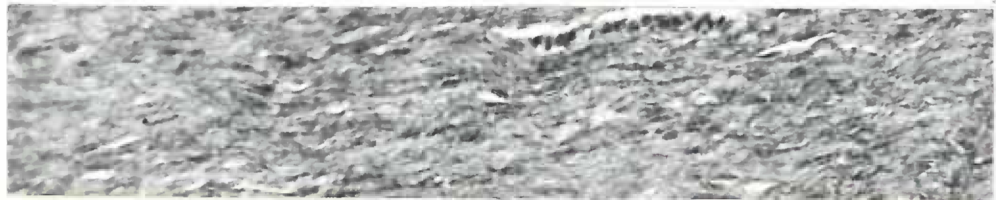
(b) Term fetus

(c) 3 month calf

(d) 1-2 year adult



(a)



(b)

(c)

(d)

described originally by Tänzer (100), and it is felt that the stain actually attaches itself to aldehydic groups on the elastin fiber (16), (34), (90). The trichrome stain imparts virtually no color to the elastin other than a very faint yellow, but the collagen stains a sky blue, with cellular nuclei staining darker blue and the cytoplasmic material a marked pink. This is an excellent stain for identification of cellular morphology.

The sections represented in Figures V and VI were selected in order to verify the chemical determinations of cellularity, collagen, and insoluble elastin as depicted in Figures II, III, and IV. Thus, the 180 day sections should represent a stage at which there would be numerous cells, very little elastin, and some collagen present. This is confirmed by examination of sections (a) of Figures V and VI. There is very little fibrous material revealed by either staining method; however, numerous cells are present as indicated by the small round nuclei. A comparison of section (a) and (b) of Figure V shows that a great increase in elastin content of ligamentum nuchae of the term fetus has occurred as compared to that present at 180 days of age. The fibers of the term fetal tissue are yet of a very fine nature. Sections (c) and (d) of Figure V, which are from 3 month calf and adult cow ligament respectively, indicate that from birth on there is a progressive thickening of the elastin fibers. At three months of post-partum age, the concentration of elastin has already reached a very high level. These sections, thus confirm the chemical findings as regards the presence of insoluble elastin, that is, the majority of elastin is laid down

between 200 days of gestation and the third month of post-partum life.

The trichrome stained sections (Fig. VI) confirm the chemical observations that collagen remains at a relatively constant concentration, because the densities of the four sections are all approximately equal. It is also apparent that cells are present in all sections, but the number is decreased considerably in the last three sections (b), (c), and (d). This would substantiate the interpretation that the DNA concentration is indicative of the extent of cellularity (Fig. II).

I wish to draw attention again to the stippled area of Figure IV which represents solid content of ligamentum nuchae, other than collagen and insoluble elastin. This area can only represent that portion of the ligamentum nuchae made up of cellular material plus soluble extracellular substances. I will refer to it henceforth as the *extrafibrillar constituent* of ligamentum nuchae. As the thesis progresses, this constituent will be seen to assume the position of the *arcanum of biological intrigue*. The hypothesis is put forward at this stage, that a portion of this extrafibrillar constituent is represented by a soluble precursor of the insoluble elastin fiber. Alternate hypothesis to consider and if possible disprove are:
1) this constituent is totally represented by cellular matter, or
2) by cellular matter plus serum-like proteins.

A decrease in cellularity of the ligament was observed both chemically (Fig. II) and histologically (Fig. VI). Thus, beyond the sixth or seventh month of fetal life some other substance or substances must be increasing in concentration to give this

extrafibrillar constituent its relatively constant value. Serum-like proteins have been demonstrated in connective tissue by Humphrey, Neuberger, and Perkins (37). Gotte and co-workers (27), (65) have identified a mucoprotein-like substance in close association with insoluble elastin, even in its purified form. This mucoprotein definitely is not elastin-like in amino acid composition. The presence of this mucoprotein must also be considered with the cellular and serum-like components.

Summary

It has been demonstrated that several constituents are present in ligamentum nuchae other than elastin. One of these is collagen, present in low and relatively constant concentration at various ages. Cellular substances are present also and appear to decrease with age. Other unknown substances must be increasing with age to balance out the decrease in cellular substances since the *extrafibrillar constituent* of ligamentum nuchae remains fixed at a relatively constant value of 6 to 10% wet weight (Fig. IV). I have not answered the question posed earlier as to the presence of a soluble precursor of elastin, but if present it would seem that the fetus in late gestation or the calf in the first few weeks of life would be the best ages at which to find this substance in maximal concentration.

Attention will next be focused on ultrastructural detail as determined by the electron microscope. Some of the problems posed above were immediately answered by this interesting study.

SECTION II

ULTRASTRUCTURAL STUDIES OF ELASTOGENESIS

Introduction

Numerous investigations have been made of the fine structure of elastin. For a survey of these, the reader can refer to the following reviews: Cox and Little (14), Partridge (72), and Ayer (1). Most investigators have utilized techniques for disruption of the mature elastin fiber purified by one means or another. Some investigators have attempted to demonstrate interconversion of collagen and elastin (30) (94). In short, almost any type of ultrastructural investigation that one could conceive of has been carried out on *purified elastins* with much significance placed on interpretation of the structures, which I believe are artifacts present in the fiber in this state.

The present study was carried out on the ligamentum nuchae of the 180 to 190 day old fetal calf. Considerable biochemical information had already been collected on this tissue by the studies described in the first experimental section (see thesis pg. 39) This information indicated the 180-200 day period of fetal development would be an optimal time to observe active elastogenesis. At this age, there would be minimal well developed elastin fibers to obscure cellular detail. There were, of course, considerable problems with fixation as is true of all fetal tissues. The observations were focused on 1) the filamentous structures associated with elastin and 2) the characteristics of the cells active in the production of elastin.

Materials and Methods

Samples of ligamentum nuchae of fetal calves, approximately 54 cm. in length (4), were obtained within 15 to 20 minutes after death of the cow. Tissues were fixed in phosphate-buffered osmium tetroxide (63) and embedded in Araldite. Sections were double stained with potassium permanganate (47) and lead citrate (82). These methods gave the best detail of the structures which were of interest, although other fixatives and stains were also tried. Electron microscopy was carried out with a Philips EM 200 Electron Microscope.

Observations and Discussion

The electron microscopy and anatomical interpretations of the electron micrographs were the work of Dr. H. Fahrenbach of the Oregon Regional Primate Research Center. This collaborative work will appear in a publication currently in press (20). It is included in this thesis because of its pertinence to the subject of the chemical composition and structure of elastin, and because the reader would not have ready access to the original manuscript.

Four types of fibers were observed in the present study of bovine fetal ligamentum nuchae. These were pre-elastin filaments, fine fibers of mature elastin, collagen fibrils, and diffuse-type filaments probably representing mucopolysaccharide-protein complexes. All these structures are found in close proximity to one cell type, the fibroblast. Each filament type will be discussed separately as regards development and morphology. The electron micrographs containing these structures are represented by Figures VII, VIII, IX, and X.

Pre-elastin Filaments

The first indication of elastogenesis consists of small extracellular accumulations of hollow-appearing filaments (pre-elastin filaments) lying adjacent to fibroblasts and more or less parallel to the long axis of the ligament (Fig. VII). In cross section, these filaments have a diameter of 130 to 140 Å with a hollow appearing core of 40 Å (Fig. IX). In longitudinal section they exhibit an indistinct segmentation (Fig. VIII). This substructure appears to consist of cylindrical or spherical units 130 Å long and of equal diameter. These are separated by more electron-opaque short sections of about 50 Å length. Thus the filaments have a periodicity of approximately 180 Å.

Mature Elastin

The fine fibers of mature elastin are first seen within clumps of pre-elastin filaments (Fig. VIII). The fibers are also oriented parallel to the long axis of the ligament. These fibers are approximately 700 to 1000 Å in diameter in the 180 day old fetal calf. In older animals these fibers can be seen to anastomose with others to form bundles which again anastomose until they reach the proportions observed in adult ligament (74). It is also noted that the pre-elastin filaments are in intimate association with the fine fibers of elastin, but are not visible within them. With the staining conditions employed the mature elastin has a coarse granular texture. This granularity appears to be due to tightly tangled and branching filaments of about 30 Å diameter (Fig. IX).

Collagen

The collagen fibril is readily identified by its 640 Å periodicity when viewed longitudinally. The 640 Å periodicity is due to the parallel arrangement of tropocollagen molecules in a staggered fashion with ends overlapping adjacent molecules approximately a quarter of their length (92). This overlapping arrangement causes the more polar portions of the molecules to be situated at regular intervals of 640 Å along the collagen fibril. It would not seem possible to confuse the 180 Å periodicity of the pre-elastin filaments with much longer periodicity of collagen (Fig. VIII).

Collagen fibrils are totally absent from the interior of the developing elastin though it appears that both are formed by the same cell (Fig. VII). This clearly demonstrates elastogenesis occurs in the absence of the direct participation of collagen and is further evidence against the thesis advocating elastin as a derivative of collagen.

Polysaccharide-protein Complexes

The diffuse "fuzz" surrounding both the developing elastin and collagen (Fig. X) is felt to be polysaccharide-protein complexes. These complexes have been readily demonstrated in these same tissues using the Schiff base reaction with basic fuchsin (56).

Polysaccharide-protein complexes are presumably of great importance in the formation of either the pre-elastin filaments or the mature elastin or both. Since there is a marked transition from the pre-elastin filaments to the mature elastin, it is tempting to speculate the "muco-polysaccharide" is active in this transition.

Elastin and lightly vulcanized rubber bear certain resemblances

FIGURE VII

Electron micrograph of a portion of a fibroblast in the ligamentum nuchae of a 180-day-old fetal calf. The fibroblast is surrounded on the left by collagen fibrils in various orientations and on the right by developing elastin fibers. Note the fine filamentous material around the entire surface of the elastin fibers. × 52,000

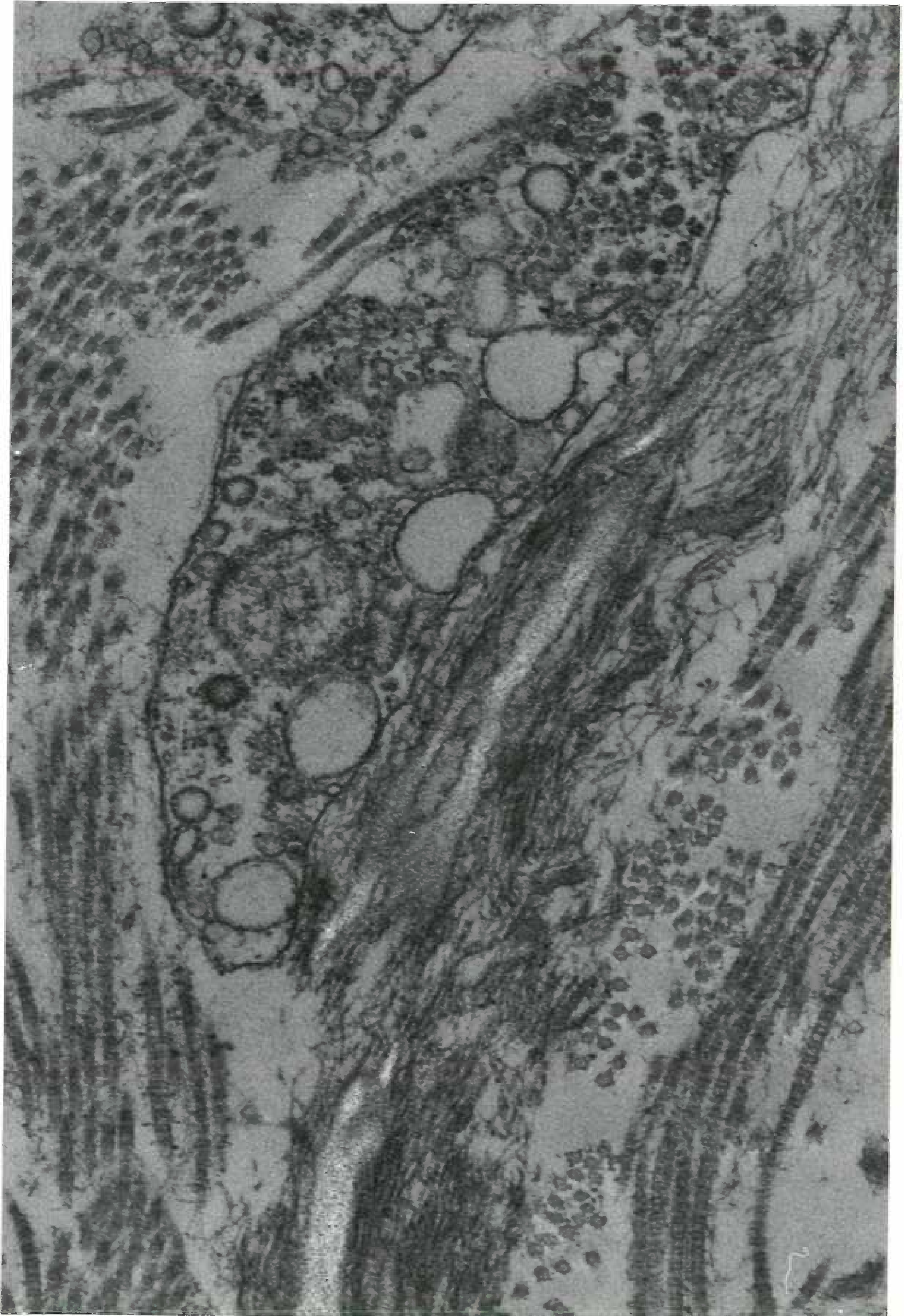


FIGURE VIII

Electron micrograph of elastin fibers sectioned longitudinally. Note the segmentation present in the filamentous material surrounding the fibers. Collagen bundles surround the elastin, but the two components do not appear to merge with each other. The 640 Å periodicity is apparent in some of the collagen fibrils. A good contrast of differences in periodicity of collagen and pre-elastin filaments is apparent in lower portion of the micrograph where these two components are in close proximity.

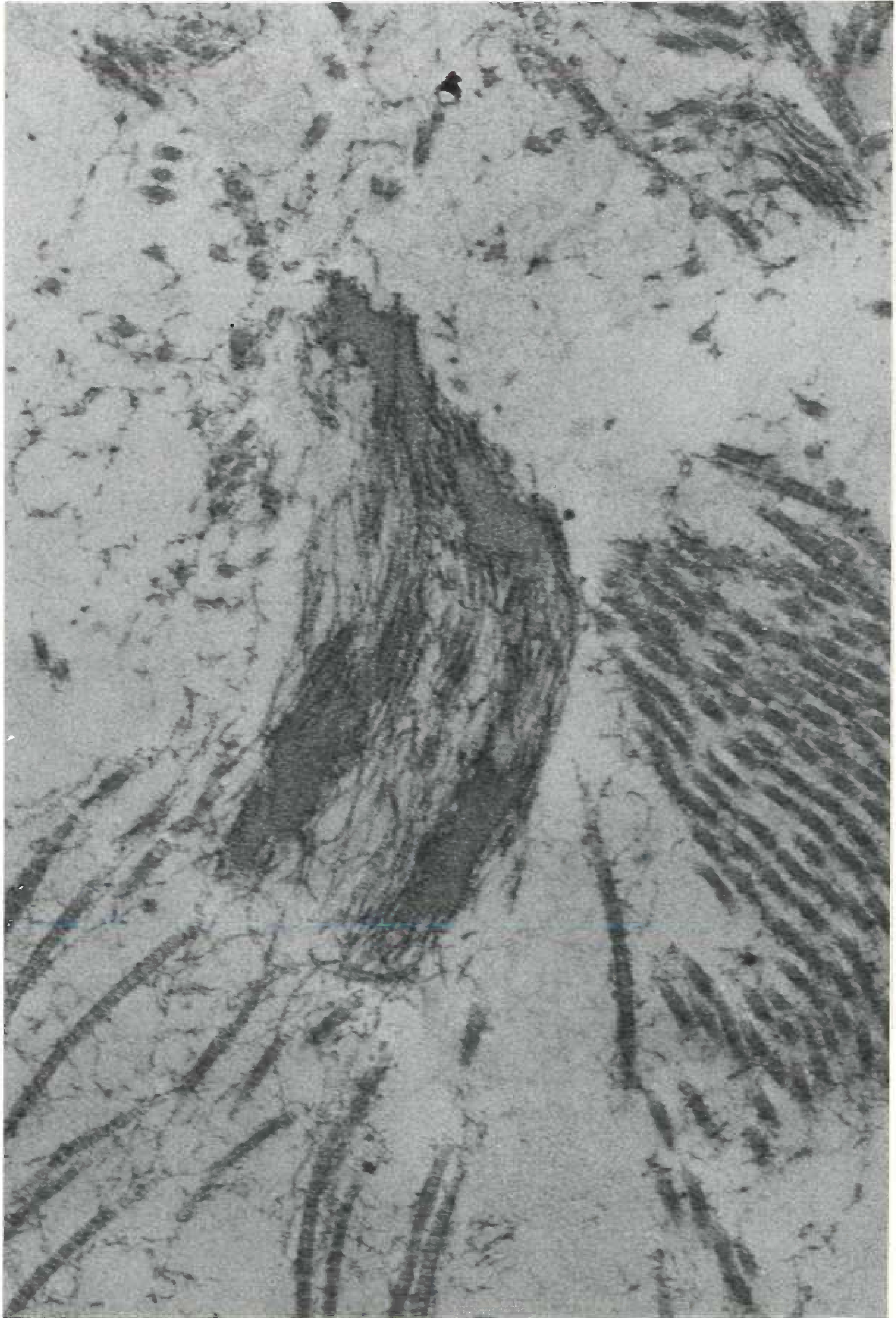


FIGURE IX

Electron micrograph of a cross-section of developing elastin fiber. Pre-elastin filaments surrounding the fiber have a definite hollow appearance (Micro-tubular). Note the texture of the mature elastin as if composed of numerous tightly packed very small filaments. Some mucopolysaccharide "fuzz" is apparent around the edges of the collagen and elastin fibrils. × 126,000

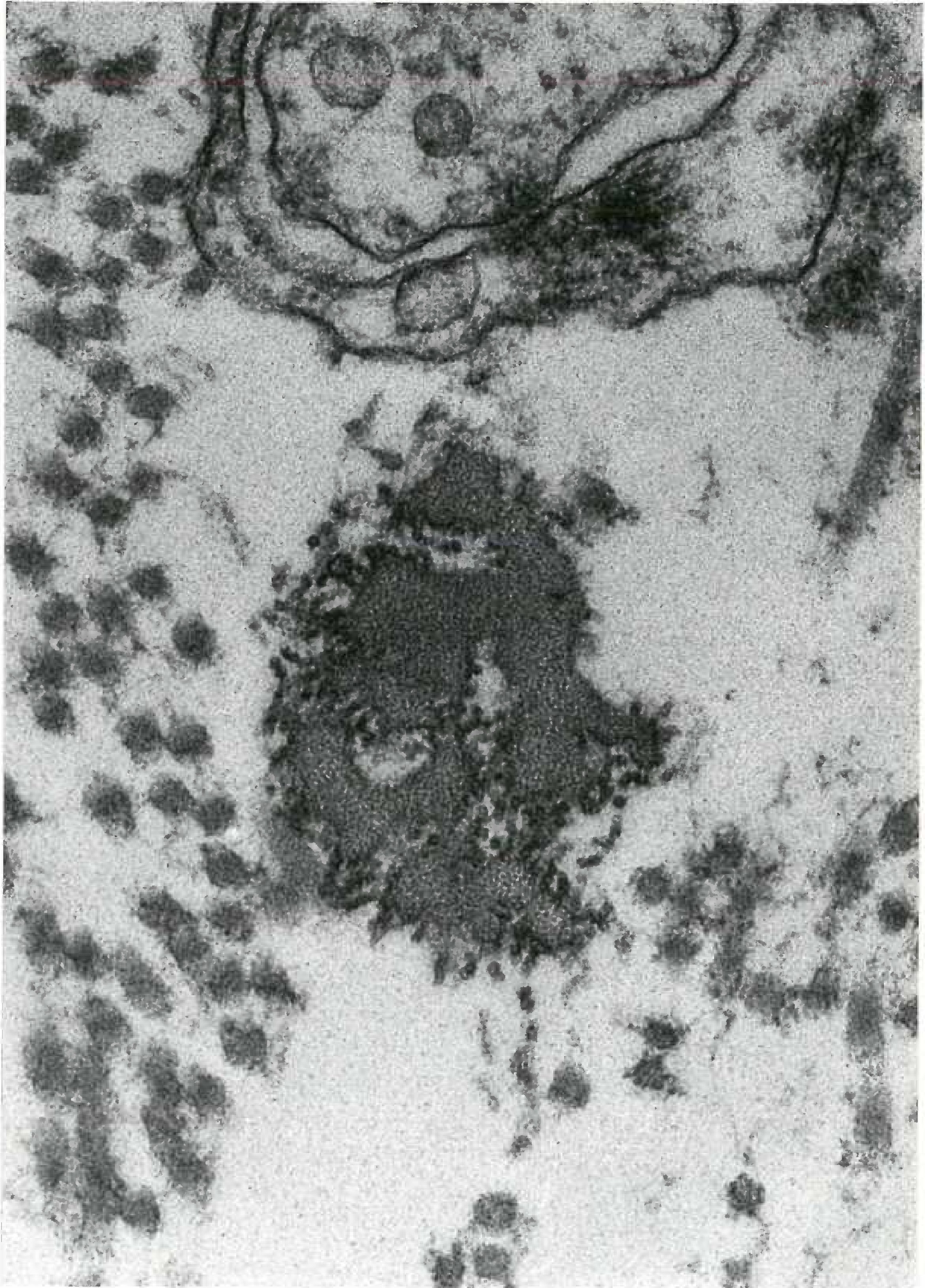
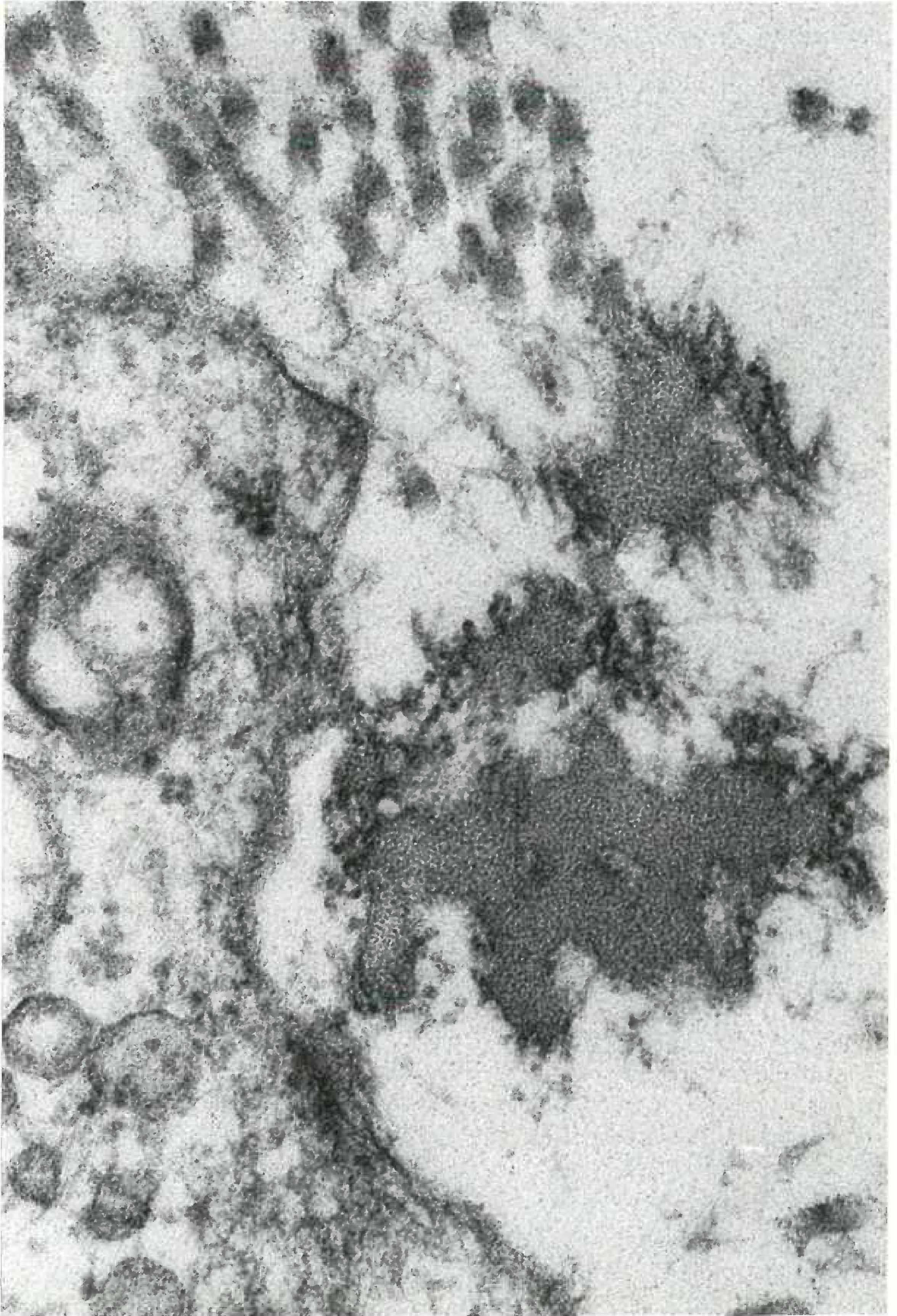


FIGURE X

Electron micrograph of a cross-section of developing elastin fibers and an adjacent fibroblast. Note the close proximity of the cell to the developing fibers to the extent that there appears to be contiguity of basement membrane material with the developing elastin (lower portion of micrograph). × 131,000.



as was pointed out by Lloyd and Garrod (50), (51) (see thesis pg. 5). Elastin, however, is not a true elastomer, because to function properly it requires a "lubricant," not inherent in its molecular structure as is the case with rubber. Elastin becomes brittle when dehydrated. Water, itself, would seem an adequate lubricant for elastin. In elastic tissue, however, water is probably in the form of a hydrated polysaccharide-protein complex. This complex may be similar to the "fuzz" observed here (Figs. IX, X) and also to that demonstrated by Gotte, et.al. (26), (27), to be in close association with the mature elastin fiber.

On the basis of the above considerations the following hypothesis is proposed. The pre-elastin filaments constitute a highly organized, hollow cylindrical arrangement of peptide chains. The short, electron, opaque portions of the filaments may represent areas in which there is greater polarity and thus greater degrees of cross-linking presumably in the form of ionic and hydrogen bonds. The longer, less dense portions may represent a more random arrangement of peptide chains with little or no cross-linking present between chains. A separation of the chains in the cross-linked area might occur by a relative state of dehydration induced by an increase in the concentration of the hydrophilic polysaccharide-protein complexes. These complexes would also act as a lubricant by being incorporated into the maturing elastin to prevent cross-linking between peptide chains. The 30 Å filaments seen in mature elastin may represent this breakdown of the pre-elastin filaments.

It is not proposed that the pre-elastin filaments represent a soluble form of elastin. Instead, this could represent the first step of polymerization of soluble elastin or *tropoelastin* to form the mature elastin fiber. *Tropoelastin*, as a generic name, should be reserved only for a homogeneous molecular species secreted by the fibroblast and which has the potential of aggregating with others of its species with the eventual formation of elastin fibers.

Summary

This study has clearly shown elastin to be an entity distinct from collagen in developing ligamentum nuchae. Both substances appear to be elaborated by the fibroblast. There is evidence for the presence of a polysaccharide-protein complex, also produced by the fibroblast, which may play an important role in maturation of elastin. Minute filaments are always seen on the periphery of developing elastin, and the hypothesis is put forth that these represent the first stage in the aggregation of tropoelastin to form the elastin fiber.

The study to follow will deal with determination of "ideal" conditions under which these observed components of early elastogenesis can be solubilized with retention of their natural chemical characteristics.

SECTION III

SWELLING STUDIES ON BOVINE LIGAMENTUM NUCHAE

Introduction

Two opposing factors are responsible for the swelling of fibrous proteins when placed in acid or alkaline solutions: 1) the swelling pressure due to the Donnan membrane effect; 2) cohesive forces present in the fibrous proteins themselves, either between fibrils and fibers, or intramolecularly (7), (49), (80). The degree of swelling will depend on the balance of these two forces. Complete solution will occur when the swelling pressure entirely overcomes the cohesive forces.

A number of studies have been made of the swelling properties of collagen and gelatin. These are adequately reviewed by Gustavson (31). In their detailed study, Bowes and Kenten (7) demonstrated the marked difference in swelling of collagen at low pOH as compared with that at low pH (Fig XI). They also showed a decrease in cohesive forces is the most important factor in determining swelling of collagen in alkaline solutions.

The present study was carried out to determine the swelling properties of a tissue rich in elastin. Very few such studies have been previously reported. Roddy and O'Flaherty (86), (87) observed the swelling of elastin which occurs in hides during the chrome tanning process. Their work has already been referred to in the historical section (see thesis pg. 5). They pointed out the difference in swelling of elastin and collagen in both acid and alkaline solutions (elastin generally swells less). Lloyd and Garrod (51)

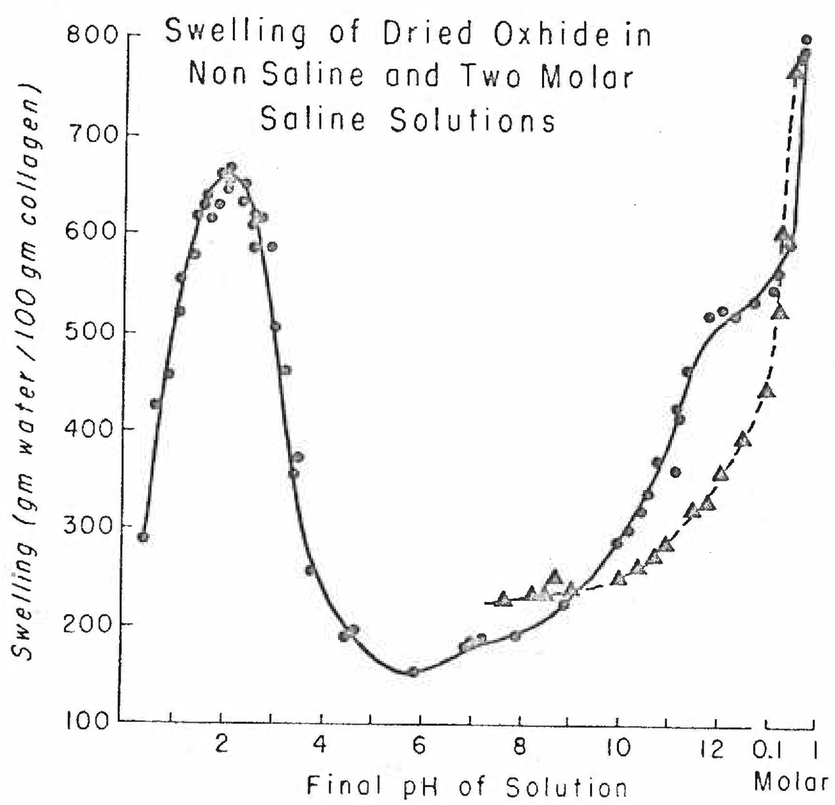
FIGURE XI

Swelling of dried ox hide in HCl-NaCl and NaOH-NaCl systems at 20° for 3 days.

● non-saline solutions

▲ 2M-NaCl solutions

Reproduced by permission of Dr. Joan Bowes, from Bowes & Kenten (6).



Swelling of Dried Oxhide in Non Saline and Two Molar Saline Solutions
Swelling (gm water / 100 gm collagen)
800
700
600
500
400
300
200
100
2
4
6
8
10
12
0.1
1
Final pH of Solution
Molar

reported briefly on the swelling of elastin in various solvents.

Three objectives were in mind at the start of this experiment:

1) To determine maximal swelling with regard to pH.

2) To determine if maximum solubilization occurs when there is maximum swelling.

3) To ascertain the effect of ionic strength on swelling and solubilization.

From the results of experiments described in sections I and II, it was considered likely that fetal ligament would contain a high concentration of soluble elastin relative to adult tissue. Therefore, nine month fetal as well as six month calf and adult ligaments were studied.

Materials and Methods

Materials

Fresh ligamentum nuchae samples were obtained from the abattoir as in the previous studies. After careful removal of all extraneous material, including surrounding fascia, the specimens were cut into 6 to 8 cm. segments and frozen, until such times as they were used for the swelling studies. Table 1 gives the composition of the materials used in the experiment.

Solutions

Swelling studies were carried out in unbuffered solutions of acid and alkali, ranging from 1N to 1μN HCl and 2N to 1μN NaOH and in distilled water. To study the effect of salt concentration, NaCl was added to these unbuffered solutions to a final concentration of 2M.

TABLE I

COMPOSITION OF BOVINE LIGAMENTUM NUCHAE

SOURCE OF LIGAMENT	NITROGEN (% dry wt)	MOISTURE (%)	TOTAL COLLAGEN (% dry wt)	INSOLUBLE ELASTIN (% dry wt)
Term Foetal Calf	15.51	75.0	15.2	47.6
6 month calf	16.71	56.2	12.3	74.0
Adult Cow	16.70	56.5	16.3	71.3

Swelling Procedure

Weighed samples (500-550 mg) of freshly thawed ligament were soaked in 200 ml. of the various solutions in beakers at room temperature, without agitation. A crystal of thymol was added to each solution to retard the growth of bacteria and molds. The beakers were sealed with Parafilm. After 3 days, the specimens were removed from solution, carefully blotted on filter paper with frequent turning for 5 minutes and reweighed. Determination of final wet weights proved difficult only at the very high pH range (greater than pH 12.5) of the non-saline solutions. In these cases the final material was very gelatinous and fragile. Final weights in this range were thus not very reliable and represent rough estimations. This problem of fragility of specimens did not exist with the solutions containing NaCl (2 molar) even at high pH values. The soaked ligament in these instances was a hard rubber-like substance which became progressively more dense as pH increased. All determinations of swelling were repeated at least twice for each type of tissue and solution.

Determination of pH

Determination of pH was carried out immediately on removal of the ligament from solution. A Beckman Zeromatic pH Meter was used with an Ag-AgCl reference electrode and a type E-2 Beckman glass electrode, to give an accurate coverage of almost the entire pH range. The meter was standardized at intervals of 2 pH units with prepared buffers as supplied by the manufacturer. Salt was added to the standard buffers to give a concentration of 2 M NaCl when determining the final pH of the saline solutions. At and above pH

13, meter readings were not considered accurate. The basicity above pH 13 was estimated by titrations of a sample of the solutions with standard 0.100 N HCl and phenolphthalein indicator after removal of the ligament remnants. High alkaline concentrations are expressed as normality rather than pH for this reason on all the graphs (Figs. XII, XIII, and XIV).

Determination of Solubilization

Nitrogen determinations were carried out on a sample of each of the solutions after removal of the ligament. The micro-Kjeldahl method of Ma and Zuazaga (53) was used. The amount of protein in solution was calculated on the basis of 16% nitrogen content.

Calculation of Swelling

Swelling was calculated for each sample as follows:

$$\text{percent swelling} = \frac{w-(a-b)}{a-b} \times 100$$

where a is the dry weight of the ligament, b is the amount of protein solubilized, and w is the wet weight of the swollen sample. Where complete solubilization of the sample occurred, swelling was assumed to be infinite, because in these cases it may be regarded that swelling has been sufficient to disperse the entire sample evenly throughout the solution. No correction for the osmotic effects exerted by solubilized protein against the unsolubilized portion was made. This was not considered to be important because the introduction of such factors would not alter the overall shape of the curves and would contribute little to the information being sought.

Results and Discussion

Swelling and Solubilization at acid pH (pH 0 - 6)

Without Salt. Adult, calf and fetal ligament have a maximal swelling (Figs. XIIa, XIIIa, and XIVa) and solubilization (Figs. XIIb, XIIIb, and XIVb) in the pH range of 2 to 4. The solubilization curve of fetal ligament shows two humps in this area but because of their small amplitude they are probably not of significance. The low degree of swelling observed at pH 4 to 6 corresponds to the isoelectric point of acid-solubilized elastin which has been reported in the pH range of 3.9 to 4.8 depending on the ionic strength of the solution (74).

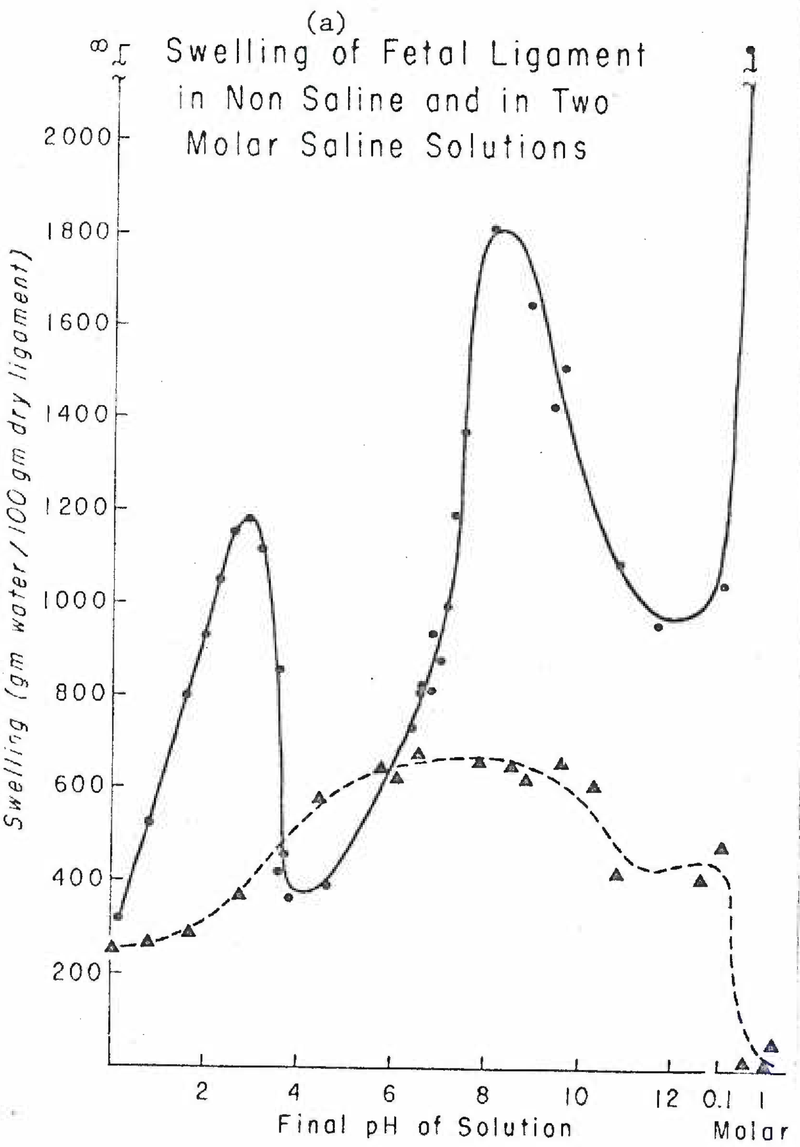
With 2M NaCl. Complete suppression of the swelling curve occurs for adult ligament (Fig. XIVa). Fetal and calf ligaments undergo a gradual increase of swelling with increase of pH, fetal ligament being more affected than calf (Figs. XIIa and XIIIa). At the isoelectric pH of these younger ligaments there is more swelling in the presence of salt than in its absence. This reversal of swelling curves at isoelectric pH is also manifest by some other proteins (70), and has been shown to occur with collagen as seen in Fig. XI (7).

Swelling and solubilization at neutral and mildly alkaline pH (pH 6 - 12)

Without Salt. Very marked swelling of fetal ligament occurs in this range, reaching a maximum in the pH range of 8 to 8.5 (Fig. XIIa). Solubilization correlates closely with swelling, both dropping off above pH 8.5 but at different rates (Fig. XIIa and b). Calf and adult ligaments (Figs. XIIIa and XIVa) show swelling properties similar to

FIGURE XII

Swelling (a) and solubilization (b) of fetal bovine ligamentum nuchae in non-saline [●] and in 2M-NaCl [▲] solutions at various pH values at 20° for 3 days.


(a)
Swelling of Fetal Ligament
in Non Saline and in Two
Molar Saline Solutions
Swelling (gm water / 100 gm dry ligament)
∞
2000
1800
1600
1400
1200
1000
800
600
400
200
2
4
6
8
10
12
0.1
1
Final pH of Solution
Molar

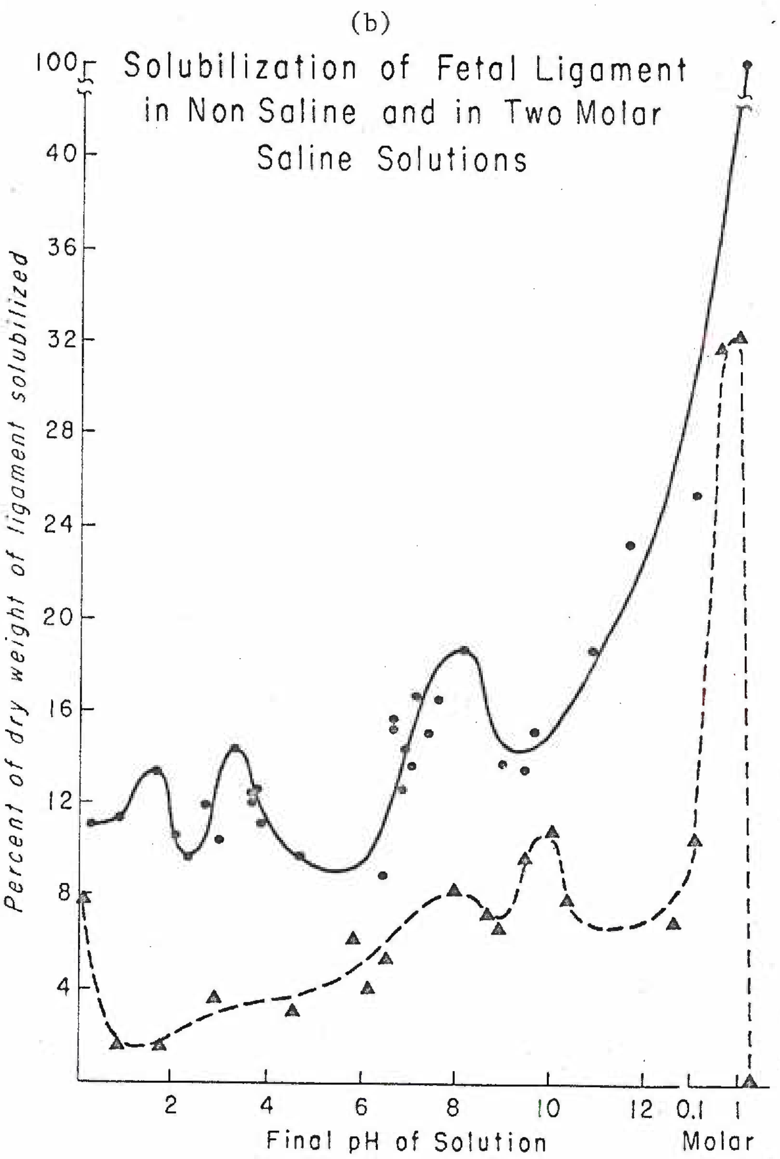
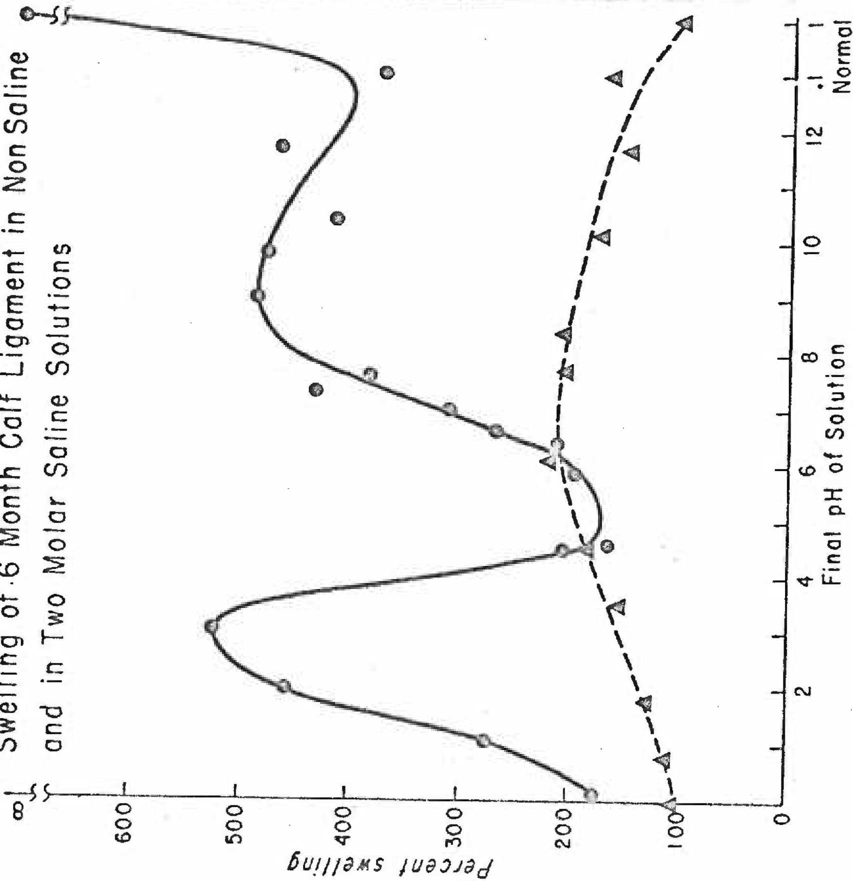

(b)
Solubilization of Fetal Ligament
in Non Saline and in Two Molar
Saline Solutions
Percent of dry weight of ligament solubilized
100
40
36
32
28
24
20
16
12
8
4
2
4
6
8
10
12
0.1
1
Final pH of Solution
Molar

FIGURE XIII

Swelling (a) and solubilization (b) of 6 month calf bovine ligamentum nuchae in non-saline [●] and in 2M-NaCl [▲] solutions at various pH values at 20° for 3 days.

(a)



(b)

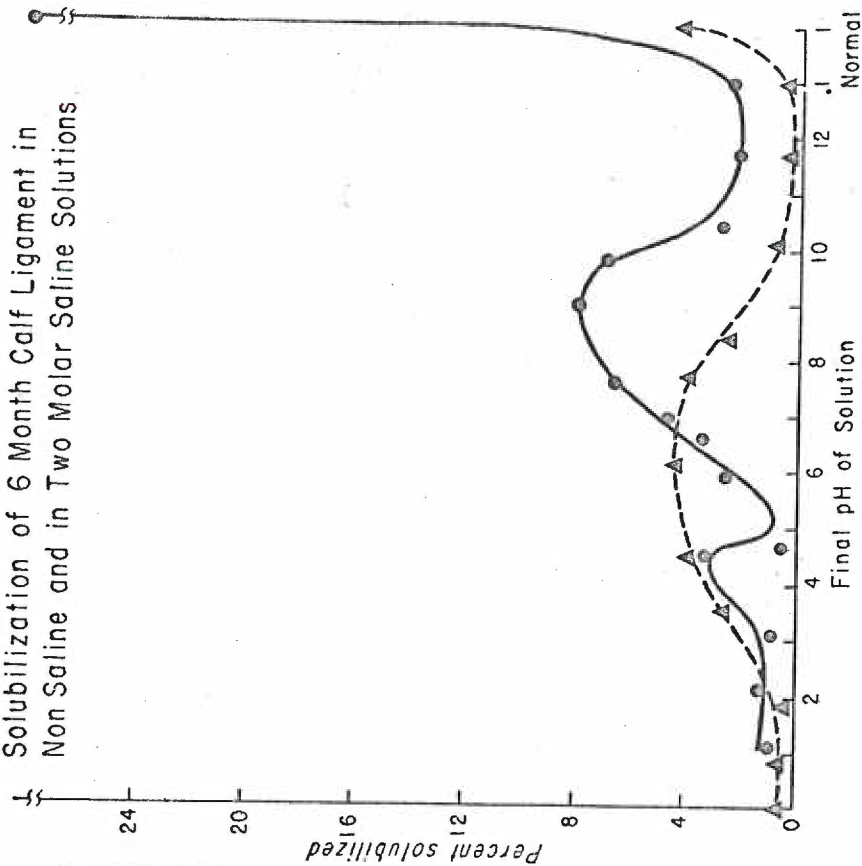
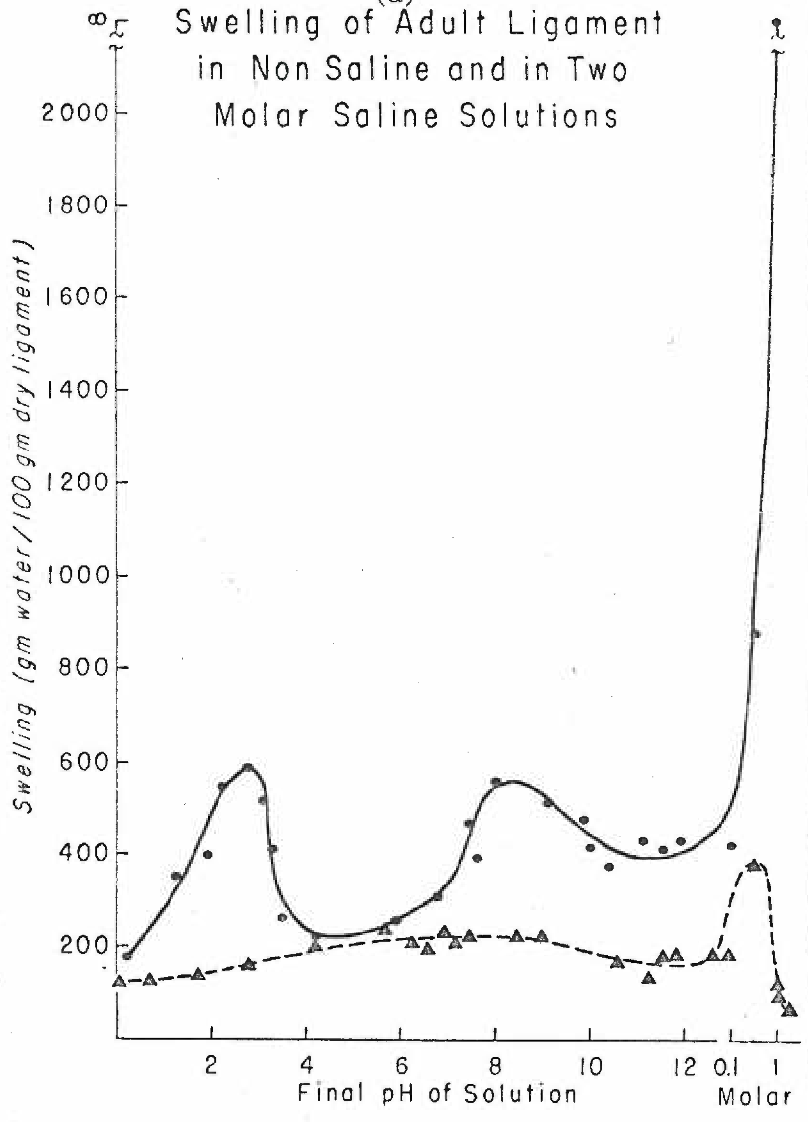
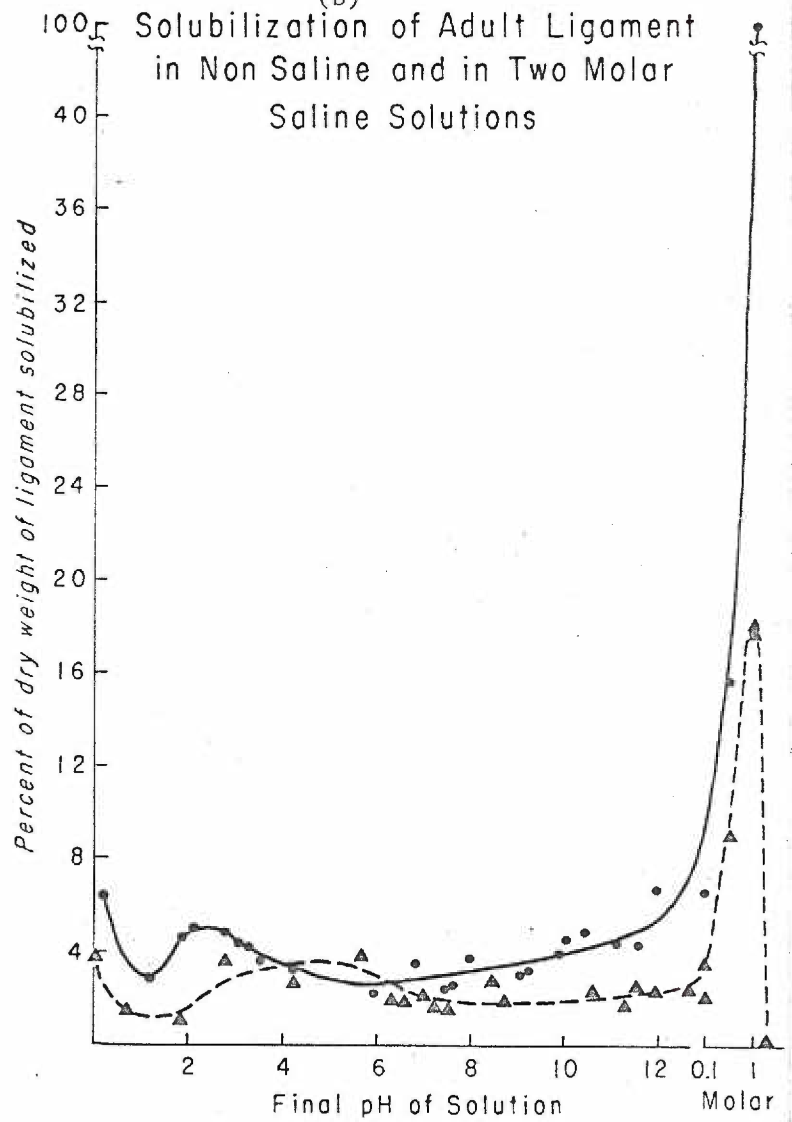


FIGURE XIV

Swelling (a) and solubilization (b) of adult bovine ligamentum nuchae in non-saline [●] and in 2M-NaCl [▲] solutions at various pH values at 20° for 3 days.


(a)
Swelling of Adult Ligament
in Non Saline and in Two
Molar Saline Solutions
Swelling (gm water / 100 gm dry ligament)
∞
2000
1800
1600
1400
1200
1000
800
600
400
200
2
4
6
8
10
12
0.1
1
Final pH of Solution
Molar


(b)
Solubilization of Adult Ligament
in Non Saline and in Two Molar
Saline Solutions
Percent of dry weight of ligament solubilized
100
40
36
32
28
24
20
16
12
8
4
2
4
6
8
10
12
0.1
1
Final pH of Solution
Molar

fetal ligament, but to a much smaller degree. Solubilization of adult ligament in this range (Fig. XIVb) is markedly different from that of fetal ligament (Fig. XIIb), in that there is virtually no increase above that observed at the isoelectric pH, whereas with fetal tissue there is twice as much solubilized protein at pH 8.4 as compared with pH 5.5. Calf ligament solubilization in this range is intermediate between the values for fetal and adult. The maximum swelling of all these tissues at pH 8.5 is not explained by the Donnan equilibrium as is the swelling at the low pH. If cohesive forces between molecules remain unaffected, the maximum swelling due to the Donnan equilibrium should occur at pH 11 to 12 (3). The observation is puzzling, and at this time I can offer no explanation of it.

With 2M NaCl. With all the ligaments there is complete suppression of the swelling that was observed in the absence of salt at pH 6 to 11 (Figs. XIIa, XIIIa, and XIVa). In general both swelling and solubilization are suppressed for all the tissues in this range but the degree of suppression is an inverse function of the age of the tissue, i.e. much more suppression in fetal tissue than adult.

Swelling and solubilization at very alkaline pH (above pH 12)

Without salt. A marked upward deflection occurs in this range in both the swelling and solubilization curves for all the ligaments (Figs. XII, XIII, and XIV). As with collagen, this may indicate weakening of the cohesive forces and probably some hydrolysis of peptide bonds, resulting in the dissolution of the elastin fibers. At

approximately 0.5 N NaOH all the tissues are completely solubilized. However, the solubilization curve for the fetal tissues has increased steadily from pH 9.0 upward whereas that of the calf and adult tissue increases sharply but not till above pH 12.

With 2M NaCl. Unusual phenomena are also manifest in this portion of the curves. There is a definite tendency towards increased solubilization and perhaps swelling also of all tissues above pH 12, but apparently the osmotic effect of the increased concentration of NaOH together with the presence of 2M NaCl is stronger than the ability of the alkali to solubilize the ligament. Thus at concentrations greater than 1N NaOH, in the presence of salt, there is complete "protection" against dissolution. Ligament taken from the 2N NaOH solution, washed and placed in a lightly buffered solution at pH 8.4 without salt, swells in the usual fashion and is not completely solubilized, as might have been expected if the presence of high salt concentration was preventing release of fragments of elastin resulting from hydrolysis of elastin at the high pH values.

The overall suppression of swelling in the presence of 2M NaCl is predictable for the Donnan equilibrium. However, the actual dehydration, which occurs above pH 12, is not predictable on this basis and is in marked contrast with the failure of salt to affect the swelling of collagen in this pH range (Fig. XI). However, it is possible the effect may be similar to the so-called "pickling" effect seen with collagen at pH 1 and salt concentrations greater than 1M (31) which also causes a dehydrating effect.

Summary

The swelling curves obtained in this study of ligamentum nuchae are markedly different from those previously obtained by Bowes and Kenten for collagen (Fig. XI). The presence of collagen in the ligaments is thus not likely to have had much effect on the behavior observed here. The main object of this study was to obtain information which would enable the choice of optimum swelling conditions for obtaining soluble elastin under mild non-hydrolytic conditions. This would allow the isolation of an elastin molecule analagous to tropocollagen. The results obtained suggest an aqueous solution at pH 8.4 would be suitable for this purpose. Under these conditions, it would be expected that soluble elastin, as well as soluble collagen and globular proteins, such as are found in other connective tissues (37) would be extracted.

On the basis of these data, the extraction procedures of Partridge, et.al., for preparation of pure elastin fibers (74) should be modified to use a low ionic strength pH 8.4 buffer rather than normal saline. In the studies to follow,this extraction procedure was utilized both for the preparation of pure elastins and for the solubilization of proteins from fetal ligament.

SECTION IV

A STUDY OF THE CHEMICAL COMPOSITION OF PURE ELASTINS

Introduction

Interest has been increasingly focused for the past three years on the effects of age on the amino acid composition of insoluble elastin. This interest was initiated by the discovery of the two cross-linking substances, desmosine and isodesmosine (103). Age change studies have shown a decrease in lysine as elastin ages and a concomitant increase in the desmosines (61) as well as X_4 (23). These studies have been carried out on aortic elastin of the chicken and the rat. However, there are no reported age change studies on ligamentum nuchae elastin even though this is the accepted standard of purity for elastins (72). As was indicated in the main thesis introduction, purified elastin is probably altered from its native form. Thus, it is unlikely the purified elastin will yield a completely accurate picture as regards the biochemistry of native elastin. However, since age changes have been reported to occur in the amino acid composition of the insoluble fiber, and since my interest in a soluble precursor of elastin has been focused on obtaining this substance from ligamentum nuchae, I felt a study of the age changes of bovine ligamentum nuchae insoluble elastins was imperative.

The questions raised at the onset of this study were as follows:

1) Do the cross-linking substances change with age in bovine ligamentum nuchae purified elastin as has been reported for aortic tissues in other animals?

2) Is it possible to use the cross-linking substances or

precursors of them as a unique marker for the identification of a soluble precursor of elastin?

3) Are there other amino acid changes, other than lysine, the desmosines, and X_4, associated with ageing elastins?

4) Can alterations in solubility of the elastins be attributed to amino acid changes which may be observed with ageing?

Materials and Methods

Preparation of Elastins

The elastins of this study were prepared by methods similar to those utilized in Study I, except that a pH 8.4 tris-buffered saline solution was used in place of the normal saline for the extraction procedure prior to autoclaving. This solution was made by preparing a 0.02M solution of tris [2-amino-2-(hydroxymethyl)-1,3-propanediol], adding salt to bring the total molarity to 0.14, and then titrating with 1.00N HCl to pH 8.4.

Hydrolysis of Elastins in Preparation for Amino Acid Analysis

Air dried elastins (10± 2 mg.) were carefully weighed into borosilicate hydrolysis tubes and 2 ml. of 6N HCl, previously degassed for 5 minutes by shaking in a flask evacuated by a water pump, was added to each tube. The air in the tubes was displaced with nitrogen, then evacuated to 50 mm Hg pressure with a water pump at which pressure the tubes were sealed. They were placed in a heating block maintained at 110±1° for 72 hours (64). Thomas et.al. have emphasized the necessity of a 72 hour hydrolysis of elastins because of slow release of lysine and cross-linking residues (103).

After completion of hydrolysis the solutions were taken to dryness on a rotary flash evaporator at 40° (64). The residues were redissolved in a small amount of water, made up to 10.0 ml. in volumetric flasks, filtered and frozen.

At the time a sample was weighed for hydrolysis, a portion was also taken (approximately 5 mg.) for evaluation of moisture content. Thus, the true dry weight of the hydrolyzed sample could be calculated, and a good measure of its purity as a protein obtained from the nitrogen value. The nitrogen determination on the hydrolysate was used to calculate the actual weight of the sample applied to the amino acid analyzer, since there was often some loss of sample at the time of hydrolysis.

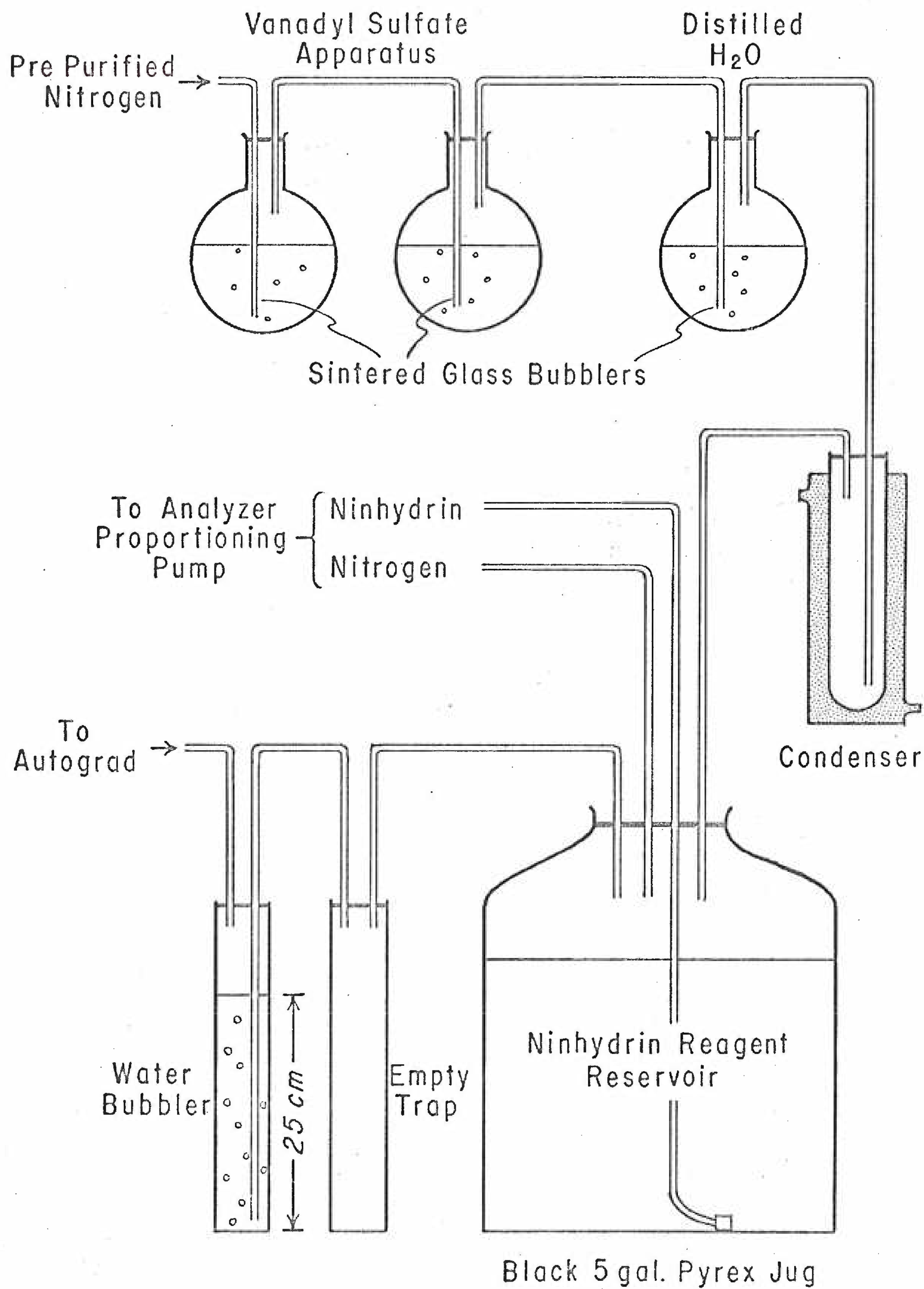
Amino Acid Analysis

Amino acid analyses were carried out on the Technicon 22 hour single channel system utilizing the identical platter set-up for the proportioning pump as described (79), (101). Several alterations were found to be necessary in the arrangement of the nitrogen supply, the buffer gradient, and the storage of ninhydrin to obtain reproducible and accurate results. Figure XV illustrates the method of storage of the working solution of ninhydrin, and the method of purification of the nitrogen supply.

The ninhydrin stock solution as described by Technicon was found to deteriorate rapidly on storage. The working solution, when properly stored, was stable for at least a month. Therefore, the following method was utilized for its preparation: 60 gm. ninhydrin, reagent grade (Pierce Chemical) and 6 gm. hydrindantin, reagent grade

FIGURE XV

Method of deoxygenation of the nitrogen supply, and storage of the ninhydrin reagent for the Technicon amino acid analysis system.


Vanadyl Sulfate
Apparatus
Distilled
H_2O
Pre Purified
Nitrogen
Sintered Glass Bubblers
To Analyzer
Proportioning
Pump
Ninhydrin
Nitrogen
Condenser
To
Autograd
Water
Bubbler
25 cm
Empty
Trap
Ninhydrin Reagent
Reservoir
Black 5 gal. Pyrex Jug

(Pierce Chemical) were added to 2250 ml. methyl cellosolve, technical grade peroxide free (Union Carbide). Nitrogen was rapidly bubbled through this mixture during dissolution. To this solution was added 850 ml. 4 M sodium acetate buffer (described in Technicon literature) to bring the total volume to 3100 ml. Nitrogen was again bubbled rapidly through the solution for 15 minutes. Twelve and four tenths liters of 50% methyl cellosolve in water was poured into a 5 gallon pyrex water jug which was completely covered on its exterior with black vinyl electrical tape to exclude all light. Nitrogen was bubbled through this for 15 minutes, after which the concentrated ninhydrin solution was added to give 15.5 liters of working solution.

A batch of working solution was found sufficient for 11 analyses. Thus, the usual procedure was to carry out 5 sample analyses, a standard analysis (18 standard amino acids supplied by Technicon), and then an additional 5 samples. New manifold tubings were installed with each batch of ninhydrin solution. Internal norleucine standards were applied to the column with all analyses of all samples and standard to correct for any changes in color yield over the 11 runs.

The buffer gradient system as described by Technicon was used except that 5 ml. methanol was added to chambers 1 and 2 of the autograd and 5 ml. less of the pH 2.875 buffer used to accomodate the methanol.[3] This was necessary to obtain a complete separation of threonine and serine.

[3]Personal communication, G. Winters of Technicon Corp.

High purity of the nitrogen with respect to oxygen was found necessary to the extent that commercially available pre-purified nitrogen, reported to contain less than 10 p.p.m. oxygen, was inadequate. Further purification was accomplished by passing the nitrogen through the vanadyl sulfate deoxygenation apparatus pictured in Fig. XV, described by Meites and Meites, (60).

All elastins were subject to at least 2 and usually 3 analyses consisting of a high load (600 μgm.) and 1 or 2 low loads (200 μgm.). This was found necessary for accurate results because of the great range of content of different amino acids which occur in elastin. The one channel colorimeter-recorder could not satisfactorily resolve this range of amino acids in a single analysis. The amino acid analysis chromatograms were integrated for determination of amino acid content as described by Spackman, Stein, and Moore (97).

Hydroxyproline and proline determinations were carried out manually because of the limitations of the single channel automated system. Hydroxyproline evaluation was carried out by the Woessner II method (110), and proline by the Troll and Lindsley method (105), with modification. Automated detection of proline appears to be a satisfactory procedure for elastin hydrolysates when using a multichannel recording system. However, hydroxyproline evaluation is best carried out manually since it is in such low concentration, and the yellow ninhydrin color yield is poor. The hydrolyzed samples prepared for amino acid analysis were utilized for estimation of hydroxyproline and proline content. The samples and standards were diluted to contain 0.5 to 4 μgm. of hydroxyproline or proline per ml.

Modified Woesner II Method for Determination of Hydroxyproline

The Woesner II method is utilized where hydroxyproline is present in a concentration of less than 2%, i.e. the remaining 98% constitutes other ninhydrin positive substances. Its greatest accuracy is in a concentration range of 1 to 3 μgm. per ml.

Twenty-four tubes were prepared at a time. These consisted of 2 water blanks, 4 standards in duplicate, and 8 samples in duplicate. Reagents and procedures were identical to the Woessner I method through the water bath stage (see thesis pg 17). The samples were divided in half after cooling, taking the 2 blanks, 4 duplicate standards and 2 of the duplicate samples in the first lot. This division was necessary because there was fading of color beginning 10 minutes after the benzene extraction step. 9.5 ml. of benzene was added to the first 12 tubes which were then covered with parafilm and shaken briskly for about 5 seconds. The bulk of the benzene was removed by use of a Pasteur pipette coupled to a water pump. The extraction was repeated with another 9.5 ml. of benzene. After shaking, most of the benzene was again removed, and the tubes were centrifuged at low speed to obtain complete separation of the two liquids. A pipette was carefully introduced into the water layer and exactly 3.5 ml. were withdrawn for transfer to a cuvette. If the solutions in the cuvettes were cloudy, momentary immersion in hot water cleared them. The absorbancy of the samples was determined at 557 mμ. on a spectrophotometer. A blank value was obtained by adding 0.2 ml. 30% hydrogen peroxide to each cuvette with mixing. Exactly 5 minutes after the addition of peroxide the absorbancies were

re-determined at the same wave-length. A standard curve was prepared by subtracting the peroxide blank absorbancy from the initial absorbancy for each standard concentration used. The second lot of samples was treated in the same way. There were no observed variations of the 2 water blanks after peroxide treatment, so these were re-used for the second lot. No more than one hour was allowed to elapse from the time the samples were taken from the water bath until all readings were completed. Twelve cuvette tubes were necessary for this test. The Woessner I method and the Troll Lindsley method were preferably carried out using only two cuvettes.

Woessner suggests the application of a small empirical correction factor for the fading of interfering chromagens produced by other amino acids. These calculations were carried out with each run. The factor usually was so low that its use did not change the answers significantly.

Troll and Lindsley Method of Proline Determination (Modified by Gilbert)

The published description of the Troll and Lindsley method (104) is inadequate, so it will be described here in full with modification.

Reagents. Permutit resin (according to Folin) was obtained commercially, washed 2 to 3 times with distilled water and dried. Citrate buffer was prepared by dissolving 20.67 gm. $Na_2 HPO_4 \cdot 7H_2O$ and 12.94 gm. citric acid in water and bringing the total volume to 1 liter. pH was adjusted to 4.0. Ninhydrin solution was prepared by dissolving 1.25 gm. ninhydrin, reagent grade (Pierce Chemical) in 30 ml. glacial acetic acid by heating to exactly 60°. After cooling, 20 ml. of 6M phosphoric acid was then added (prepared by diluting 69.2 ml. 85% phosphoric acid

to 100 ml. with water). Proline standards of 1 to 5 μgm. per ml. were prepared from stock in a similar manner to the preparation of hydroxyproline standards for the Woessner methods (see thesis pg 17). A lysine standard of 10 μgm was also prepared in this way.

Procedure. Samples and standards of proline and lysine were diluted to proper concentrations with the citrate buffer. Fifteen ml. of the standards and samples were placed in 16 × 150 screw top tubes fitted with teflon lined caps. Three quarters ml. of Permutit resin, dry volume measured in a 15 ml. conical centrifuge tube, was added to each tube. The tubes were tightly capped and shaken *vigorously* for 15 minutes on a horizontally oscillating mechanical shaker. Two 5.0 ml. portions of each of the samples and standards were transferred to 25 × 200 mm. screw top tubes. Two citrate buffer blanks were also included. Glacial acetic acid and ninhydrin reagent were added to each tube in 5.0 ml. quantities. The tubes were then capped loosely and placed in a 95° water bath for one hour. After cooling, 5.0 ml. benzene was added to each tube followed by 15 minutes of *vigorous* shaking. During this stage color which had developed transferred to the benzene layer. Good separation of phases was effected so there was no need for centrifugation. The benzene layer of each tube was withdrawn and its absorbancy determined at 515 mμ. on a spectrophotometer. The two blanks were first read against each other, one blank was then discarded and the empty cuvette used for reading the remaining samples. If the lysine standards gave a reading above the blank value, the entire test was repeated, because

this indicated the Permutit resin had not adequately removed the other amino acids responsible for the interfering chromagens. The reproducible accuracy was better than ±2% using the above modifications.

Determination of Total Nitrogen Content of Elastins and Hydrolysates of Elastins

The Technicon automated nitrogen method was utilized with modification. This is an adaption of the alkaline phenol-hypochlorite reaction described by Jane Russel (88). Digestions were carried out manually using 2 ml. concentrated sulfuric acid and 2 gm. potassium sulfate (low N content) to raise the boiling temperature of the acid. No oxidants were used. Digests were refluxed for a full 2 hours after the initial clearing period. After cooling the digests were diluted to a nitrogen concentration of 3 to 12 µgm. per ml.

Nitrogen standards were prepared by dissolving ammonium sulfate in 10% sulfuric acid to give a nitrogen concentration of approximately 150 µgm. per ml. 1.00 ml. of this solution was pipetted into each of 100 10 × 75 mm acid washed pyrex test tubes. Tubes were then sealed with a rounded end to allow ease of washing out the standard when opened. Randomly selected tubes were standardized by the micro-Kjeldahl distillation apparatus (53). Immediately prior to a series of nitrogen determinations an ampoul of standard was opened and diluted with double distilled, deionized, boiled water to give four standards ranging from 2 to 15 µgm. nitrogen per ml. This "special water" was also used to dilute the samples appropriately.

The technicon platter arrangement for development of the chromagen was modified by adding an additional long mixing coil and changing the tubing sizes in the proportioning pump so that only

1 ml. of standard or sample was utilized from each sample cup. This platter set up is diagrammed in Figure XVI. The concentrated sodium hydroxide and alkaline phenol reagents were prepared as described in the Technicon publication. Household Chlorox bleach was used for the sodium hypochlorite solution. Reagents appeared to be stable indefinitely when stored in polyethylene screw-top bottles. The analyzer was operated at a rate of 40 samples per hour. Samples were arranged in sets of 3 (triplicate determinations). Two water blanks were placed between each set of samples. Slightly better results seemed to occur when the reagents were chilled. Manifold tubing in the proportioning pump was changed with each 300 to 400 determinations.

Amide Nitrogen and Ash Determinations of Elastins

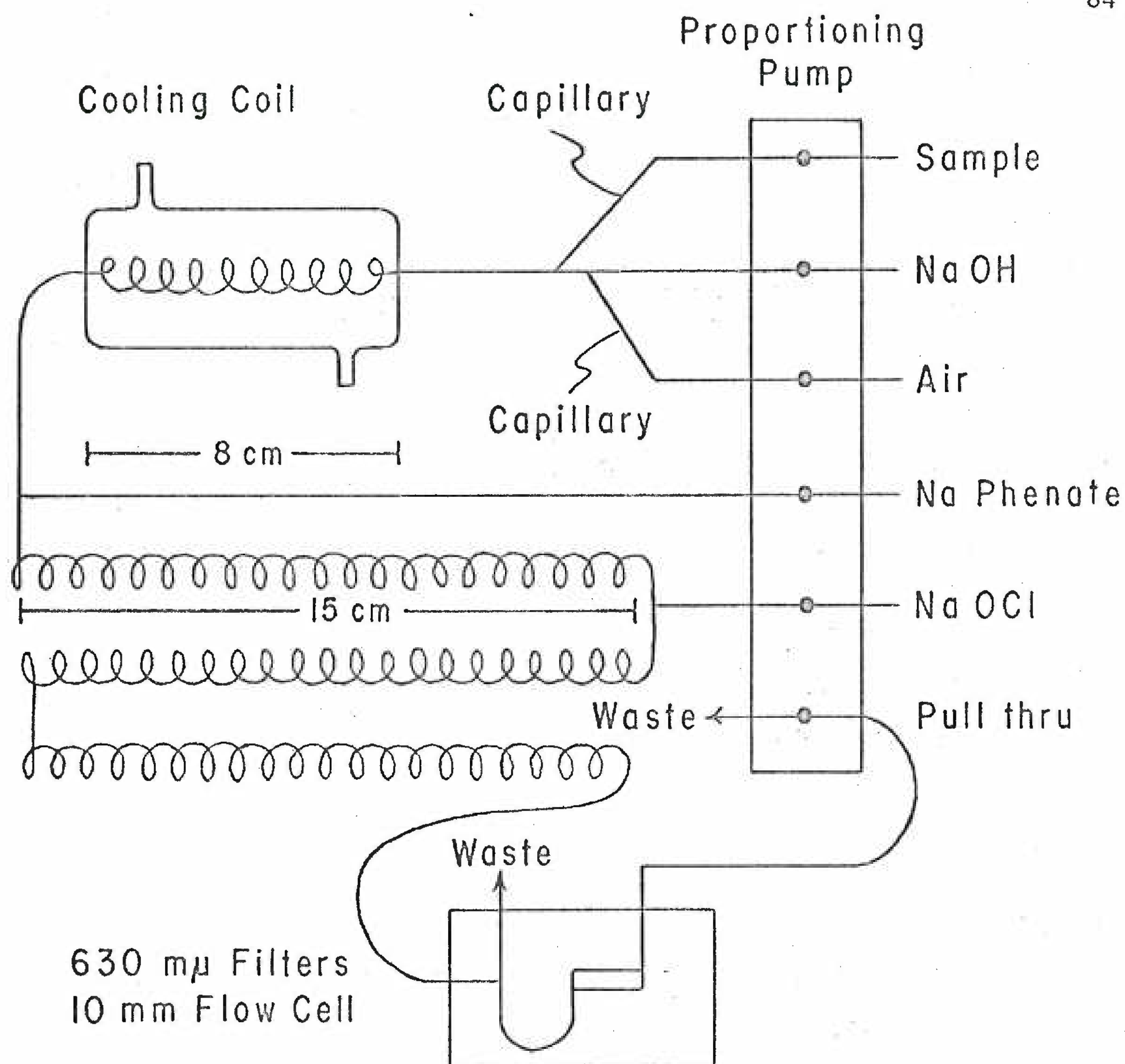
Eastoe and Courts (17) have given a very good description of these determinations. Their methodology was followed except that small samples were utilized (approximately 100 mg.). All samples had been subject to Wiley Mill treatment with a 60 mesh screen. Distillations for the amide nitrogen determinations were carried out on the same apparatus used for micro-Kjeldahl distillations.

Sodium Hydroxide Treatment of Purified Elastins

Finely powdered elastins (previously put through a Wiley Mill with a 60 mesh screen) were weighed out in 500 mg. quantities at room humidity and suspended in 10.0 ml. of 0.1N NaOH. Samples were simultaneously weighed for determination of dry weight. The NaOH suspensions were heated in a water bath at 98° for 50 minutes. After cooling they were centrifuged, the supernatants discarded, and the residues washed twice with cold 0.1N NaOH and twice with distilled

FIGURE XVI

Platter arrangement for automated determination of nitrogen on the Technicon analyzer using the Na phenate-hypochlorite reaction.



	Tygon Tube Color Code	Flow Rate ml / min
Sample	Yellow	1.20
NaOH	Green	2.00
Air	White	0.60
Na Phenate	White	0.60
NaOCl	Black	0.32
Pull thru	Purple-Black	2.90

water. They were then dehydrated by the usual method of 50% ethanol-ether followed by pure ether. After air drying, the elastin residues were transferred to weighing bottles for oven drying at 100° for 24 hours to determine dry weight.

Results and Discussion

Automated Determination of Total Nitrogen

Because the amino acid analyses were being carried out on substances of varying amino acid composition, the determination of total nitrogen content was necessary as a measure of purity. It will be noted later that the recoveries of total amino acids during the analyses varied considerably. To be assured that a portion of the sample had not been lost during the hydrolysis procedure, nitrogen determination was also carried out on the HCl hydrolysates of elastin. The original Technicon system for nitrogen determinations involves an automatic digestion apparatus using a mixture of sulfuric acid, perchloric acid, and selenium oxide as a digestant. The sensitivity of this system is reported to range from 10 to 100 μgm. nitrogen per ml. Preliminary tests using the Technicon digestion mixture for the Kjeldahl method gave poor agreement between identical samples (±10%) regardless of digestion times or the amount of digestant solution used. No doubt the automated system would improve these results, but not to the level of accuracy obtainable with the simple Kjeldahl digestion and distillation procedure. Other oxidants and accelerators were tried with sulfuric acid giving somewhat better results. Bradstreet (8) has stressed that there is no substitute in the Kjeldahl digestion procedure for prolonged boiling with sulfuric acid and

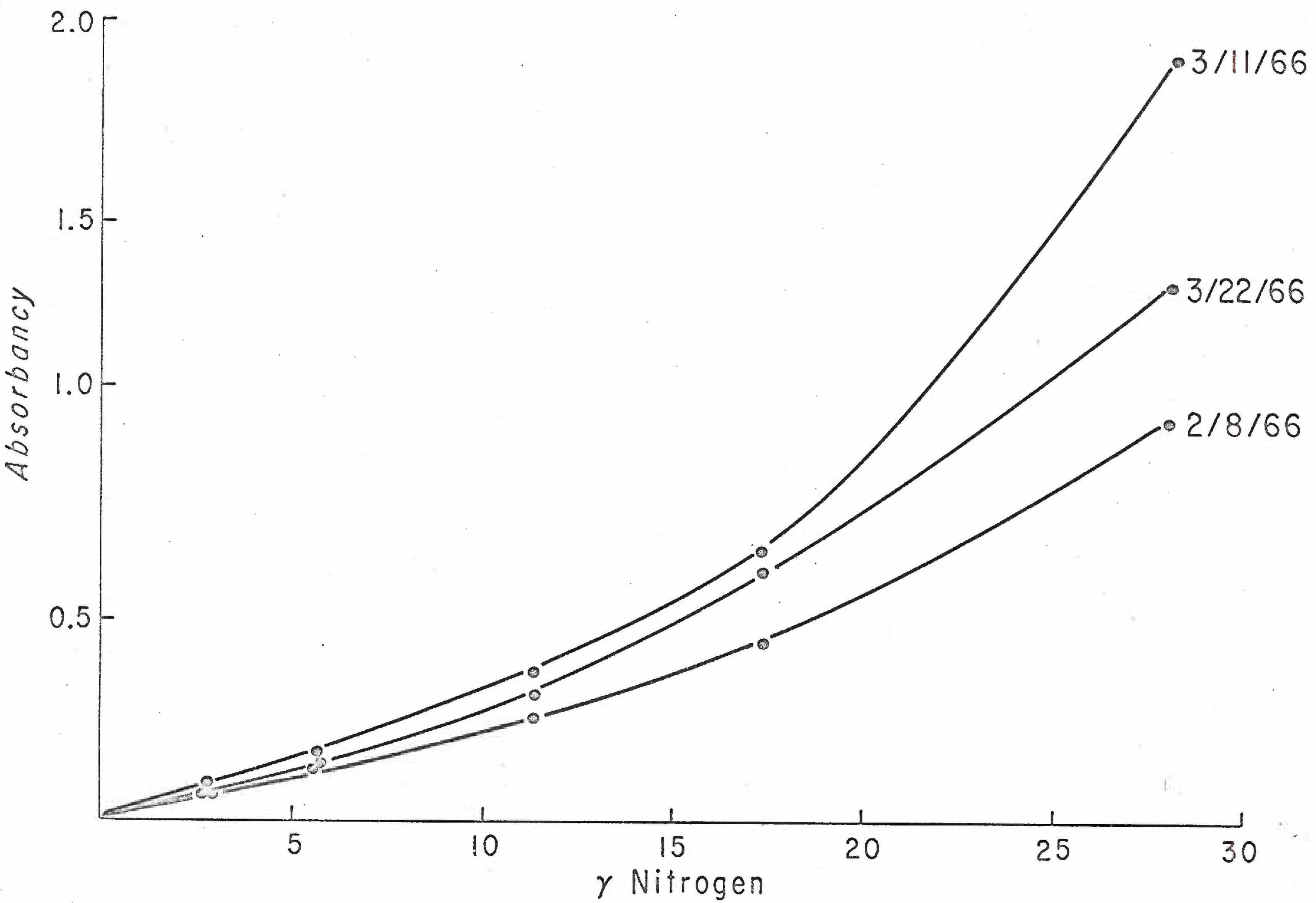
potassium sulfate. This statement is confirmed by my results in which 5 samples of elastin (10 mg. per sample) were digested for 2-1/2 hours in 2 ml. of sulfuric acid containing 2 gm. of potassium sulfate to elevate the boiling temperature approximately 50°. Using the sodium phenate-hypochlorite colorimetric reaction, results agreed to ±0.1% when the nitrogen concentration of the diluted digests was between 2 and 12 μgm. per ml.

Figure XVII contains the curves from the standards of 3 consecutive nitrogen determinations (3 days of operation) using the same reagents, but with fresh standards prepared for each days analyses. It is noted that there is considerable spread of the curves at the highest standard concentration. The same manifold tubings were used for the 2/8 and 3/11 runs. The old tubing may account for the high values of the 3/11 run. With the use of fresh tubing for each days operation, standards fall in the range of the 2/8 and 3/22 values. The 3/22 run was tested for accuracy at the high concentrations. In the range of 2 to 28 μgm. per ml., accuracy was only ±3%. In the range of 2 to 17 μgm. per ml., accuracy increased to ±0.9%. Although tests were not carried out extensively at the higher concentrations, it would seem possible to improve the readings in this area with further modification of the platter design.

Because of the great sensitivity of this test, it was difficult to store standards for more than a few days. The preparation of a large batch of standards in sealed vials seemed the best solution to this problem.

FIGURE XVII

Curves from the standards of three consecutive series of nitrogen determinations on the Technicon analyzer using the Na phenate-hypochlorite reaction. These series were three days of operation using the same reagents with fresh standards prepared for each day.


2.0
1.5
1.0
0.5
Absorbancy
5
10
15
20
25
30
γ Nitrogen
3/11/66
3/22/66
2/8/66

It was most feasable to begin digestion of samples several days prior to the actual colorimetric determination, providing the digests were stored in glass stoppered vials. Operation of the analyzer at 40 samples per hour allows approximately 300 single determinations in a days time. Samples were determined in triplicate with two water blanks. This amounted to 50 samples per day plus two series of standards. Preparation of digests and calculations involved approximately one days work for 50 samples. The determination of a similar number of samples in duplicate by the Kjeldahl method required 8 to 9 days, and gave poorer reproducibility. Thus, this method would appear to be an advantage in any laboratory requiring a large number of precise nitrogen analyses.

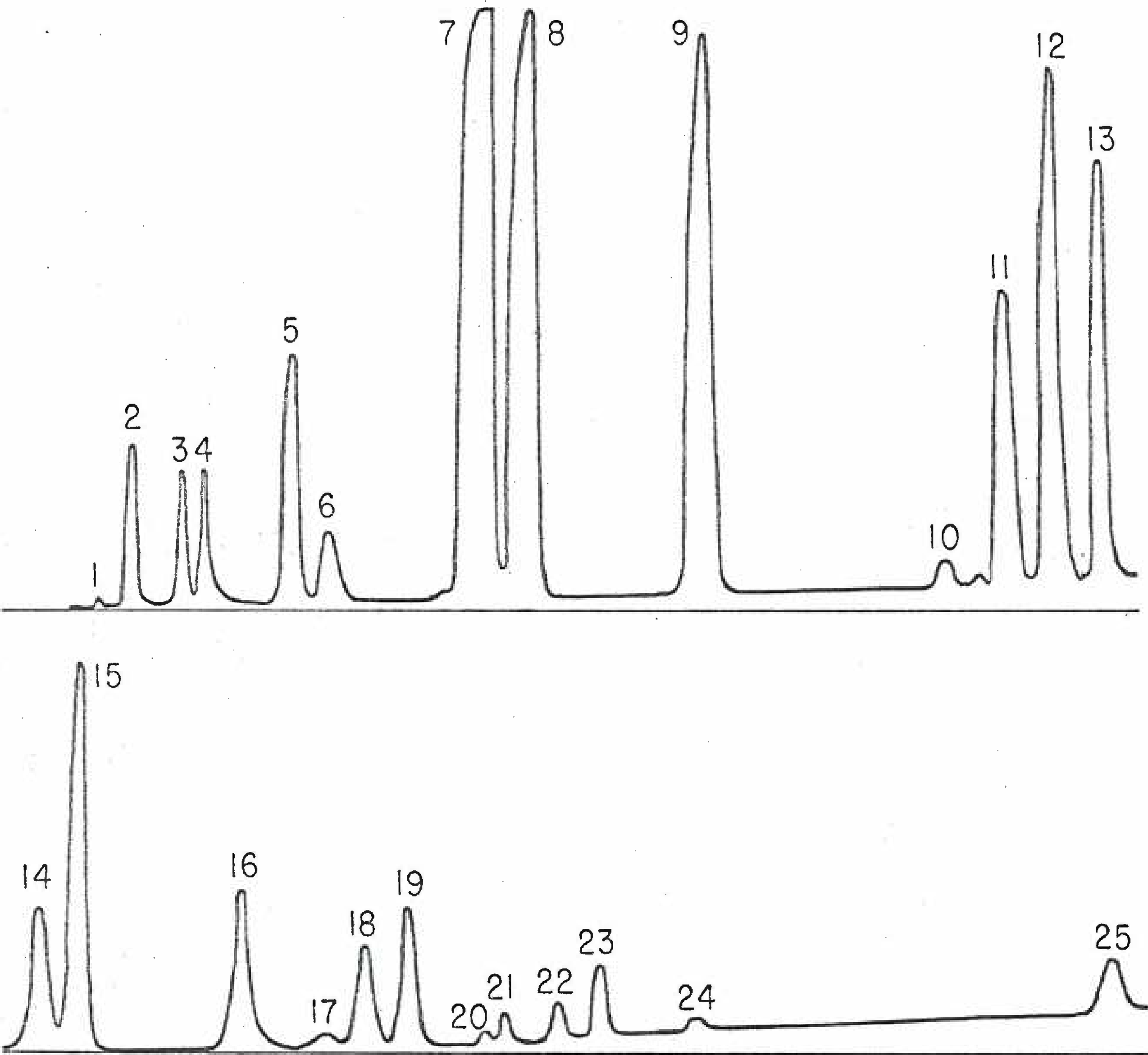
Method of Amino Acid Analysis

A typical amino acid analysis on elastin, represented by 9 year old cow ligamentum nuchae elastin, in a load of 600 μgm. is reproduced in Figure XVIII. There is complete separation of all the standard amino acids as well as the recognized "cross-linking substances" which are eluted between phenylalanine and lysine. The Technicon 6-1/2 hour system (102) as used by some investigators in connective tissue research gives incomplete separation of desmosine and isodesmosine. The "X_3" substance (discussed in Section V, pg 111) is not recognizable on the accelerated system.

The results of amino acid analysis can be expressed in gm. amino acid per 100 gm. protein (gm. %), gm. residues amino acid per 100 gm. protein (gm. res. %) or residues amino acid per 1000 total residues (res./1000). Most investigators involved in structural

FIGURE XVIII

A typical amino acid analysis represented by nine year old cow ligamentum nuchae elastin, in a load of 600 µgm. using the Technicon 22 hour analysis system with alterations as described in the text.


1
2
3 4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
1. Hydroxyproline
2. Aspartic acid
3. Threonine
4. Serine
5. Glutamic acid
6. Proline
7. Glycine
8. Alanine
9. Valine
10. Alloisleucine
11. Isoleucine
12. Leucine
13. Norleucine (internal standard)
14. Tyrosine
15. Phenylalanine
16. Ammonia
17. X_2
18. Isodesmosine
19. Desmosine
20. X_3
21. Ornithine
22. Lysinonorleucine (X_4)
23. Lysine
24. Histidine
25. Arginine

studies of proteins choose to express their results in res./1000. This enables the frequence of occurrence of a particular amino acid to be easily appreciated. It must be remembered, however, these investigators are dealing with proteins of homogenous composition and a known molecular weight. Such is not the case with elastin. A further point of consideration is that recoveries of the amino acids from elastin hydrolysates, particularly those of early fetal origin, is often poor (Table II). Eastoe and Leach (18) state the following regarding expression of amino acid composition in terms of res./1000: "This method of expressing analytic values is clearly valid only where the recovery of amino acids is substantially complete as indicated by total nitrogen recovery close to 100%." For these reasons amino acid analyses results in this thesis are expressed in terms of gm. res. %. In this way a rapid assessment of recoveries can be made by simply totaling the values for all the amino acids present in a particular analysis.

Results of Amino Acid Analysis

Amino Acid Analyses results are expressed in Tables II and III. Table II is a comparison of the amino acid analysis of various ages of fetal bovine ligamentum nuchae elastins. Also included are analyses of adult elastins. One adult analysis is from tissues which I collected; the other is that reported by Partridge for a similar substance (73). Table III represents a comparison of amino acid analyses of purified elastins of bovine ligamentum nuchae origin before and after hot alkali treatment. Each column in the above tables represents an average of three determinations. Agreement between

TABLE II

AMINO ACID COMPOSITION OF BOVINE LIGAMENTUM NUCHAE ELASTINS IN GM. RES. %

Amino Acid	4 mo. Fetal	6 mo. Fetal	7 mo. Fetal	8 mo. Fetal	9 mo. Fetal	1-2 yr. Adult	Partridge Adult (73)
OHpro	1.18	1.71	2.45	0.86	1.38	0.73	1.4
Asp	3.58	2.87	1.79	0.77	1.05	0.67	1.0
Thr	1.61	1.28	1.21	0.70	0.72	0.52	1.0
Ser	0.73	0.54	0.62	0.36	0.44	0.42	0.8
Glu	6.70	5.19	3.97	2.86	2.43	2.08	2.3
Pro	7.35	12.40	9.39	11.65	12.77	13.52	10.8
Gly	9.52	13.45	17.36	22.59	17.37	20.99	20.5
Ala	8.59	11.69	14.99	18.63	14.21	17.16	18.4
Val	8.49	10.66	12.69	15.53	13.58	16.00	14.4
Cys	Trace	-----	-----	-----	-----	-----	----
Met	0.69	-----	0.20	-----	-----	-----	----
Ileu	3.46	3.38	3.35	3.39	2.78	3.13	3.2
Leu	6.97	7.49	7.71	7.96	7.08	7.72	7.7
Tyr	1.97	2.04	1.79	1.36	1.14	1.36	1.1
Phe	3.92	4.13	4.37	4.62	4.26	4.99	5.5
NH_3	0.57	0.73	0.67	0.58	0.47	0.62	
Ides	0.25	0.43	0.62	0.85	0.81	0.69	
Des	0.30	0.52	0.72	1.13	0.88	0.98	
X_3						-----	
Orn	0.27		0.16			-----	
X_4	Trace	Trace	0.16	0.23		0.18	
Lys	4.06	3.06	1.75	0.69	1.03	0.44	1.0
His	0.98	0.62	0.36	Trace	0.18	-----	0.3
Arg	4.41	3.43	2.05	0.94	0.86	0.71	1.1
Totals	75.60	85.62	88.38	95.70	83.44	92.91	90.5
% Total Nitrogen	16.16	16.67	15.72	17.00	15.51	16.70	

TABLE III

AMINO ACID COMPOSITION OF BOVINE LIGAMENTUM NUCHAE ELASTINS
FROM VARIOUS AGES IN GM. RES. %
A.E. = AUTOCLAVED ELASTIN
A.+N.E. = AUTOCLAVED & NaOH TREATED ELASTIN

Amino	6 mo. Fetal		9 mo. Fetal		3 mo. Calf		1-2 yr. Adult		9-10 yr. Adult	
Acid	A.E.	A.+N.E.	A.E.	A.+N.E.	A.E.	A.+N.E.	A.E.	A.+N.E.	A.E.	A.+N.E.
OHpro	1.71	1.81	1.38	0.79	0.92	0.98	0.73	1.31	1.44	1.49
Asp	2.87	2.68	1.05	0.89	0.72	0.72	0.67	0.67	0.67	0.66
Thr	1.28	1.22	0.72	0.56	0.49	0.57	0.52	0.59	0.93	0.54
Ser	0.54	0.83	0.44	0.42	0.19	0.48	0.42	0.30	0.85	0.44
Glu	5.19	4.44	2.43	2.22	2.50	2.04	2.08	2.01	2.34	2.06
Pro	12.40	9.21	12.77	11.91	11.69	13.02	13.52	13.47	12.77	12.81
Gly	13.45	14.28	17.37	20.20	20.74	21.01	20.99	19.23	20.95	22.51
Ala	11.69	12.24	14.21	16.44	17.33	17.51	17.16	16.13	17.18	18.68
Val	10.66	11.20	13.58	14.68	14.99	15.30	16.00	13.73	15.98	15.75
Met										
Ileu	3.38	3.60	2.78	2.94	2.87	3.04	3.13	2.49	3.29	3.04
Leu	7.49	7.94	7.08	7.56	7.76	7.74	7.72	6.93	8.27	8.02
Tyr	2.04	2.08	1.14	1.34	1.31	1.30	1.36	1.02	1.50	1.26
Phe	4.13	4.56	4.26	4.84	4.49	4.96	4.99	4.19	4.71	4.99
NH_3	0.73	0.88	0.47	0.61	0.59	0.52	0.62	0.60	0.72	0.52
Ides	0.43	0.47	0.81	0.87	0.82	0.87	0.69	0.61	0.55	0.61
Des	0.52	0.50	0.88	1.06	0.88	1.26	0.98	0.81	0.82	0.91
X_4	Trace	0.10		0.20	0.23	0.22	0.18	0.25	0.14	0.15
Lys	3.06	3.16	1.03	0.86	0.69	0.66	0.44	0.45	0.37	0.38
His	0.62	0.55	0.18			Trace				Trace
Arg	3.43	2.83	0.86	0.82	0.94	0.76	0.71	0.78	1.06	0.71
Total	85.62	84.58	83.44	89.21	90.15	92.96	92.91	85.57	94.54	95.53

runs on the same sample was generally better than ±2%. However, some of the amino acids present in small quantity, e.g. the desmosines, X_4, etc., were only reproducible to within ±5%. It will be noted there is good agreement between the analysis of the two adult elastins reported in Table II.

Total amino acid nitrogen recoveries in the 4 and 6 month fetal elastins is very low. As age progresses, recoveries improve (Tables II and III). This in itself is justification for the expression of results in gm. res. %, since I am representing the actual amount of an amino acid present in an elastin sample and not the ratio of the amino acid residues to the total number of residues present. The significance of these lower recoveries is not understood at this time. There may be some properties of early elastin which makes them extremely resistant to acid hydrolysis. Also they may contain other nitrogenous substances which do not react with ninhydrin.

A comparison of the contents of desmosine and isodesmosine with the contents of lysine in the autoclaved elastins represented in Table II is summarized below.

Age of Animal	Totals of Des. & Ides.	Lysine
4 mo. fetal	0.55 gm. res. %	4.63 gm. res. %
6 mo. fetal	0.95	3.06
7 mo. fetal	1.34	1.75
8 mo. fetal[4]	1.98	0.69

[4]The results of this analysis are not consistent with the trends observed in the other analyses. Although these values represent an average of 3 runs, they were carried out on only one elastin sample. This 8 month fetal analysis should therefore be disregarded.

Age of Animal	Totals of Des. & Ides.	Lysine
9 mo. fetal	1.69	1.03
Adult	1.67	0.44
9 y/o cow	1.37	0.37

It is apparent that the content of the desmosines in bovine ligament elastin increases during fetal development but has reached adult levels by the end of the eighth fetal month. There seems to be a fall in this value in the older adult samples. The lysine content continues to decrease throughout life so that in the very old animal its value is less that 10% of that present in the 4 month fetal elastin. These results are at variance with those of other investigators reporting age changes in aortic elastins of the rat (23), and the chicken (61). Both these animals are reported to show a progressive increase in desmosine and isodesmosine concentration during the first year of life. Miller, Martin, Mecca, and Piez (62) demonstrated a quantitative conversion of lysine to the desmosines in chick embryo aortic organ cultures. The results shown in Figures II and III would indicate the metabolism of lysine, at least in bovine ligamentum nuchae elastin, is more complex than the simple relationships demonstrated by organ culture studies.

Preliminary studies on post-partum porcein aortic elastin (upper thoracic) verify the observations made on bovine ligamentum nuchae. The results of this study are summarized below in terms of desmosine, isodesmosine and lysine concentration changes with age.

Age of Animal	Totals of Des. & Ides.	Lysine
10 days	1.83 gm. res. %	0.93 gm. res. %
52 days	1.89	0.91
90 days	1.89	0.74
180 days	1.84	0.70

A progressive decrease in lysine content is also observed in the elastin of this species over the first 180 days of life even though the desmosine and isodesmosine contents are stable. It would thus appear that lysine is being utilized for other purposes, perhaps for the production of other types of cross links, this utilization continuing after cross-linking via desmosines is completed.

Amide Nitrogen Determinations

These results are tabulated in Table IV in gm. res. % and at the bottom of Table V in terms of moles per 100 gm. elastin. The data in Table V are the easiest to interpret. It can be seen that the total molar quantities of aspartic and glutamic acids are very close to the amide nitrogen concentrations. This would indicate these amino acids are present in their amide form only. The amount of ammonia from sources other than amide nitrogen is very low and probably of no significance.

The Effects of Sodium Hydroxide Treatment on Purified Elastins

It was implied earlier in this section (pg. 95) that the composition of early bovine fetal elastic tissue differs from that of the term fetus and the adult cow because of differences in amino acid recovery during analysis. To test this hypothesis, sodium

TABLE IV

RESULTS OF CHEMICAL STUDIES ON PURIFIED ELASTINS FROM BOVINE LIGAMENTUM NUCHAE

All determinations were based on oven dried samples.

	% Residue After 50 min. Hot NaOH Treatment	% Ash	Gm. Res. % Amide N (NH_3) Average of Duplicate Determinations
6 mo. Fetal	79.64		
9 mo. Fetal	87.24	0.152	0.458
	89.52	0.124	
3 mo. Calf	93.92	0.054	0.390
	94.15	0.065	
6 mo. Calf	93.99	0.072	0.370
	94.07	0.058	
Adult (Young)	92.83	0.078	0.430
	92.38	0.073	
Adult Old (9 yr.)	92.77	0.161	0.376
	92.80	0.164	

hydroxide treatment similar to that used for treatment of aortic elastin (44) was carried out on the purified ligamentum nuchae elastins. Table IV lists the weight recoveries of these elastins after treatment. Sodium hydroxide treatments were carried out on 6 and 9 month fetal elastins, 3 month calf elastins, and 1 and 9 year old adult elastins. It is noted that a greater amount of undefined material is removed from the fetal elastins. From the 2 fetal samples studied, it appears that loss is an inverse function of age. All elastins, however, show the loss of some material. This may consist of, at least in part, amino acid residues and peptides from the elastin itself due to the hydrolytic nature of the treatment. The increased loss in fetal tissues would suggest that either this elastin is different in amino acid composition or there is a contaminant not removed from fetal tissues by the usual methods of extraction and autoclaving.

A comparison of amino acid analyses of elastins before and after sodium hydroxide treatment is given in Table III. Assessment of results of this treatment are complex. It is immediately apparent, however, that desmosine, isodesmosine, and X_4 concentrations are not significantly altered by this treatment. The calf and adult elastins appear to have reacted similarly to the treatment with respect to changes of other amino acid concentrations. A comparison of polar and non-polar residues in 6 month fetal, 9 month fetal, and 3 month post-partum calf elastins points up the differences in response to treatment of fetal versus calf and adult elastins. Gm. res. % values (Table III) have been converted to moles of amino

acids per 100 gm. elastin and represented in Table V to aid in this comparison. Polar amino acids were considered to be hydroxyproline, aspartate, threonine, serine, glutamate, lysine, histidine, and arginine. Non-polar amino acids were glycine, alanine, valine, isoleucine, leucine, tyrosine, and phenylalanine.

Figure XIX is a graphic representation of the totals of polar and non-polar amino acids before and after hot alkali treatment of purified bovine ligamentum nuchae elastins as obtained from Table V. A 70% reduction in the concentration of polar amino acids is seen to occur from the sixth month of gestation to the third month of life post-partum. Concomitantly there is a 25% increase in the non-polar residues. It is also noted that sodium hydroxide treatment has its greatest effect on the amino acid composition of elastin of the term fetus. The content of polar amino acids is decreased and that of the non-polar ones increased by the treatment at this age bringing the ratio of these two groups close to those of the calf elastin. This change may represent the loss of a protein or peptide having a high content of polar residues. The data of experiments in Section 1 pointed out that at this age elastin deposition was progressing at a very rapid rate. This loss may thus actually represent a portion of the newly formed elastin fiber, perhaps that which is poorly cross-linked.

An increase of approximately 6% occurs in the non-polar amino acid residues in the treated elastin of the 3 month calf. No polar changes are observed, but this is probably not significant since these residues make up a very small portion of the total residues

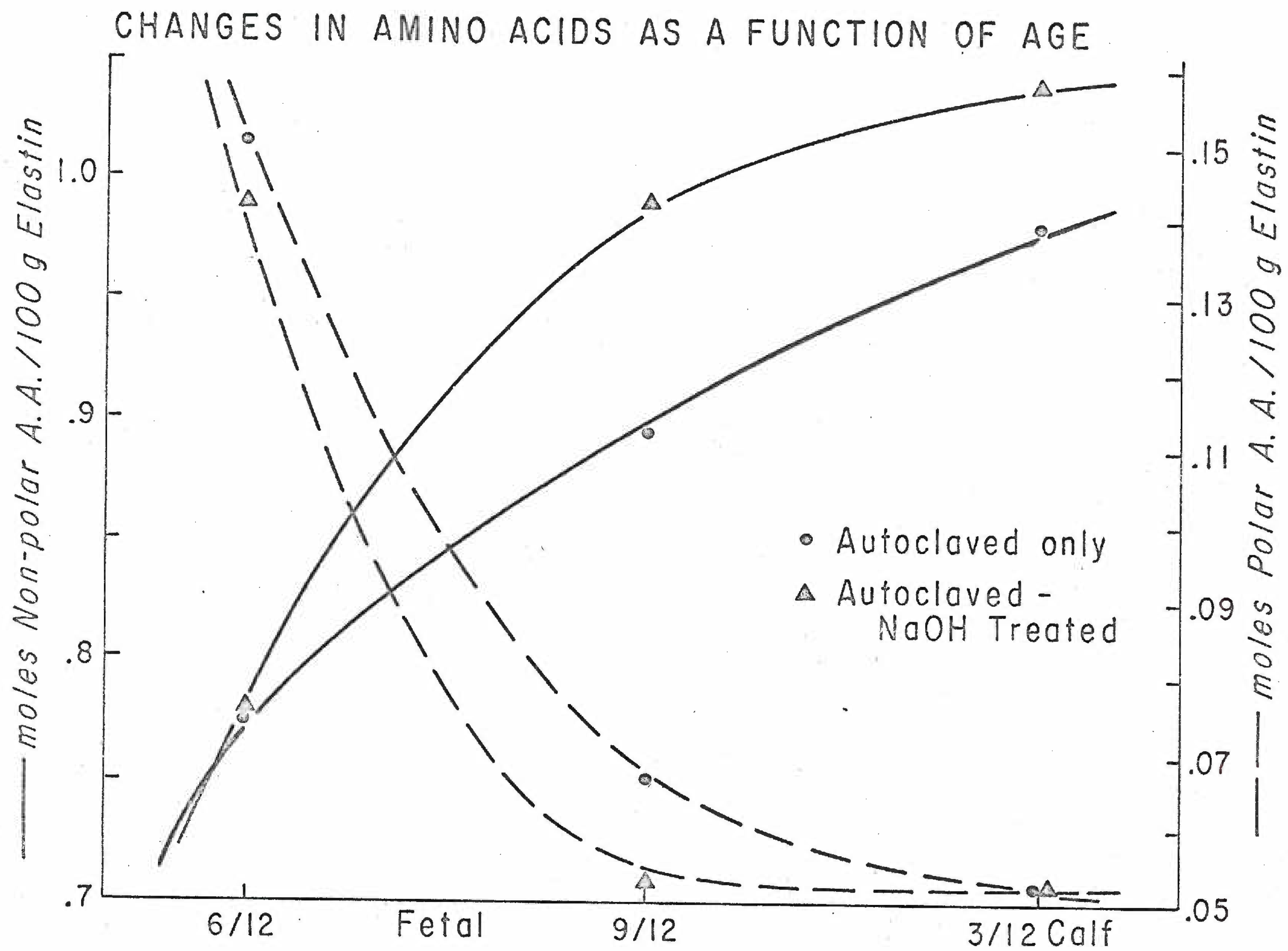
TABLE V
AMINO ACID COMPOSITION OF BOVINE LIGAMENTUM NUCHAE ELASTINS
FROM VARIOUS AGES IN MOLES/100GM ELASTIN
A.E. = AUTOCLAVED ELASTIN
A.+N.E. = AUTOCLAVED & NaOH TREATED ELASTIN

Lines divide Polar and Non-polar components

Amino	6 mo. Fetal		9 mo. Fetal		3 mo. Calf		1-2 yr. Adult		9-10 yr. Adult	
Acid	A.E.	A.+N.E.	A.E.	A.+N.E.	A.E.	A.+N.E.	A.E.	A.+N.E.	A.E.	A.+N.E.
OHpro	.0151	.0160	.0122	.0070	.0081	.0087	.0114	.0065	.0127	.0132
Asp	.0249	.0233	.0091	.0077	.0063	.0063	.0058	.0058	.0058	.0057
Thr	.0127	.0121	.0071	.0055	.0048	.0056	.0057	.0051	.0092	.0053
Ser	.0062	.0095	.0051	.0048	.0022	.0055	.0039	.0048	.0098	.0051
Glu	.0402	.0344	.0188	.0172	.0194	.0158	.0156	.0161	.0181	.0160
Pro	.1277	.0948	.1315	.1227	.1204	.1341	.1387	.1392	.1315	.1319
Gly	.2356	.2501	.3042	.3538	.3632	.3680	.3368	.3676	.3669	.3942
Ala	.1644	.1722	.1999	.2312	.2108	.2463	.2269	.2414	.2416	.2627
Val	.1075	.1129	.1369	.1480	.1511	.1542	.1384	.1613	.1611	.1588
Ileu	.0299	.0318	.0246	.0260	.0254	.0269	.0276	.0220	.0291	.0269
Leu	.0662	.0701	.0625	.0668	.0686	.0684	.0612	.0682	.0731	.0708
Tyr	.0125	.0127	.0070	.0082	.0080	.0080	.0083	.0062	.0092	.0077
Phe	.0281	.0310	.0289	.0329	.0305	.0337	.0339	.0285	.0320	.0339
Lys	.0239	.0246	.0080	.0067	.0054	.0051	.0035	.0034	.0029	.0030
His	.0045	.0040	.0013							
Arg	.0220	.0181	.0055	.0052	.0060	.0049	.0050	.0045	.0068	.0045
NH_3 as amide N			.027		.023		.025		.022	
Asp + Glu			.028		.026		.021		.024	
NH_3 other than amide N			.001		.012		.011		.020	

FIGURE XIX

A graphic representation of the totals of polar and non-polar amino acids of elastins before and after treatment with 0.1N hot NaOH. The autoclaved bovine ligamentum nuchae elastins were obtained from a 6 month fetus, a term fetus, and a 3 month calf. The data for this chart are derived from Table V.


CHANGES IN AMINO ACIDS AS A FUNCTION OF AGE
moles Non-polar A.A./100 g Elastin
moles Polar A.A./100 g Elastin
1.0
.9
.8
.7
.15
.13
.11
.09
.07
.05
6/12
Fetal
9/12
3/12 Calf
Autoclaved only
Autoclaved - NaOH Treated

present. This 6% increase of non-polar residues is suspiciously similar to the 6 to 8% loss of total weight observed in these tissues with the hot alkali treatment (Table IV). If a correlation exists, the 6% must represent loss of an unidentified substance which is not ninhydrin positive.

Table IV also lists the results of ashing studies on these elastins. Since all ash values are extremely low, the possibility of a mineral substance as the unidentified component is ruled out. Carbohydrate content of ligamentum nuchae elastins is also too low to account for any significant part of this unidentified component (65), (73). The undefined yellow pigment of elastin increases with age (42) and possibly represents a portion of this lost component. More concrete evidence on this matter must await a better chemical definition of elastin.

The apparent resistance of early fetal "elastin," in terms of altered ratios of polar and non-polar amino acids, to the alkali treatment is as yet unexplained (Figure XIX). It would be inconsistent with modern biochemical concepts of protein synthesis to think of this as a different tissue. From the work of Slack (96), and Lansing (44), (45) it is known that once elastin has attained its insoluble form it is biochemically inert and thus this "elastin" of high polar residue content (16% as opposed to 5% in the 3 month calf) must be somehow represented in the more aged elastin. The most logical conclusion seems to be that a contaminating protein of high polar residue content constitutes an appreciable share of the "elastin" isolated at this early age. This protein is impervious

to the hot alkali treatment. Perhaps it is a cellular constituent rendered insoluble by the extraction procedures. If present in the 9 month fetal ligament, the protein contaminant must constitute only a small portion since this "elastin" was significantly altered by the treatment.

Summary

The "elastin" of bovine ligamentum nuchae, even in its most purified form, does not appear to be a homogeneous substance. Several contaminating substances, some proteinaceous and others not, are present. Also, some of these substances appear to increase with age while others decrease. "Elastin" from young fetal tissue is the most heavily contaminated as evidenced by its high content of polar amino acids. The progressive increases in desmosine and isodesmosine with post-fetal age reported by other investigators have not been observed in the elastic tissues I have studied. Lysine, however, undergoes a steady decrease through the entire age period studied (early fetal to 9 year old adult). This change may indicate the formation of other types of cross-linking agents as yet unidentified.

I have not answered the question raised in the introduction to this section regarding the use of the cross-linking substances, or precursors of them, as unique markers for the identification of a soluble precursor of elastin. The metabolic study to follow will throw more light on this subject and further point up the key roll which lysine plays in the biochemistry of elastin.

SECTION V

METABOLIC STUDIES OF LYSINE INCORPORATION INTO GROWING ELASTIN

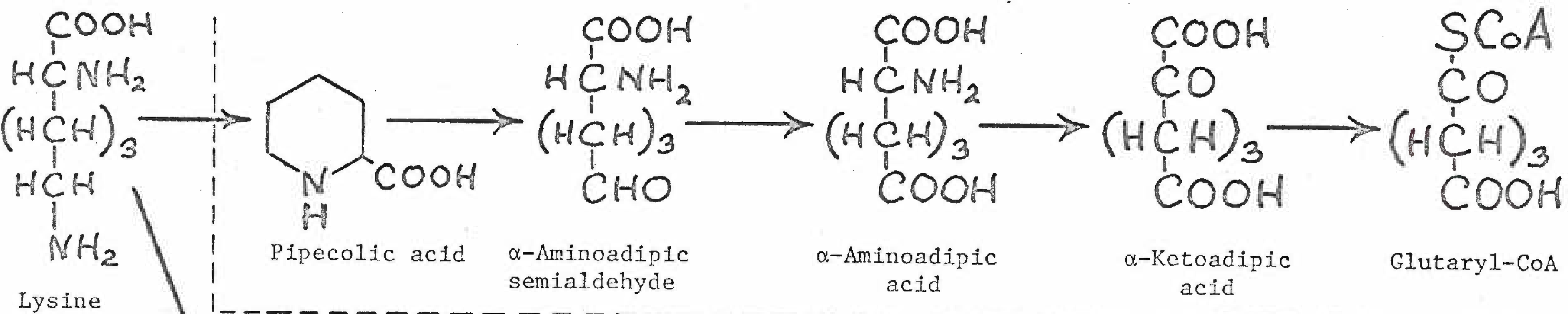
Introduction

With the uncertain fate of lysine incorporated into elastin demonstrated by the previous experiment, it seemed necessary to study the uptake of this amino acid in radioactive form into a rapidly growing elastic tissue. There have recently been several substances reported to be present in elastin hydrolysates, other than desmosine and isodesmosine, which are suspected to be derived from lysine. No isotopic studies in living animals have, as yet, been reported on these derivatives. One of these substances is lysinonorleucine, or X_4, first described by Franzblau, Sinex, and Faris (22). Its structure was verified in a later publication (23) in which a synthetic lysinonorleucine was shown to have identical properties to that occurring in elastin. Its metabolism in growing rats was followed over a six-month period showing it increases with age. Franzblau (23) has postulated it may be an intermediate product in the formation of the desmosines from lysine.

Figure XX is a summary of the known degradative pathways of lysine in rat liver (59) as well as the steps which are believed to occur in conversion of lysine to the desmosines (76). It will be noted that lysinonorleucine (X_4) is visualized as a reduced Schiff base formed by the coupling of α-amino-adipic-semialdehyde with the ε-amino group of an unaltered lysine molecule. Very recently several other lysine-related substances have been partially identified in elastin hydrolysates which are apparently different from

FIGURE XX

A summary of the known degradative products of lysine which occur in rat liver (59), and also the steps which are believed to occur in the conversion of lysine to desmosine (76) and X_4 (22) in elastin.



Lysine
Pipecolic acid
α-Aminoadipic semialdehyde
α-Aminoadipic acid
α-Ketoadipic acid
Glutaryl-CoA
Liver Pathway
Elastin-bound Lysine
Elastin-bound X_4
One lysine and three semialdehydes in apposition
Elastin-bound Desmosine

X_4 (71), (95). Thus, the postulated complexity of lysine metabolism in elastin has some verification. To test this further, the uptake of this amino acid in a uniformly labeled C^{14} form was studied in two groups of young chickens.

Materials and Methods

Study 1: long-pulse study

Six newly hatched Leghorn (male and female) chicks were observed for uniform growth for a 10-day period. One animal, representing the average growth rate of the group, was injected during the second week of life with 100 µc. of uniformaly labeled C^{14} L-lysine (New England Nuclear) in four intravenous doses given daily. Two animals of similar weight were injected simultaneously with saline for control studies. The remaining three chicks were not utilized. Five weeks after the final injections, the animals were killed by decapitation. The weights of the injected and controlled animals at the time of death were almost identical (2% variation). Their thoracic aortae were dissected free of loose adventitial tissue, minced, and extracted for 24 hours with saline. Collagen was removed from the aortae by autoclaving in water at 30 p.s.i. for 6 hours. The residue remaining was divided; half was treated with 0.1N NaOH at 98° for 25 minutes; the remainder was treated for the usual 50 minutes. After washing and drying, the elastins were hydrolyzed in 6N HCl for amino acid analysis as described previously (see thesis pg. 72). The yields of elastin from control and experimental animals showed no significant differences.

The Technicon amino acid analyzer was modified for this metabolic study by coupling a Packard anthracene scintillation flow cell (19), a modification of the original apparatus described by Rapkin and Gibbs (81), between the ion exchange column and the proportioning pump. In this way the eluates of the column were forced through the flow cell before being subjected to the ninhydrin reaction. Radioactivity was detected by the Tricarb scintillation counter. It was set to give a printed readout every 5 minutes. Also, a continuous tracing was obtained by coupling a time-constant device to the Tricarb which averaged activity over 100-second intervals and recorded this on a chart paper moving at the same speed as the paper in the Technicon recorder. Once the time delay between the Tricarb and Technicon systems had been determined, the two charts could be superimposed to give a simultaneous evaluation of radioactivity and ninhydrin positivity. An additional delay of 1.5 ml. due to an isotope effect (78) was noted for most amino acids. Integration of the total activity in an area was estimated from the 5-minute print-out tape by the method described by Elwyn (19). Calibration of the system was carried out using a norleucine standard of known specific activity.

Study 2: short-pulse study

Four animals were utilized for this study. The experiment was begun during the second week of life. Two chicks received two intravenous doses of labeled lysine eight hours apart. The other two were utilized for saline control injections with the same dose schedule. The dose of C^{14} L-lysine was the same as that used in

study 1. The experiment was terminated 12 hours after the last injection. The elastins were prepared as previously, except that the 25-minute, hot-alkali treatment was deleted, using only the 50-minute treatment. The amino acid analyses of all these samples (Study 1 and 2) were carried out at a load of 2 to 4 mg. of hydrolyzed elastin per analysis. This high load was necessary to detect the presence of any small amounts of activity in the eluted amino acids which might otherwise be unrecognized.

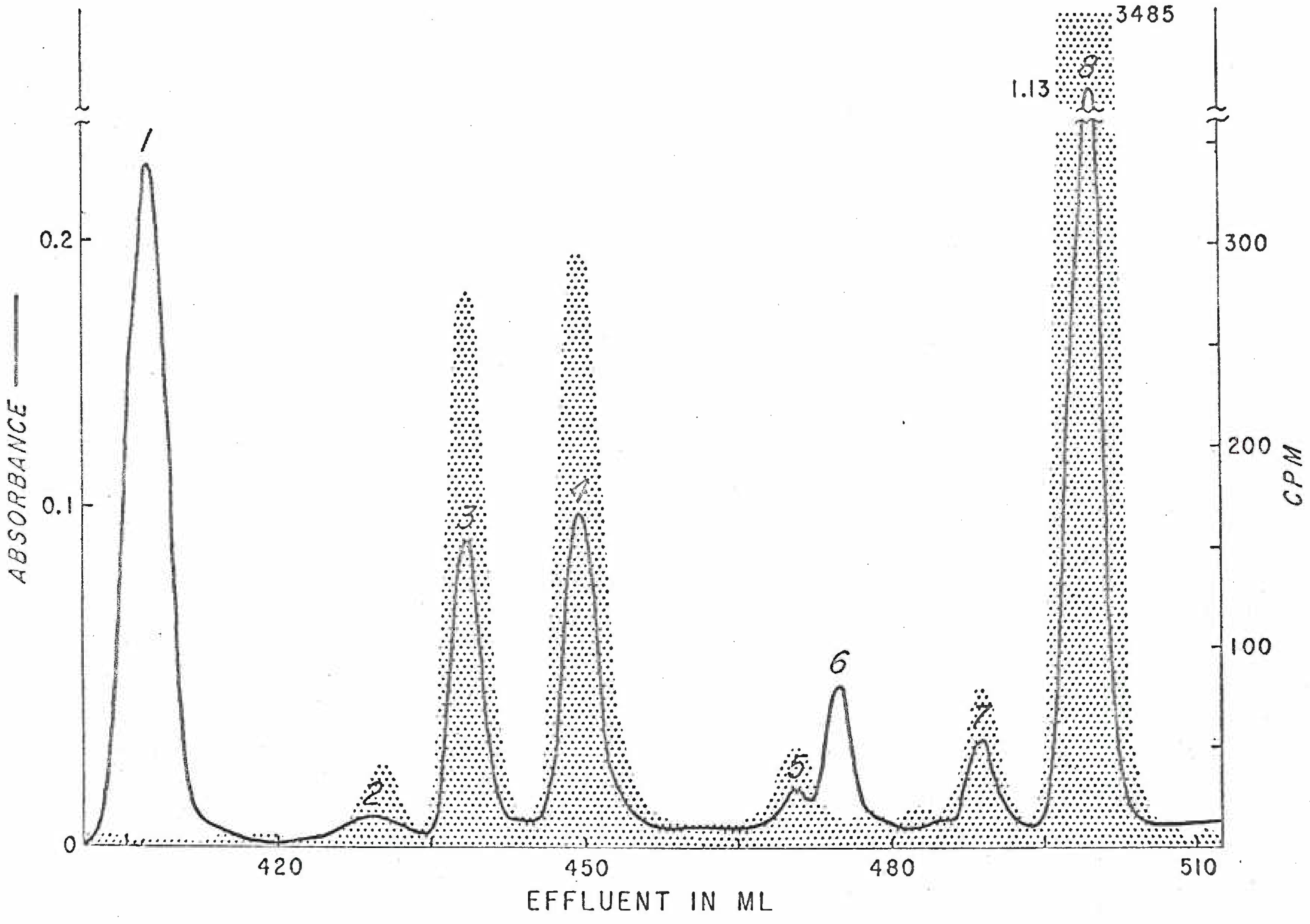
Results and Discussion

The initial, long-time point, first studied in this experiment, was undertaken before the short one because of the reported metabolic inertia of elastin (45). Only one previous radiolysine uptake study had been reported in living animals (76). Incorporation of activity in the study of Partridge was extremely low so that few conclusions could be drawn from the experimental results. However, with the confirmation of adequate lysine uptake in moderate doses from my long-pulse study, the short-pulse experiment was undertaken.

In both the long and short-pulse studies, radioactivity from the labeled lysine was found in lysine in the chromatogram as well as in 8 or 9 other areas. This result came as a complete surprise, since the known metabolism of lysine in vertebrates is quite limited (Fig. XX). Radioactive label was observed in X_4, a small peak in front of this (arbitrarily named X_3), desmosine and isodesmosine, a small peak in front of isodesmosine (arbitrarily named X_2), as well as 3 or 4 areas in the neutral group of amino acids. Figure XXI is a portion of the amino acid chromatogram from a lysine uptake study in rat aortic elastin (13). It is included as an example because it is virtually identical to the long-pulse

FIGURE XXI

A portion of the amino acid analysis chromatogram from a lysine uptake study in rat aortic elastin (13). The radioactivity scan is superimposed allowing for a delay of 1.5 ml. due to an isotope effect (78). The rat aortic elastin was prepared by autoclaving and treating with hot 0.1N NaOH for 25 minutes. These tracings are almost identical to the amino acid analysis chromatogram obtained from the long-pulse chick aortic elastin treated for 25 minutes with hot alkali. They demonstrate the excellent separation of the radioactive components in this area of the chromatogram. Peak numbers refer to: 1. NH_3 2. X_2 3. Isodesmosine 4. Desmosine 5. X_3 6. Ornithine 7. X_4 8. Lysine.



ABSORBANCE
CPM
EFFLUENT IN ML
0
0.1
0.2
100
200
300
420
450
480
510
3485
1.13
1
2
3
4
5
6
7
8

25-minute alkali-treated chick elastin. The chromatographic tracing from ammonia to lysine has been reproduced with the tracing from the Tricarb superimposed. Resolution of the radioactivity is quite good, and there appears to be no impaired resolution of the ninhydrin peaks, even though the column eluate has passed through the flow-cell.

Since there were no known derivatives of lysine which could give the activity observed with the neutral amino acids, some attention was focused on this area. Of particular interest was the activity associated with the glycine peak, since this constituted over 20% of the total activity present in the elastin of the long pulse chick. It was suspected the activity under this peak was not glycine. Listed below is a summary of the activity observed in the 25-minute alkali-treated elastin from Study 1.

% activity under glycine peak	22.6
% activity in leucine area	3.2
% activity under tyrosine peak	6.7
% activity in X_2 and desmosine area	29.2
% activity in X_3 and X_4 peaks	4.1
% activity under lysine	13.8
Total activity accounted for by above	79.6%

The glycine peak from a subsequent chromatogram of labeled elastin was isolated and taken to dryness in a flash evaporator at 40°. Extraction of the amino acid(s) from the salt residue was achieved by the use of 90% ethanol for a 20 minute period. This alcoholic solution was again taken to dryness in the flash evaporator, and the

residue disolved in 0.5 ml. water. This solution was spotted on 6 thin layer, cellulose chromatographic plates which were then subjected to various solvent systems to attempt a separation of the radioactivity from the glycine. It was noted that using a methanol-water-pyridine system 8:2:4, a faint band of ninhydrin positivity occurred a considerable distance above glycine. The Rf value of the unidentified material was 0.7, and that of glycine 0.2. Several thin layer chromatograms were repeated with this solvent system and the cellulose divided into 8 thin horizontal sections. These sections were scraped off and eluted separately for evaluation of radioactivity by scintillation counting. It was conclusively shown the radioactivity was associated exactly with the ninhydrin positive material moving ahead of glycine.

Because of the high content of methanol in the solvent system used for thin layer chromatography, indications were strong that this effected the chromatographic separation of the radioactive substance from glycine. Therefore varying amounts of methanol were added to chambers 3 and 4 of the Technicon Autograd, these being the chambers which have the greatest influence on the elution of glycine (77). With 20 ml. of methanol in each of these chambers, a complete separation of the radioactive material was achieved from glycine on the Technicon system. The radioactive substance was observed to chromatograph as a small ninhydrin peak, 15 ml. ahead of glycine.

Also, it was noted this altered gradient (with methanol in chambers 3 and 4) had an influence on the other areas of radioactivity

observed previously. The results of high load runs of the long and short-pulse studies using the methanol gradient are summarized in Table VI and demonstrate the influence of methanol. Of most remarkable interest was the appearance of a "new peak" which separated from lysine, chromatographing approximately 10 ml. behind it. It was associated with radioactivity. This substance was arbitrarily called X_5. The altered gradient also gave much better definition of the substances chromatographing with the neutral amino acids. Eight distinct areas of activity could be recognized. Most of these were associated with ninhydrin positive substances though this was usually quite small. The only common neutral amino acids which had activity directly associated with their ninhydrin positivity were norleucine and tyrosine. When possible, an attempt was made to evaluate the activity of these new substances in dpm/micromole by assuming the ninhydrin color yield to be the same as that of leucine. These specific activities are listed in Table VII.

Although this study is only in its preliminary stages, several inferences can be made by a comparison of the early and late specific activities (Table VII). Attention will be focused on the pre-glycine activity and the activities between X_2 and X_5, since these are the only points where specific activity could be consistently calculated. It is noted that the pre-glycine substance more than doubles its specific activity between the two time points. This would imply this substance is an end-product of lysine, metabolism, i.e. a stable "cross-linking" substance and not an unstable intermediate. The constant specific activities of desmosine and

TABLE VI

A COMPARISON OF ACTIVITIES IN AORTIC ELASTIN OF LONG AND SHORT-PULSE LYSINE UPTAKE STUDIES IN THE GROWING CHICK

Amino acid analysis performed on Technicon 22 hour system with altered gradient (20 ml. methanol in chambers 3 and 4). Anthracene flow cell coupled between ion exchange column and proportioning pump to determine radioactivity.

	50 MIN. HOT ALKALI TREATED SHORT PULSE CHICKS SPECIFIC ACTIVITY = 3139 DPM/mg ELASTIN		50 MIN. HOT ALKALI TREATED LONG PULSE CHICKS SPECIFIC ACTIVITY = 1423 DPM/mg ELASTIN	
Peak	Counts	% of Total	Counts	% of Total
pre-Glycine	192	2.8	689	19.2
Cystine-Methionine Area	249	3.7	22	0.6
Isoleucine-Leucine Area	20	0.3	60	1.7
Under Norleucine	103	1.5	54	1.5
Under Tyrosine	72	1.1	217	6.0
#1 Behind Phenylalanine	244	3.6	169	4.7
#2 Behind Phenylalanine	68	1.0	117	3.3
#3 Behind Phenylalanine	44	0.6		
X_2	30	0.4	90	2.5
Isodesmosine	551	8.1	497	13.8
Desmosine	471	6.9	617	17.2
X_3	162	2.4	111	3.1
X_4	132	1.9	125	3.5
Lysine	3668	53.9	278	7.7
X_5	366	5.4	10	0.3
% Recovery		93.6		85.1

TABLE VII

LONG AND SHORT-PULSE LYSINE UPTAKE STUDIES EXPRESSED IN μMOLES AND DPM/μMOL

Peak	50 MIN. HOT ALKALI TREATED SHORT PULSE CHICKS		50 MIN. HOT ALKALI TREATED LONG PULSE CHICKS	
	μMoles/100 mg. Elastin	DPM/μMol	μMoles/100 mg. Elastin	DPM/μMol
*pre-Glycine	0.443	20,000	0.564	48,484
Cystine-Methionine Area				
Isoleucine-Leucine Area				
Under Norleucine				
Under Tyrosine				
*#1 Behind Phenylalanine	0.955	11,801		
#2 Behind Phenylalanine				
*#3 Behind Phenylalanine	1.946	1,047		
*X_2	0.575	2,441	0.257	1,278
Isodesmosine	1.086	23,417	1.058	18,640
Desmosine	1.249	17,391	1.198	20,422
*X_3	0.932	8,010	0.623	7,063
X_4	0.502	12,162	0.533	9,343
Lysine	9.489	17,840	6.144	1,799
X_5	0.977	17,269	0.716	543

Where specific activity is not calculated there was no ninhydrin peak or activity was associated with amino acids not known to be products of lysine metabolism.

*Color yield of Leucine used to evaluate specific activity.

isodesmosine would substantiate the concept of lysine being continuously converted to desmosine and isodesmosine. This data taken by itself is in agreement with the data of Miller, et.al. (61) and Franzblau, et.al. (23), and in disagreement with my previous observations made in Section IV (see thesis pg. 96) regarding the fixed concentrations of the desmosines after late fetal life. However, the micromolar quantities of these two substances were approximately equal in concentration in these two chicks (Table VII). The two logical conclusions one can arrive at from this limited data are: 1) the discrepency is a result of species difference; or 2) lysine does undergo conversion to desmosines after fetal life, but most of this occurs in elastin layed down while the animal was very young. Elastin layed down during later stages of development does not form as many cross-links. These same conclusions may be drawn for X_3 and X_4.

It is noted also in Table VII, lysine specific activity decreases markedly with age. This is in agreement with my previous observations (see Section IV).

The behavior of the X_5 substance is of great interest. Note its activity is very low in the long-pulse study. It has the same activity as lysine in the short study. This may indicate that this substance is an early intermediate in the conversion of lysine to cross-linking agents. This marked decrease in specific activity definitely indicates it is not an end-product of lysine metabolism. The same may be said for the X_2 substance, although judging from the magnitude of its ninhydrin peak, it is present in an extremely

low concentration.

As a side light of this study, I have also made a comparison of the results of the two treatments given elastin in Study 1, i.e. 25 and 50 minute hot alkali treatment. The results of these treatments are summarized in Table VIII. Total specific activity decreased with the longer treatment indicating some elastin or contaminating substance is removed by this process. The pre-glycine and post-tyrosine substances are not altered by the alkali treatment which would suggest these are cross-linking substances. The desmosines behave similarly. Lysine and X_5 undergo a marked decrease in activity. Two possible explanations for this decrease are: 1) these two substances are present in contaminants which are removed by the additional treatment; or 2) these two substances are present primarily in poorly cross-linked elastin easily removed by the additional treatment. Because there is such a great decrease in specific activity, and because experiments in Section IV suggested newly formed elastin was solubilized by hot alkali treatment, the latter possibility seems the most likely.

It is very probable that some of the activity observed in the chromatograms represent degradative products formed during the purification procedure and hydrolysis of elastin. There is no way of determining which these are at present. Additional time points are necessary to complete this study and then perhaps these questions will be answered. The possibility of X_5 being lysinoalanine should be investigated. This substance is formed during hydrolysis of certain proteins in which lysine and cystine are situated adjacent to

TABLE VIII

EFFECTS OF HOT ALKALI TREATMENT ON RECOVERIES OF RADIOACTIVITY FROM C^{14} LYSINE LABELED CHICK AORTIC ELASTIN (LONG-PULSE STUDY)

Amino acid analysis performed on Technicon 22 hours system with altered gradient (20 ml methanol in chambers 3 and 4). Counts in individual areas expressed as % of total counts in that analysis.

Area of Activity	25 min. Treatment	50 min. Treatment
Total specific activity in DPM/mg elastin	1612	1423
Peak before glycine	18.6%	19.2%
Leucine & Tyrosine Areas	2.6%	9.8%
Area Behind Tyrosine	7.6%	8.0%
X_2, Isodesmosine & Desmosine	27.3%	33.5%
X_3, X_4	3.3%	6.6%
Lys	19.6%	7.7%
X_5	6.0%	0.3%

each other in the peptide chain. Bohak (5) has observed it in enzymes subjected to alkali treatment before hydrolysis. It appears near the lysine area of chromatograms.

Summary

The study of incorporation of labeled lysine into aortic elastin of growing chicks has demonstrated the metabolism of this amino acid in this tissue to be complex. It would appear that several other cross-linking agents are present in addition to the desmosines and lysinonorleucine. Alteration of the Technicon elution gradient by the addition of methanol to chambers 3 and 4 of the Autograd has revealed the presence of at least 2 previously unrecognized ninhydrin positive substances. It is believed that one of these may represent an early intermediate in the conversion of lysine to the cross-linking agents. This intermediate may be present in the tropoelastin molecule and thus serve as a unique marker for this substance.

SECTION VI

STUDIES ON THE ANTIGENIC PROPERTIES OF ELASTIN

Introduction

In this study attention was turned to the antigenic properties of elastin with the hope of using immunochemical methods for detecting a soluble precursor of elastin. Previous reports (2), (57), (107) indicated that elastin was non-antigenic. However, through a personal communication from Dr. S.M. Partridge, it was learned that his group had succeeded in developing antibodies against α elastin, a soluble product of partial hydrolysis of elastin. They felt, however, that the specificity of their antisera was questionable.

α and β elastin were first described by Partridge and co-workers in 1955 (73), (74). These products of partial hydrolysis of elastin were shown to have the same amino acid composition as the whole purified material. β elastin, which is released first during hydrolysis, has a molecular weight of 6,000, while α elastin has a molecular weight of 80,000. Partridge at one time proposed that fibrillar elastin might consist of polymers of the α units, and the surrounding matrix of β units, possibly alternately linked with polysaccharide material (75).

Recently Robert and co-workers (85) have described the development of rabbit antibodies against high molecular weight peptides obtained by alcoholic potassium hydroxide digestion of bovine aortic elastin (84).

Materials and Methods

Preparation of α and β Elastin

α and β elastin were prepared from adult bovine ligamentum nuchae elastin by partial hydrolysis of the purified elastin with 0.25M oxalic acid (74). Deionization of the α and β elastin was then carried out with the use of Permutit resin as follows: Five volumes of anion exchange resin (resin S-1) and 2 volumes of cation exchange resin (resin Q) were mixed and poured into a 40 × 2.5 cm. glass column. Total volume of the column was 170 ml. It was washed with 200 to 300 ml. distilled water. Then, 300 ml. of the oxalic hydrolysate of elastin was quickly poured through. The presence of elastin in the eluate was detected by holding an ultraviolet lamp close to the collecting tube with the room slightly darkened. Elution of the soluble elastins was marked by a blue-white fluorescence in the tube. The exclusion volume for this column was 80 ml., and its capacity sufficient to handle about 350 ml. of the hydrolysate. The deionized α-β solution was lyophilized and stored at -20° in screw-top jars.

Development of Anti-α Elastin Antibodies

A 2% saline solution of the α-β elastin mixture was prepared from the lyophilized material obtained above. This was thoroughly mixed to an emulsified state with equal parts of a complete Freund's Adjuvant by the use of a Virtis homogenizer. For primary immunization, the emulsion was injected weekly into 3 rabbits of approximately 4 pounds weight. A dose of 1.0 ml., divided into 4 portions in the back, was given subcutaneously. After 5 weeks of primary immunization,

the animals were allowed to rest for 2 months. A series of 4 secondary injections were then given at weekly intervals. This secondary treatment, including 2 months of rest between injections, was repeated 2 additional times. Fourteen days after eliciting the third secondary response the rabbits were exsanguinated by cardiac puncture.

Precipitin Tests

Interfacial tests (10) were carried out in 8 cm. capillary tubes by filling the tubes with equal quantities of antigen and antibody solutions and then sealing the lower end with plasticine. Tubes were incubated at 37° for one hour and then at 4° for 48 hours. Readings of precipitins were graded 1+ to 4+ on the basis of precipitins obtained from a calf serum/anti-calf serum system. The results of an interfacial test are shown in Figure XXII.

Passive Cutaneous Anaphylaxis Test

The PCA test was carried out on two, 600 gm. guinea pigs, using the method described by Ovary (69). Two sites were injected with the anti-α elastin, one concentrated antiserum and the other a 1:10 dilution. A third site was injected with saline as a control. Five hours after injection of the antibodies, 1.0 ml. of a saline solution containing 4 mg. α-β elastin and 10 mg. Evans Blue was given intravenously. One hour later the animals were killed and their skins examined.

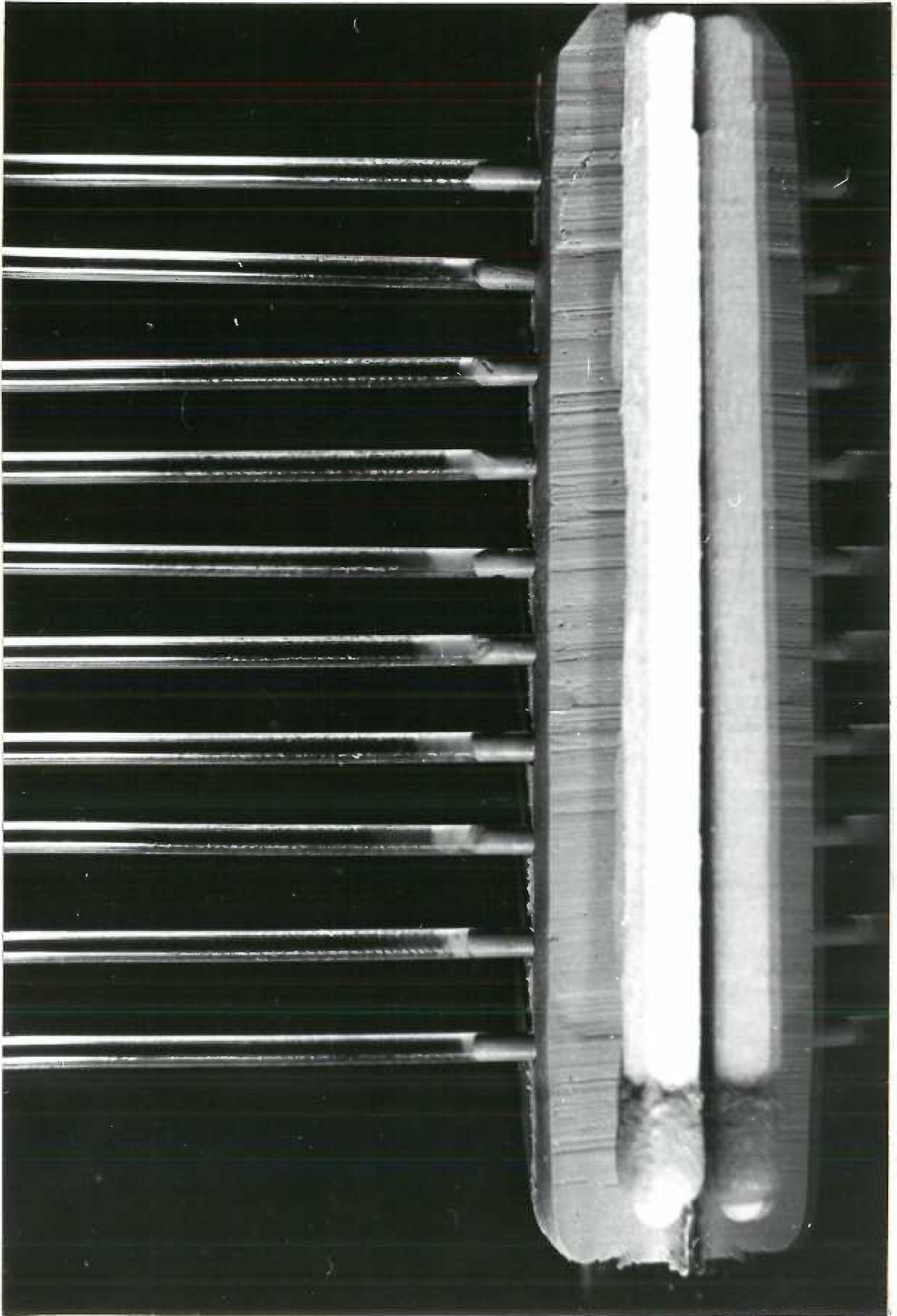
The Hemagglutination Reaction for Detection of Antigen-antibody Reactions Using Tanned Formalinized Cells

Type O, Rh positive human cells collected in Alsever's solution were formalinized after the method of Butler (9). A 4% solution of

FIGURE XXII

An interfacial test using various dilutions of calf serum against anti-calf serum diluted 1:2. The results are tabulated below. Tubes are numbered from left to right. The prozone phenomenon, manifest by the large precipitates in tubes 4 and 5, indicates antigen-antibody concentrations were optimal in these tubes. Antigen excess occurred in tubes 1, 2, and 3. Antibody excess occurred in tubes 6, 7, 8, and 9.

Tubes	1	2	3	4	5	6	7	8	9	10
mg. Antigen (protein) per ml.	65	32	16	8	4	2	1	0.5	0.25	Saline Control
Readings	1+	2+	3+	4+	4+	3+	2+	1+	1+	---



these cells was incubated with an equal volume of *fresh* 1:20,000 tannic acid solution for 30 minutes at room temperature. After washing 3 times with pH 7.4 phosphate buffered normal saline, a 2% saline suspension was made, and 5 ml. aliquots quick-frozen in liquid nitrogen. Aliquots were stored at -20° in sealed tubes until used for the hemagglutination studies.

Immediately prior to testing, sufficient quantities of cells were thawed and incubated at room temperature for one hour with equal volumes of the serially diluted antigen. After 3 washes with a 1:200 normal rabbit serum in buffered saline, 0.1 ml. portions of the coated, tanned, cell suspension, in 2% concentrations were mixed with an equal volume of serially diluted antibody. The presence of hemagglutination was read after incubation for 3 hours at room temperature. All tubes were reshaken and read again after a total incubation time of 12 to 18 hours. Sharper readings were obtained after 12 hours; controls were also more uniform.

Immunodiffusion and Immunoelectrophoresis Studies

The micro-apparatus produced by LKB was utilized for immunodiffusion and immunoelectrophoretic studies (48). This is a modification of the methods as described by Scheidegger (91) and Ouchterlony (68). Gels were allowed to incubate at room temperature for 96 hours and then photographed by indirect transmitted light.

Results and Discussion

The primary immunization of rabbits with elastin resulted in a very weak percipitin reaction when testing the sera of all 3 animals. A prozone phenomenon (15) could not be documented (an example of a

prozone phenomenon is shown in Figure XXII). Sera obtained during the secondary treatments gave progressively stronger reactions with each treatment. The results of the primary and secondary immunization of one of the rabbits is summarized in Table IX. During the third series of secondary treatments, a maximum amount of precipitate was obtained at 0.5 and 0.25 mg. per ml. of the antigen when reacted with the undiluted rabbit serum. Although this is not a marked reaction, it is of the magnitude often encountered when immunizing rabbits with such substances as thyroglobulin.[5] Precipitins were never of a marked flocculant nature, but areas of antigen and antibody excess were apparent. The precipitin bands obtained by Ouchterlony gel diffusion were not distinct. Only one band was seen, the sharpest occurring with a 0.125 mg. per ml. antigen concentration reacted with undiluted antiserum. It was suspected, therefore, that this was a system in which the antigen-antibody complex existed largely in the soluble state.

Results were more uniform with the immunoelectrophoretic techniques. Using large quantities of sera in repeated application, which was necessary to give visible results, one diffuse band was obtained when the original antigen was reacted with the antiserum from any one of the 3 test rabbits. All control sera obtained from these rabbits prior to primary immunization were negative. Attempted cross reactions of the anti-α elastin with bovine serum and collagen were also negative. α-β elastin prepared from fetal bovine ligamentum nuchae was reacted against the antisera of all 3 animals. It gave

[5]Personal communication Dr. Pirofsky

TABLE IX

PRECIPITATING ANTIBODY RESPONSE OF RABBIT AFTER REPEATED INJECTIONS OF 2 PER CENT ALPHA-ELASTIN WITH EQUAL PARTS OF FREUND'S ADJUVANT

Undiluted rabbit anti-sera plus	ANTIGEN CONCENTRATIONS mg/ml								
	1.0	0.5	0.25	0.125	0.062	0.031	0.016	Saline Control	Anti-Serum Control
Primary Response 1st & 2nd month	+/-	+/-	+/-	1+	1+	+/-	+/-	-	-
Secondary Responses									
Set 1 - 5th month	+/-	+/-	+/-	1+	+/-	+/-	+/-	-	-
Set 2 - 7th month	+/-	+/-	1+	1+	+/-	+/-	+/-	-	-
Set 3 - 9th month	+/-	1+	2+	2+	1+	+/-	+/-	-	-
Set 4 - 11th month	1+	2+	2+	1+	1+	+/-	-	-	-

Precipitin tests were read after incubating at 37° for 1 hour and then in cold for 18 hours.

a reaction similar to that obtained with the original antigen.

Antigen-antibody interaction was also demonstrated by passive cutaneous anaphylaxis using guinea pigs. Strong positive tests were obtained with the undiluted and the 1:10 diluted antisera.

Hemagglutination scoring was read in a fashion similar to that of Stavitsky (98). Marked 4+ reactions were obtained with 1.0 and 0.5 mg. per ml. concentrations of the antigen and a 1:50 dilution of the anti-serum. Hemagglutination was evident at dilutions down to 0.125 mg. per ml. of antigen using this same concentration of anti-serum. Table X summarizes representative results of the hemagglutination studies. Figure XXIII represents these same results photographically.

Collagen was found to interfere with hemagglutination studies. All tubes, including control, antigen-tagged cells without anti-sera, gave 1+ readings. However, incubation at 4° rather than at room temperature prevented this non-specific agglutination phenomenon. Strong positive cross reactions were also obtained when the anti-sera were incubated with the α-β elastin from fetal tissue. The above results indicated a strong antigen-antibody reaction with specificity.

Extracts were made of term fetal bovine ligamentum nuchae using the tris-HCl buffered saline described previously (see thesis pg. 70). Because of the interference of collagen noted earlier, the collagen was precipitated from this extract by adding cold absolute ethanol to a total concentration of 20%. Ethanol was removed by dialysis. Hemagglutinations were then carried out at 4° using the ligament extract as antigen and the anti-α elastin as

TABLE X

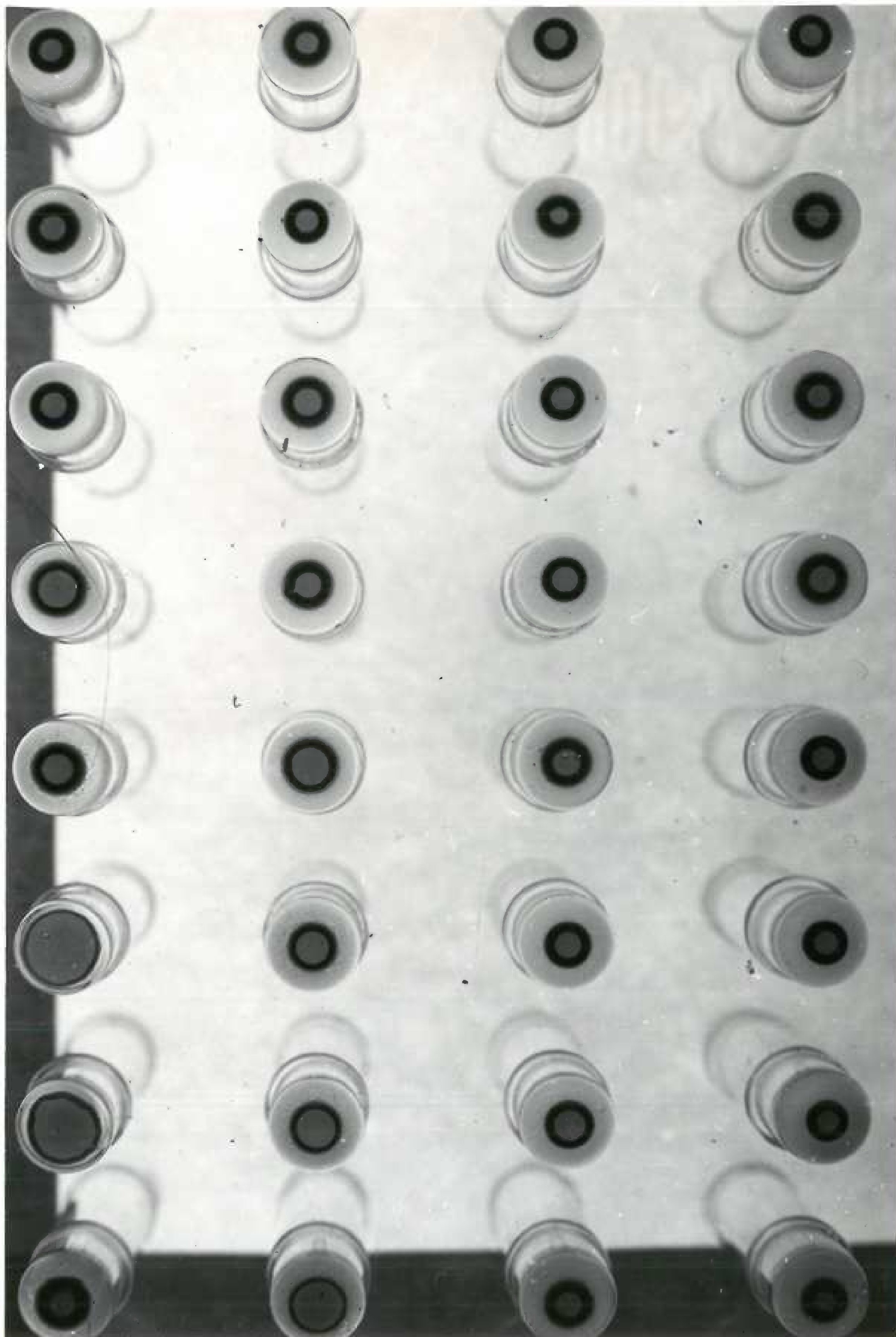
HEMAGGLUTINATIONS OBSERVED WHEN ALPHA ELASTIN WAS REACTED WITH ANTI-ALPHA ELASTIN

READING MADE AFTER 12 HR INCUBATION AT ROOM TEMPERATURE

	Concentration of Alpha Elastin Incubated with Formalinized, Tanned, Red Cells (mg/ml)							
Anti-Alpha Elastin	2.0	1.0	0.5	0.25	0.125	0.0625	0.031	Control Formalinized, tanned, red cells
1:50	4+	4+	3+	2+	1+	-	-	-
1:250	2+	1+	1+	+/-	-	-	-	-
1:1250	+/1	-	-	-	-	-	-	-
Saline Control	-	-	-	-	-	-	-	-

FIGURE XXIII

Hemagglutinations observed when alpha elastin was reacted with anti-alpha elastin. Readings were photographed after 12 hours incubation at room temperature. Scorings are recorded in Table X (page 132).



antibody. Strong cross reactions were observed. Table XI summarizes these results.

These ligament extracts were utilized in another study to immunize rabbits using the same regime as that described for α elastin. The anti-sera obtained were found to cross react with the α-β elastin using the hemagglutination technique with formalinized, tanned, red cells and also the PCA test. However, no cross reactions were detected by Ouchterlony diffusion or immunoelectrophoresis using these same anti-sera.

Throughout this section I have referred to an antibody formed against a mixture of α and β elastins as "anti-α elastin." It is most likely that antibodies are only being formed against α elastin because it is known that in only a few instances laboratory animals are capable of forming antibodies against substances having molecular weights less than 10,000 (15), (43). Considering the relatively low antigenicity of the soluble elastins, it would seem highly improbable that any antibodies were formed against the β component.

The availability of an anti-serum to α elastin together with the hemagglutination technique described provides an amply sensitive method for specifically detecting small amounts of soluble elastin in the presence of other proteins. Testing of preliminary DEAE cellulose chromatographic fractions of ligamentum nuchae extracts, using the method of Gelotte, Flodin, and Killander (25) has given positive results in 2 of 6 fractions obtained. These fractions have not as yet been fully analyzed, but it is anticipated that they may be elastin-like in composition, or that they may contain one or more of the lysine

TABLE XI

HEMAGGLUTINATIONS OBSERVED IN A 20 PER CENT ETHANOL TREATED EXTRACT OF FETAL BOVINE LIGAMENTUM NUCHAE WHEN REACTION WITH ANTI-ALPHA ELASTIN.

READINGS MADE AFTER 3 HR INCUBATION AT +4°C.

Anti-Alpha Elastin Dilutions	Concentrations of Extract Incubated with Formalinized, Tanned, Red Cells (mg/ml)							
	2.0	1.0	0.5	0.25	0.125	0.062	0.031	Saline Control
1:50	4+	4+	3+	3+	2+	1+	-	-
1:100	3+	4+	3+	3+	1+	-	-	-
1:200	2+	3+	3+	2+	1+	-	-	-
Saline Control	-	-	-	-	-	-	-	-

metabolites discussed in Section V. Heating of the fractions to 60° and then cooling resulted in coacervation of the 2 fractions which gave positive hemagglutinations. This phenomenon has been observed in solutions of α elastin (74).

Summary

It was found possible to develope antibodies against partial hydrolysates of elastin by repeated injections with Freund's Adjuvant. These antibodies were readily detected by the use of the PCA test and hemagglutination reaction. The antibodies seemed to be specific for elastin. The anti-elastin was found to react with crude extracts of fetal ligamentum nuchae. Chromatographic separation of this extract with DEAE cellulose has given 6 fractions, two of which gave positive hemagglutination tests using the anti-elastin serum.

GENERAL DISCUSSION

The general aims of these studies of the *Biochemical Properties of Elastic Tissues* have been oriented towards the identification of a soluble precursor of the insoluble elastin fiber. The magnitude of this task was appreciated to some extent at its onset, because a review of the elastin literature revealed very little progress had been made regarding the "true biochemical nature of elastin," even though there had been an active interest in this subject for over half a century. The objective of my studies has not as yet been realized, although I feel considerable progress has been made in the right direction. The purpose of this general discussion is to highlight what I feel are the important achievements gained in these studies.

I believe the contributions of experiments in *Section I* are primarily the establishment of ligamentum nuchae of the fetal calf as an ideal model for the study of elastin synthesis. There are, however, several problems unanswered regarding this tissue, the primary being the nature of the *extrafibrillar constituent*. This appears to be a complex substance as indicated by the fractionation of the extracts of ligamentum nuchae (Section VI) yielding approximately 6 proteinaceous substances. Some of these were serum-like; others were definitely of another nature. Studies of Section I also pin-pointed the term fetus as the appropriate age at which to study the soluble precursor of elastin because this is the age at which elastogenesis is progressing at its most rapid rate. It is estimated approximately one gram of insoluble elastin per week is being formed

in the ligamentum nuchae at this age.

The ultrastructural studies discussed in *Section II* gave visual demonstration of the *extrafibrillar constituent* of ligamentum nuchae. The pre-elastin filaments represent an interesting and previously unobserved biological phenomenon.[6] Probably the main contribution of this study is the demonstration of the complexity of elastin fiber formation and the inter-relationship of the pre-elastin filaments to the polysaccharide-protein complexes. These complexes appear to be of extreme importance in the formation of elastin. This study has also made it clear that the *extrafibrillar constituent* is composed in part, of the polysaccharide-protein complexes, the pre-elastin filaments, and cellular matter. This would speak for "true elastin" being a non-homogeneous entity which is a concept generally agreed upon by most investigators in the connective tissue field (32).

The results of the swelling studies carried out in *Section III* are largely unexplainable on the basis of the present understanding of proteins and their relationship to the Donnan membrane effect. Thus, the complete significance of this swelling study is at present unappreciated. Some of the phenomenon observed, particularly the inhibition of swelling in the presence of high salt concentration, will probably become more significant as a better understanding of the molecular structure of elastin is achieved. The most practical aspect of this study was the establishment of a method by which a

[6]Dr. J. Waisman of Salt Lake City has very recently reported filamentous structures in aortae of copper deficient pigs similar to these filaments.

large quantity of protein could be solubilized in undenatured form from fresh ligamentum nuchae of fetal origin.

The majority of the effort represented in the experimental sections of this thesis comprises the experiments of *Section IV*. The experiments were felt to be a necessary ground-work in the better understanding of factors which would influence the composition of purified elastin. Of greatest significance in this study are: 1) a substantiation of observations made in Sections I and II, pointing out that elastin even in its "purified" form is not a homogeneous substance, and 2) the observations of changes with age of the known cross-linking substances of elastin as well as their common precursor, lysine, demonstrating a more complex role as regards lysine metabolism in elastin than has previously been appreciated.

The steady decrease in the content of lysine in purified elastin with age is an extremely interesting observation, and led to the lysine uptake studies in *Section V*. Metabolically lysine was seen to be incorporated actively into growing elastic tissue, and once incorporated, to undergo a number of changes. Thus the "inertia" of this tissue does not appear to be as real as previously believed (44), (45), (96). It would appear most probable that cross-linking agents are present in elastin other than desmosine, isodesmosine, and lysinonorleucine.

The present day conception of the elastin fiber is one of a mass of loosely organized, randomly arranged peptide chains linked at infrequent intervals by the cross-linking agents. From the amino acid analysis data, as represented in Tables III and V, it would

appear that cross-linking occurs at an average interval of 80 to 90 residues. At a first consideration, this may appear to be an appropriate amount of cross-linking. If, however, one considers rubber as a model of an elastomer, one realizes this is an unrealistically low figure. There may possibly be other types of bonding than covalent linkages between elastin molecules, but this would seem unlikely considering the stresses which the fiber is subject to in the natural physiology of vertebrate tissues. It is possible to stretch elastin to 150% of its original length without disruption of its elastic properties (36). Bonding of the hydrogen and ionic nature would not hold up under stresses such as these. Vulcanized rubber contains approximately 5 to 6% sulfur which acts as a cross-linking substance by presumably forming -S-S- bonds between the isoprenoid units (21). This 5 to 6% sulfur content represents a much greater degree of cross-linking than is known, at present, to exist in elastin. I feel this evidence is indication that there must be other types of cross-linking occurring between elastin molecules.

The studies of lysine metabolism in elastic tissue and also those of the antigenicity of elastin, *Section VI*, had as their primary aim the establishment of some method of identification of tropoelastin. The study of the antigenic properties of elastin coupled with the hemagglutination reaction has established a reliable test for the identification of what seem to be soluble forms of elastin. It is not known at present if these forms represent tropoelastin, solubilized newly formed elastin, or elastin as

present in the micro-filaments. The ultimate aim, of course, is to identify a single molecular species which is the soluble precursor of the insoluble elastin fiber. It may well be that this precursor actually consists of 2 or more substances. The two peaks identified on fractionation of ligament extract may contain 2 soluble forms of elastin which must polymerize with each other in some undetermined fashion to form the elastin fiber.

SUMMARY AND CONCLUSIONS

A study of the physical and chemical properties of fetal, calf, and adult bovine ligamentum nuchae has revealed the following: 1) Maximum elastin deposition occurs during the later few weeks of gestation and early post-partum life. 2) Ultrastructurally, it is apparent elastin deposition is a complex process involving a preliminary polymerization of tropoelastin into filamentous structures which are then depolymerized to form the final random arrangement of elastin peptide chains. 3) An optimum extraction medium for the solubilization of proteins from ligamentum nuchae appears to be one of low ionic strength buffered at pH 8.4. 4) An anti-elastin antibody, developed for the identification of soluble elastins, can be used for the identification of "elastin-like" substances in the protein extracts of ligamentum nuchae.

A study of elastins purified from fetal, calf and adult bovine ligamentum nuchae has revealed: 1) Elastin is a non-homogeneous substance even in a so called "highly purified" state. 2) The amino acid composition of elastin changes with age, a marked decrease occurring in the number of polar residues. 3) Cross-linking substances are only observed to increase in concentration during fetal age.

A metabolic study of aortic elastin in young chicks has shown: 1) Elastin is a metabolically active substance even after its deposition as a fiber. 2) Lysine is incorporated very rapidly into elastin, and once incorporated may undergo a number of changes. 3) Incorporation of lysine in vivo occurs into the cross-linking substances desmosine, isodesmosine, and X_4. 4) Several other amino

acids, not as yet identified, are present in elastin from metabolically altered lysine. It is suggested that some of these new amino acids represent additional cross-linking substances in elastin.

ACKNOWLEDGMENTS

I wish to express particular gratitude to Dr. E. G. Cleary for his advice and guidance during my studies of elastic tissue, to Dr. D. S. Jackson for introducing me to the connective tissue field, and to Dr. R. T. Jones for his helpful criticisms and counsel during the later portion of my experimental work and the writing of this thesis.

I wish to thank the following for their collaborative efforts on the various experiments:

Dr. J. P. Bentley

Dr. E. G. Cleary

Dr. W. H. Fahrenbach

Dr. D. S. Jackson

Dr. B. Pirofsky

Dr. Z. T. Wirtschafter

The skilled technical assistance of Mrs. J. Wilson, Mrs. A. Quilici, Mr. G. Davies, and Mrs. C. Head is sincerely appreciated.

Mrs. J. Fawcett and my wife, Joyce, are responsible for the excellent appearance of the final manuscript.

The histologic sections and photographs in Experimental Section I were very graciously done by Dr. Z. T. Wirtschafter.

The experimental work reported herein was carried out during the tenure of a Public Health Service Post-doctoral Fellowship F2-HE-24,271 from the National Heart Institute.

REFERENCES

1. Ayer, J.P. Elastin tissue. Int. Rev. Connective Tissue Res., 1964. 2, 33-100.

2. Ayer, J.P., & Feldmanis, L. Lack of antigenicity of elastin isolated with formic acid. Fed. Proc., 1958, 17, 1674. (Abstract)

3. Bendall, J.R. The titration curves of elastin and of the derived alpha and beta proteins. Biochem. J., 1955. 61, 31-32.

4. Bogart, R. Improvement of livestock. New York: McMillan Co., 1959.

5. Bohak, Z. N^{ε}-(DL-2-amino-2-carboxyethyl)-L-lysine, a new amino acid formed on alkaline treatment of proteins. J. Biol. Chem., 1964. 239, 2878-2887.

6. Bowes, J.H., & Kenten, R.H. Some observations on the amino-acid distribution of collagen, elastin and reticular tissue from different sources. Biochem. J., 1949. 45, 281-285.

7. Bowes, J.H., & Kenten, R.H. The swelling of collagen in alkaline solutions. Biochem. J., 1950. 46, 1-8.

8. Bradstreet, R.B. The Kjeldahl method for organic nitrogen. New York: Academic Press, 1965.

9. Butler, W.T. Hemagglutination studies with formalinized erythrocytes: effect of bis-diazo-benzidine and tannic acid treatment on sensitization of soluble antigen. J. of Immunol., 1963. 90, 663-671.

10. Campbell, D.H., Garvey, J.S., Cremer, N.E., & Sussdorf, D.H. Methods in immunology. New York: W.A. Benjamin, Inc., 1963.

11. Cleary, E.G. A correlative and comparative study of the nonuniform arterial wall. Unpublished M.D. dissertation, University of Sydney, 1963.

12. Cleary, E.G., Jackson, D.S., & Sandberg, L.B. Changes in amino acid composition of bovine ligament elastin with ageing. Proc. Int. Symp. Biochem. & Physiol. of Connective Tissue. Lyons, France, 1965. In Press

13. Cleary, E.G., Sandberg, L.B., & Jackson, D.S. The incorporation of lysine into growing elastin. Biochem. Biophys. Res. Comm., 1966. 23, 139-144.

14. Cox, R.C., & Little, K. An electron microscope study of elastic tissue. Proc. Roy. Soc. London, 1961. 155 (Series B), 232-242.

15. Cushing, J.E., & Campbell, D.H. Principles of immunology. New York: McGraw-Hill, 1957.

16. Dempsey, E.W., & Lansing, A.I. Int. Rev. Cytol., 1954. 3, 437-453.

17. Eastoe, J.E., & Courts, A. Practical analytical methods for connective tissue proteins. London: E. & F. N. Spon Ltd, 1963.

18. Eastoe, J.E., & Leach, A.A. A survey of recent work on the amino acid composition of vertebrate collagen and gelatin. In C. Stainsby (Ed.) Recent advances in gelatin and glue research. London: Pergamon Press, 1958. pp. 173-178.

19. Elwyn, D.H. Continuous scintillation counting of amino acid analyzer column eluates: Atomlight (New England Nuclear Corp.) April, 1964.

20. Fahrenbach, W.H., Sandberg, L.B., & Cleary, E.G. Ultrastructural studies on early elastogenesis. Anat. Rec., In Press.

21. Fieser, L.F., & Fieser, M. Organic chemistry (3rd Ed.) New York: Reinhold Publishing Corp., 1956.

22. Franzblau, C., Sinex, F.M., & Faris, B. Isolation of an unknown component from hydrolysates of elastin. Nature, 1965. 205, 802-803.

23. Franzblau, C., Sines, F.M., Faris, B., & Lampidis, R. Identification of a new crosslinking amino acid in elastin. Biochem. Biophys. Res. Comm., 1965. 21, 575-581.

24. Garrison, F.H. An introduction to the history of medicine. Philadelphia: W. B. Saunders Co., 1929.

25. Gelotte, B., Flodin, P., & Killander, J. Fractionation of human plasma proteins by gel filtration and zone electrophoresis or ion-exchange chromatography. Arch. Biochem. Biophys., 1962. Suppl. 1, 319-326.

26. Gotte, L., & Serafini-Fracassini, A. Electron microscope observations on the structure of elastin. J. Atheroscler. Res., 1963. 3, 247-251.

27. Gotte, L., Serafini-Fracassini, A., & Moret, V. The chemical composition of the NaCl-soluble fraction from autoclaved elastin. J. Atheroscler. Res., 1963. 3, 244-247.

28. Gotte, L., Stern, P., Elsden, D.F., & Partridge, S.M. The chemistry of connective tissues. 8. The composition of elastin from three bovine tissues. Biochem. J., 1963. 87, 344-351.

29. Grant, R.A. Distribution of aortic collagen, elastin, and carbohydrate in different species. Biochem. J., 1965. 95, 53p.

30. Grant, R.A. Preparation of elastin-like material from collagen by cross-linking followed by heat treatment. Biochem. J., 1965. 97, 5c.

31. Gustavson, K.H. The chemistry and reactivity of collagen. New York: Academic Press Inc., 1956.

32. Hall, D.A., Reed, R., & Tunbridge, R.E. Electron microscope studies of elastic tissue. Exp. Cell Res., 1955. 8, 35-48.

33. Harkness, L.R., Harkness, R.D., & McDonald, D.A. The collagen and elastin content of the arterial wall in the dog. Proc. Roy. Soc. (b), 1957. 146, 541-551.

34. Hass, G.M. Elastic tissue. Arch. Path., 1939. 27, 334-365 & 583-613.

35. Henle, J. Allgemeine anatomie. Leipzig: L. Voss, 1841. Cited by Hass, G.M. in Arch. Path., 1939. 27, 334-365.

36. Hoeve, C.A.J., & Flory, P.J. The elastic properties of elastin. J. Amer. Chem. Soc., 1958. 80, 6523-6526.

37. Humphrey, J.H., Neuberger, A., & Perkins, D.J. Observations on the presence of plasma proteins in skin and tendon. Biochem. J., 1957. 66, 390-399.

38. Jackson, D.S., Leach, A.A., & Jacobs, S. Amino acid composition of the collagen fractions of rabbit skin. Biochem. Biophys. Acta., 1958. 27, 418-420.

39. Jackson, D.S., Sandberg, L.B., & Cleary, E.G. The swelling of bovine ligamentum nuchae as a function of pH. Biochem. J., 1965. 96, 813-817.

40. Jackson, D.S., Sandberg, L.B., & Cleary, E.G. Antiserum to α-elastin and its use in the detection of soluble elastin in extracts of foetal ligaments. Biochem. J., 1965. 98, 13p.

41. Kissmeyer, A., & With, C. Clinical and histological studies on the pathological changes in the elastic tissues of the skin. Brit. J. Dermat. and Syph., 1922. 34, 175-194 & 221-237.

42. LaBella, F.S., & Lindsay, W.G. The structure of human aortic elastin as influenced by age. J. Gerontol., 1963. 18, 111-118.

43. Landsteiner, K. The specificity of serological reactions (revised edition). New York: Dover Publications, Inc., 1962.

44. Lansing, A.I. Chemical morphology of elastic fibers. In C. Ragan (Ed.) Connective tissue. Trans. Second Conference. New York. Josiah Macy Jr. Foundation, 1951. pp. 45-85.

45. Lansing, A.I. Elastic tissue in atherosclerosis. In I.H. Page (Ed.) Connective tissue thrombosis and atherosclerosis. New York: Academic Press, 1959. pp. 167-179.

46. Lansing, A.I. Elastic tissue. In A.I. Lansing (Ed.) The arterial wall. Baltimore: Williams & Wilkins, 1959. pp. 136-160.

47. Lawn, A.M. The use of potassium permanganate as an electron-dense stain for sections of tissue embedded in epoxy resin. J. Biophys. Biochem. Cytol., 1960. 7, 197-198.

48. LKB operating manual for immunoelectrophoresis equipment LKB 6800A, LKB-Produkter AB, Stockholm, Sweden.

49. Lloyd, D.J. Protein structure and water absorption. J. Phys. Chem., 1938. 42, 1-10.

50. Lloyd, D.J., & Garrod, M. The rubber-like condition of the fibres of animal skin. J. Soc. Dyers. Col. (Symp. on Fibrous Proteins), 1946. pp. 24-29.

51. Lloyd, D.J., & Garrod, M. A contribution to the theory of the structure of protein fibres with special reference to "thermal shrinkage" of the collagen fibre. Transactions of the Faraday Society, 1948. 44, 441-451.

52. Lowry, O.H., Gilligan, D.R., & Katersky, E.M. The determination of collagen and elastin in tissues, with results obtained in various normal tissues from different species. J. Biol. Chem., 1941. 139, 795-804.

53. Ma, T.S., & Zuazaga, G. Micro-Kjeldahl determination of nitrogen; a new indicator and an improved rapid method. Industr. Engng. Chem., 1942. 14, 280-282.

54. Mall, F.P. Reticulated tissue, and its relation to the connective tissue fibrils. Johns Hopkins Hosp. Reports, 1896. 1, 171-208.

55. Mall, F.P. On the development of the connective tissues from the connective-tissue syncytium. Am. J. Anat., 1902. 1, 329-365.

56. Mallory, F.B. Pathologic techniques. Philadelphia: W.B. Saunders Co., 1942.

57. Marceau, G. Immunological properties of elastin and elastase. Laval Med., 1963. 34, 882-911.

58. Masson, P.J. Some histologic methods: trichrome stainings and their preliminary technique. J. Tech. Methods, 1929. 12, 75-90.

59. Meister, A. Biochemistry of the amino acids. (2nd Ed.) New York: Academic Press. 2 vols.

60. Meites, L., & Meites, T. Removal of oxygen from gas streams. Anal. Chem., 1948. 20, 984-985.

61. Miller, E.J., Martin, G.R., & Piez, K.A. The utilization of lysine in the biosynthesis of the elastin crosslinking molecule. Biochem. Biophys. Res. Commun., 1964. 17, 248-253.

62. Miller, E.J., Martin, G.R., Mecca, C.E., & Piez, K.A. The biosynthesis of elastin cross-links. The effect of copper deficiency and a lathyrogen. J. of Biol. Chem., 1965. 240, 3623-3627.

63. Millonig, G. Further observations on a phosphate buffer for osmium solutions in fixation. Fifth Int. Congr. for Electron Microscop., 1962. 2, P-8.

64. Moore, S., & Stein, W.H. Chromatographic determination of amino acids by the use of automatic recording equipment. In S.P. Colowick & N.P. Kaplan (Eds.) Methods in enzymology. New York: Academic Press, 1962. Vol. VI, pp. 819-831.

65. Moret, V., Serafini-Fracassini, A., & Gotte, L. The carbohydrate composition of the NaCl-soluble fraction from autoclaved elastin. J. of Atherosclerosis Res., 1964. 4, 184-188.

66. Neuman, R.E., & Logan, M.A. The determination of hydroxyproline. J. Biol. Chem., 1950. 184, 299-306.

67. O'Dell, B.L., Elsden, D.F., Thomas, J., Partridge, S.M., Smith, R.H., & Palmer, R. Inhibition of desmosine biosynthesis by a lathyrogen. Biochem. J., 1965. 96, 35p.

68. Ouchterlony, O. Antigen-antibody reactions in gels. Acta. Pathol. Microbiol. Scand., 1949. 26, 507-515.

69. Ovary, Z. Immediate reactions in the skin of experimental animals provoked by antibody-antigen interaction. Progr. Allergy, 1958. 5, 459-508.

70. Page, A.W., & Page, R.O. Influence of neutral salts on the plumping of hides. Ind. Eng. Chem., 1927. 19, 1264-1268.

71. Page, R.C., & Benditt, E.P. Beta aminopropionitrile (BAPN) inhibition of amino oxidase. Fed. Proc., 1966. 25, 2685. (Abstract)

72. Partridge, S.M. Elastin. Advances in Protein Chem., 1962. 17, 227-302.

73. Partridge, S.M., & Davis, H.F. The chemistry of connective tissue 3. Composition of the soluble proteins derived from elastin. Biochem. J., 1955. 61, 21-30.

74. Partridge, S.M., Davis, H.F., & Adair, G.S. The chemistry of connective tissue 2. Soluble proteins derived from partial hydrolysis of elastin. Biochem. J., 1955. 61, 11-21.

75. Partridge, S.M., Davis, H.F., & Adair, G.S. The composition of mammalian elastin. In R.E. Tunbridge (Ed.) Connective tissue. Symposium of C.I.O.M.S. Oxford: Blackwell, 1957. pp. 222-237.

76. Partridge, S.M., Elsden, D.F., & Thomas, J. Biosynthesis of the desmosine and isodesmosine cross-bridges in elastin. Biochem. J., 1964. 93, 30c-33c.

77. Peterson, E.A., & Sober, H.A. Variable gradient device for chromatography. Anal. Chem., 1959. 31, 857-862.

78. Piez, K.A. Continuous scintillation counting of carbon-14 and tritium in effluent of the automatic amino acid analyzer. Anal. Biochem., 1962. 4, 444-458.

79. Piez, K.A. & Morris, L. A modified procedure for the automatic analysis of amino acids. Anal. Biochem., 1960. 1, 187-201.

80. Procter, H.R., & Wilson, J.A. XXXV. - The acid-gelatin equilibrium. J. Chem. Soc., 1916. 109, 307-319.

81. Rapkin, E., & Gibbs, J.A. A system for continuous measurement of radioactivity in flowing streams. Nature, 1962. 194, 34-36.

82. Reynolds, E.S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 1963. 17, 208-211.

83. Richards, A.N., & Gies, W.J. Chemical studies of elastin, mucoid, and other proteins in elastic tissue, with some notes on ligament extractives. Amer. J. Physiol., 1902. 7, 93-134.

84. Robert, L., & Poullain, N. Studies on the structure of elastin and the mode of action of elastase. 1. A new method of preparation of soluble derivatives of elastin. Bull. Soc. Chim. Biol., 1963. 45, 1317-1326.

85. Robert, L., Parlebas, J., Poullain, N., & Robert, B. Recent studies on the immunochemistry of fibrous proteins of the connective tissue. Protides of the Biological Fluids, 1963. 2, 109-113.

86. Roddy, W.T., & O'Flaherty, F. Some notes on the elastic tissue of calf skin during the manufacture of chrome leather. J. Amer. Leather Chem. Ass., 1938. 33, 512-525.

87. Roddy, W.T., & O'Flaherty, F. The behaviour of reticular tissue of animal skin. J. Amer. Leather Chem. Ass., 1939. 34, 671-687.

88. Russell, Jane A. The colorimetric estimation of small amounts of ammonia by the phenol-hypochlorite reaction. J. Biol. Chem., 1944. 156, 457-461.

89. Sandberg, L.B., Cleary, E.G., & Jackson, D.S. Antigenic properties of soluble elastins and their use in detecting soluble elastin in ligament extracts. Nature, 1966. 210, 195.

90. Scarselli, V. Some improvements in colorimetric determination of elastin. Nature, 1959. 184, 1563.

91. Scheidegger, J.J. A micro-method of immuno-electrophoresis. Int. Arch. Allergy Appl. Immunol., 1955. 7, 103-110.

92. Schmitt, F.O. The macromolecular basis of collagen structure in connective tissue. In I.H. Page (Ed.) Thrombosis and atherosclerosis. New York: Academic Press, 1959. pp. 43-65.

93. Schneider, W.C. Phosphorous compounds in animal tissues. J. Biol. Chem., 1945. 161, 293-302.

94. Schwarz, W. Elekgronenmikroskopische untersuchungen über die bildung elastischer fasern in der gewebekultur. Z. Zellforsch., 1964. 63, 636-643.

95. Sinex, F.M., Franzblau, C., & Faris, B. Isolation of an unusual ultraviolet absorbing component of elastin. Fed. Proc., 1966. 25, 2974. (Abstract)

96. Slack, H.G. Metabolism of elastin in the adult rat. Nature, 1954. 174, 512-513.

97. Spackman, D.H., Stein, W.H., & Moore, S. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem., 1958. 30, 1190-1206.

98. Stavitsky, A.B. Micromethods for the study of proteins and antibodies. 1. Procedure and general applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. J. of Immunol., 1954. 72, 360-367.

99. Stein, W.H., & Miller, E.G., Jr. The composition of elastin. J. Biol. Chem., 1938. 125, 599-614.

100. Tänzer, P. Monatschr. Prakt. Dermatol., 1891. 12, 394. Cited by Dempsey, E.W., & Lansing, A.I. Int. Rev. Cytol., 1954. 3, 437-453.

101. Technicon bulletin regarding operation of 22 hour amino acid analyser system. (no number or date available)

102. Technicon bulletin regarding operation of 6-1/2 hour amino acid analyser system. (no number or date available)

103. Thomas, J., Elsden, D.F., & Partridge, S.M. Degradation products from elastin: Partial structure of two major degradation products from the cross-linkages in elastin. Nature, 1963. 200, 631-632.

104. Troll, W., & Lindsley, J. A photometric method for the determination of proline. J. Biol. Chem., 1955. 215, 655-660.

105. Unna, P.G. Histochemie der haut. Leipzig: Franz Deuticke, 1928; The histopathology of diseases of the skin, translated by N. Walker, New York: Macmillan & Co., 1896. Cited by Hass, G.M. in Arch. Path., 1939. 27, 334-365.

106. von Ebner. Rollet's Unters. an der Inst. für Physiol. u. Histol. in Gvaz. Leipzig: 1870. Cited by Dempsey, E.W., & Lansing, A.I. Int. Rev. Cytol., 1954. 3, 437-453.

107. Walford, R.L., Hirose, F.M., & Doyle, P. A chemical and immunologic study of elastin and elastase. Lab. Invest., 1959. 8, 948-960.

108. Webb, J.M., & Levy, H.B. New developments in chemical determination of nucleic acids. In D. Glick (Ed.) Methods of biochemical analysis. New York: Interscience Publishers, Inc., 1958. Vol. 6, pp. 1-30.

109. Weissman, N., Reay, D.T., Coulson, W.F., & Carnes, W.H. Cardiovascular studies on copper-deficient swine. VIII. Elastin content of aorta determined by elastase digestion and formic acid extraction. Lab. Invest., 1965. 14, 372-380.

110. Woessner, J.F., Jr. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys., 1961. 93, 440-447.