IDENTIFICATION AND ROLE OF DITYROSINE IN CONNECTIVE TISSUE PROTEINS

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ABSTRACT

A fluorescent substance was detected in elastin and in an alkali-soluble, hydroxyproline-free protein (ALSP) extracted from elastic tissue during the purification procedure for elastin. This fluorescent substance had ion exchange and paper chromatographic properties identical to those of dityrosine, the o,o'-biphenol analog of tyrosine, which has been proposed to be an interchain crosslink in the rubber-like insect protein, resilin. Radioisotopic experiments indicated its derivation from tyrosine. This fluorescent compound was isolated in pure form from ALSP from adult bovine ligamentum nuchae. A comparison of both the gel permeation, ion exchange and paper chromatographic properties, as well as the ultraviolet absorption and fluorescence characteristics of the isolated compound with those of synthetic dityrosine confirmed its identity as dityrosine. Both in the case of bovine ligamentum nuchae and day-old chick aorta, dityrosine was present in greater quantities in ALSP as compared to elastin; dityrosine could not be detected in elastin from adult bovine ligamentum nuchae.

Solubility, gel permeation, and ion exchange characteristics of ALSP indicated that, like similar glycoproteins reported by others from a variety of connective tissues, ALSP exists in various states of aggregation. The dityrosine content of ALSP was found to be directly

related to the size, extent of aggregation and insolubility of the protein. Introduction of additional dityrosine into ALSP by treatment with horseradish peroxidase was accompanied by an increase in aggregation and a decrease in solubility of the protein. After extensive enzymic hydrolysis of ALSP, dityrosine was concentrated in the enzyme resistant portion of the protein. On the basis of these observations, dityrosine was proposed to be an interchain crosslink in ALSP. Other crosslinks may also be present in ALSP, some of which may be derived from dityrosine.

Studies of the amino acid compositions of aortic elastin from fetal and young chicks of various ages indicated that alkali-purified elastin was associated with a considerable amount of a more polar protein. The amino acid composition of this polar, alkali-insoluble contaminant appeared to be similar to that of ALSP. Moreover, this polar contamination was most persistent in the youngest of tissues. The morphology of developing elastic fibres and the persistence of the polar contamination suggested that ALSP may be involved in the biosynthesis of elastin. A hypothesis for this role is presented.

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Three unusual, lysine-derived amino acids, desmosine, isodesmosine and lysinonorleucine have been identified in elastin, where they probably function as the interchain crosslinks necessary for the rubber-like properties of the protein. Because of steric considerations and the slow rate of formation especially of the desmosines, it is possible that other, more rapidly formed crosslinks may be present in developing elastin. That these proposed crosslinks may be of aromatic origin is suggested by the fact that the fluorophore responsible for the blue-white fluorescence of elastin has been proposed to be involved in an age-related crosslinking process, and by the established role of aromatic amino acids in the crosslinking of insect cuticular protein, plant lignin and the rubber-like insect protein, resilin.

Ground substance proteins have been demonstrated in close association with elastic fibres, and require vigorous procedures such as extraction with hot alkali for their removal. Depending on the staining procedure used, these associated proteins have been visualized either as an amorphous coating on the fibrillar elastic elements, or as a microfibrillar structure surrounding the weakly staining core of the elastic fibres. Because of the changing proportion of microfibrillar to elastin components, the involvement of this microfibrillar material in the biosynthesis and laying down of elastic fibres has been proposed. The observation that these alkali-extractable substances show a blue-white fluorescence similar to that of elastin also raises the possibility of the presence of unusual substances of aromatic origin, perhaps with a crosslinking role, in this protein or proteins.

The purpose of this investigation was:

(1) to investigate the possible involvement of aromatic amino acids, especially tyrosine, in the formation of crosslinks early in the development of elastin, (2) to study the fluorescent protein(s) closely associated with elastin and its (their) possible role in the biosynthesis of elastin.

II. GENERAL INTRODUCTION

GENERAL INTRODUCTION

ELASTIN

The components of the extracellular spaces of connective tissue are readily differentiated into fibrous elements and an amorphous ground substance, although the ease of this distinction should not imply that these are isolated components without important interactions between them. The fibrous elements are elastin, collagen and reticulin, the last thought to be closely related to collagen. Collagen fibres, possessing a high tensile strength and low extensibility, are concentrated in tissues such as tendons, cartilage and skin, where these mechanical properties are desirable. On the other hand, elastic fibres are more prominent in tissues such as ligaments and the large blood vessels, where extensibility and rubber-like elasticity are required. Because of their yellow coloration, these elastin-rich tissues have been referred to as "yellow connective tissue".

Much of the early work on elastin consisted of histological studies, and the well-characterized staining properties of elastic fibres have been extensively reviewed (Dempsey and Lansing, 1954; Hall, 1959; Fullmer, 1965). Because of the intimate association of the elastic elements with proteins and carbohydrates of the ground substance, it is sometimes difficult, in these histological studies, to differentiate between the staining properties of the elastic fibre itself and those of the associated carbohydrates and proteins. For this reason we must draw a careful distinction, as did Hall in 1959, between the properties of the morphologically intact elastic fibre *in situ* and those of the elastic protein, elastin.

Both elastin and collagen are unusual among proteins in that, once laid down, they are subject to little or no metabolic turnover. Slack (1954), labelling the elastin of adult rat aortas with ^{14}C-glycine, found no measurable metabolic turnover of the protein for at least 9 days after the administration of the labelled amino acid. Kao et al. (1961) obtained similar results using ^{14}C-lysine and, at the same time, showed that the rate of synthesis of elastin is much greater in younger animals as compared to adults. Walford et al. (1964), studying young rats labelled with ^{14}C-glycine, showed that, after an initial decline due to growth and maturation, the specific activity of aortic elastin remained constant for up to 930 days.

As a consequence of their inertness, any chemical alterations in elastin or collagen, occurring by either random or physiologically controlled mechanisms, will persist and accumulate throughout life. These alterations may be related to the progressive changes in physical properties of these proteins apparent during aging.

1. Isolation and Purification of Elastin

Elastin is insoluble in all reagents except those that hydrolyze peptide bonds. Advantage is taken of this property in the purification of elastin; most isolation methods are based on the solubilization of all contaminating substances, leaving pure elastin as an insoluble residue. Many isolation methods have been proposed, each attempting to obtain maximum purification of elastin with minimum degradation of the protein. Elastins purified in these diverse ways often show different amino acid compositions and properties. Thus, varied purification methods have resulted in ambiguity in the definition of the elastic protein. This is

reflected in the definition of elastin offered by Hall in 1959: "elastin is the name given to the derived protein obtained from elastic tissue by techniques aimed at the removal of as much extraneous material as possible, without causing undue degradation of the protein".

(a) Methods for the Removal of Collagen

The use of warm 88% formic acid for the extraction of collagen from connective tissue was first suggested by Hass (1942), although he conceded that the elastic fibres were "not wholly spared" by the procedure. Ayer et al. (1958) and Carton et al. (1960) have also used this treatment, suggesting that it results in the removal of all microscopically visible traces of collagen while the morphological integrity of the elastic fibre remains intact.

Boiling in dilute acetic acid, first used by Tilanus in 1844 (cited by Richards and Gies, 1902), was recommended by Gross (1949) and Loeven (1960b) for the removal of collagen from connective tissues although they, along with Hall (1951) and Hall et al. (1952), recognized that non-collagenous proteins still remained in the elastic tissue after this treatment. On the other hand, Partridge and Davis (1950) suggested that prolonged boiling of proteins in dilute acetic acid resulted in hydrolysis of susceptible peptide bonds.

Extraction with hot water as a method for collagen removal was described by Horbaczewski (1882) and Schwartz (1894) (Horbaczewski and Schwartz cited by Richards and Gies, 1902). This method was later used by Lowry et al. (1941) and Neuman and Logan (1950). Extensive autoclaving with water has been widely employed for the complete removal of collagen (Partridge et al., 1955; Partridge, 1962).

(b) Extraction with Hot Alkali

It was evident to even the earliest investigators (Chittenden and Hart, 1887; Schwartz, 1894; cited by Richards and Gies, 1902) that removal of all contaminating substances from elastin required more vigorous extraction procedures. This was also recognized by Lowry et al. (1941). Lansing et al. (1952) recommended a 45 minute treatment with 0.1M NaOH at 98^{o} as a general purification method for elastin. Although Partridge et al. (1955) considered autoclaving with water to be sufficient to yield a pure elastin residue from bovine ligamentum nuchae, Gotte et al. (1963b) demonstrated that further treatment with hot alkali extracted 2%, by weight, of a polar, non-collagenous, non-elastin protein. Collagen-free aortic elastic tissue was more extensively contaminated by this protein. While extraction with cold 0.5M NaOH was partially successful in removing the polar contaminant, complete extraction required treatment with hot alkali.

The principle objection to the purification of elastic tissues by hot alkali extraction has been the fear that the elastin itself would be hydrolytically damaged by the procedure. However, Lansing et al. (1952), studying the time course of the loss of weight of elastic tissue during hot alkali extraction, found that, up to 45 minutes at least, this pattern was consistent with the removal of contaminating substances; general dissolution of the elastin occurred only after more prolonged treatment. Moreover, Gotte et al. (1963b) were unable to detect any increase in the number of N-terminal amino acid residues in elastin after a 45 minute hot alkali extraction, indicating that there had been little or no peptide bond cleavage by this treatment.

Lansing's method (Lansing et al., 1952) has been widely employed, especially in studies in which a chemically pure elastin preparation is desired. Since, with few exceptions, the amino acid compositions of elastins from all tissues are identical after this treatment, the composition of the protein remaining after the Lansing extraction has been generally accepted as that of pure elastin.

(c) Other Methods

Enzymic methods, using trypsin, pepsin and collagenase (Hospelhorn and Fitzpatrick, 1961; Czerkawski, 1962) have, in general, been inadequate for the removal of all contaminating substances from elastic tissue, although Steven and Jackson (1968) have suggested that this can be accomplished by treatment with crude bacterial α-amylase and collagenase. Boiling with 40% urea (Hall, 1951) and prolonged extraction with peracetic acid and KOH (Cox and Little, 1961) have also been recommended, but have not found widespread favour.

(d) Nature of the Contaminating Materials

The ease of purification of elastin will depend on the properties and proportions of the various contaminating materials and, therefore, will vary with the source of the tissue. In general, the substances contaminating the elastic tissues and extracted by the various procedures will be discussed as ground substance components. At this point, however, it would be useful to indicate the relationship between some of these contaminants and the elastic fibre that has been suggested as a result of electron microscopic observations.

Gross (1949) reported that collagen-free elastic fibres appeared to be embedded in an amorphous matrix which could be removed by digestion

with trypsin. Lansing et al. (1952) proposed a similar model for the elastic fibre and suggested that elastase treatment first dissolved the amorphous matrix, revealing the fibrous elements, and subsequently digested the fibres themselves. Hall et al. (1952) and Hall (1955) proposed that this amorphous component, which they called "elastomucin", acted as a cementing substance between the fibrous elements, and was the material removed by hot alkali extraction. Aortic and ligamentum elastic fibres differed only in their proportions of these amorphous and fibrillar elements. Similarly, Gotte and Serafini-Fracassini (1963) and Gotte et al. (1963a) demonstrated that this amorphous matrix could be removed, in part, by prolonged extraction with 2M NaCl, or completely extracted by hot alkali treatment. Later, pancreatic elastase was resolved into two components, elastoproteinase and elastomucase (Hall and Gardiner, 1955; Banga and Balo, 1957; Hall, 1957a, 1957b; Loeven, 1960a; Loeven, 1963), whose principal substrates were the fibrillar and amorphous components, respectively.

The heroic methods necessary for the removal of some of these contaminants has led to suggestions that they are involved in the structure of the elastic fibre itself. Thus, Czerkawski (1962) regarded elastin as a sialoprotein. Similarly, LaBella (1957, 1958) demonstrated that extensively defatted and hot alkali-extracted elastin still contained at least two lipid components as well as 0.1% phosphorous. Lansing et al. (1952) reported the release of a lipid material from alkali-purified elastin during the course of digestion with elastase, and Loomeijer (1958, 1961) isolated from elastin a peptide-bound fluorescent lipid which he characterized as a saturated fatty acid.

(e) Special Difficulties in the Purification of Very Young and Very Old Elastins

It is generally true that elastins from various sources can be purified to identical amino acid compositions by the Lansing method, the only known exception for adult tissue being bovine ear cartilage elastin which dissolves in the hot alkali before all of the contaminant can be removed (Gotte et al., 1963b). However, several investigators have reported difficulty in the purification of very young and very old elastins.

Cleary et al. (1965, 1967) demonstrated that a large amount of a non-collagenous protein was removed from collagen-free bovine fetal ligamentum nuchae by hot alkali treatment. The quantity of this extracted protein decreased with increasing fetal age. At the same time, although all of this non-collagenous,polar contaminant could be removed from adult ligamentum by hot alkali extraction, fetal tissues still retained a significant amount of the contaminant protein even after this treatment. Similarly, Steven and Jackson (1968) reported that hot alkali extraction was insufficient to purify bovine fetal ligamentum nuchae and aortic elastin unless the tissue had been previously treated with a crude preparation of bacterial α-amylase. These results are in contrast to those of Miller et al. (1964) who reported that, except for an initial fall in lysine content, there were no changes in the amino acid composition of hot alkali-purified chick aortic elastin from the 14 day embryo to the adult chicken.

Several investigators have also reported an increase in the polar amino acid content of elastins from very old tissues, despite purification with hot alkali (Lansing et al., 1951; Lansing, 1955; Fitzpatrick

and Hospelhorn, 1965b; Gotte et al., 1965). LaBella et al. (1966) demonstrated that only very vigorous extraction procedures, dissolving most of the tissue, could result in a residue having the normal amino acid composition of elastin. Even these extreme procedures failed to purify completely the oldest of tissues. From these results, LaBella concluded that the increased polarity of elastin from old tissues was due to the presence of a polar contaminant protein which became extremely firmly bound to the elastic fibres with age.

2. Solubilization of Elastin

Although the insolubility of elastin is a useful property for its isolation, it is a decided disadvantage in attempts to characterize the protein. Thus, several methods have been employed to solubilize elastin, while at the same time doing as little hydrolytic damage as possible.

Treatment of elastin with hot 0.2M oxalic acid dissolves the protein, hydrolyzing it into two major components (Adair et al., 1951; Partridge et al., 1955; Wood, 1958). One of these components, designated α-elastin, has a molecular weight of approximately 80,000 and reversibly coacervates on warming. The second component, β-elastin, has a molecular weight of approximately 6,000 and does not coacervate. Because β-elastin was the principal hydrolytic product in early stages of oxalic acid treatment, while α-elastin predominated in later stages, it was initially suggested that elastin was a heterogeneous mixture of these two proteins. However, Partridge and Davis (1955) demonstrated that the amino acid compositions of these two proteins were very similar, indicating that they were hydrolytic fragments of the same protein.

Other methods, including refluxing in 40% urea, mild acid and alkaline hydrolysis and pepsin treatment, have also been used to solubilize elastin, all of these resulting in a mixture of coacervating and non-coacervating components (Bowen, 1953; Hall and Czerkawski, 1961). Treatment of α-elastin with elastase destroyed its ability to coacervate (Hall and Czerkawski, 1961), suggesting that a minimum molecular size of the protein was necessary for this phenomenon to occur.

3. Biosynthesis of Elastin

Although the formation of mature collagen fibres in vivo is now generally accepted to proceed via the condensation of soluble precursor proteins elaborated from the fibroblast, no such causal relationship has been firmly established among cells, precursors and mature fibres in the case of elastin. Indeed, the "elastoblast", first postulated by Loisel in 1897 (cited by Partridge, 1962), has never been recognized as a morphological entity. This difficulty in definition of the specific cell type responsible for the synthesis of elastin led to suggestions that both collagen and elastin were derived from the same precursor protein, differing only in their states of organization. These proposals were encouraged by observations that collagen fibres appeared to become morphologically and histologically elastin-like as a result of pathological disorders (Tunbridge et al., 1952) or various chemical or enzymic treatments (Burton et al., 1955; Banga et al., 1956; Keech et al., 1956; Keech and Reed, 1957). Furthermore, after pathological or chemical degradation collagen fibres could be digested with pancreatic elastase, which was at that time thought to be specific for elastin. A more general proteolytic character has since been demonstrated for this enzyme (Mandl, 1961). Thus, Hall et al.

(1955) proposed a scheme for the biosynthesis of elastin involving its formation either from the same precursor protein as collagen or from degradation products of collagen. The evidence for this hypothesis was thoroughly reviewed by Hall (1959). With the advent of reliable amino acid analyses for collagen (Bowes and Kenten, 1948; Bowes et al., 1955; Eastoe, 1955) and elastin (Stein and Miller, Jr., 1938; Neuman, 1949; Neuman and Logan, 1950; Lansing et al., 1951) it became clear that the amino acid compositions of these two proteins were sufficiently different that the possibility of a collagen-to-elastin conversion or a common precursor protein became remote (Partridge, 1962).

With the demise of this concept, it became generally accepted that elastin, like collagen and, indeed, most of the fibrillar and non-fibrillar components of the ground substance, is elaborated from the fibroblast (Partridge, 1962; Branwood, 1963; Fahrenbach et al., 1966; Hashimoto and Dibella, 1966; Takagi, 1969a). During the synthesis of connective tissues, either under conditions of wound repair or in rapidly developing embryonic tissue, the fibroblasts are elongated or stellate in shape, contain a well-developed rough endoplasmic reticulum with many cisternae and have an abundance of the Golgi apparatus, demonstrating all of the characteristics of cells engaged in rapid synthesis (Haust et al., 1965; Haust and More, 1967).

During the synthesis of embryonic connective tissue, a fibrillar, electron dense material is often seen in the intracellular spaces, especially in vesicles pinched off from the Golgi regions (Jackson, 1954; 1956; Fahrenbach et al., 1966; Haust and More, 1967; Takagi and Kawase, 1967). The suggestion that these vesicles fuse with the cell membrane

and extrude their contents into the extracellular space (Fahrenbach et al., 1966) is supported by observations of dense bundles of these fibrils situated along the outside of the cell membrane (Low, 1962; Haust, 1965; Fahrenbach et al., 1966; Takagi and Kawase, 1967), sometimes in U-shaped depressions in the membrane (Fahrenbach et al., 1966; Takagi, 1969a). Occasionally the intra- and extracellular fibrils appear to be continuous with one another with the absence of a cell membrane in that area (Hashimoto and Dibella, 1966; Haust and More, 1967).

Extracellular microfibrillar material was reported by Jakus (1954) in rat cornea, and has since been observed in peridontal membranes (Fullmer and Lillie, 1958), where they are called oxytalan fibrils, in aorta (Jensen, 1962; Bierring and Kabayasi, 1963; Fyfe et al., 1968), in ligamentum nuchae and tendon (Fahrenbach et al., 1966; Greenlee, Jr., et al., 1966), in cartilage matrix (Jackson, 1954, 1956) and in other developing connective tissues.

These microfibrils have been reported to be approximately 100 A in diameter, often observed to be tubular in structure with an electron dense periphery and a lucid core (Low, 1962; Fahrenbach et al., 1966; Takagi and Kawase, 1967; Takagi, 1969a) and sometimes appear to have a beaded or vesicular substructure (Haust, 1965, Fahrenbach et al., 1966; Haust and More, 1967), especially when stained with potassium permanganate (Takagi, 1969a). They stain deeply with uranyl acetate and/or lead, but only weakly or not at all with phosphotungstic acid. In contrast, elastin stains well with phosphotungstic acid but not with uranyl acetate or lead, and collagen is stained by uranyl acetate and phosphotungstic acid but not by lead (Greenlee, Jr. et al., 1966, Takagi and Kawase, 1967).

The role of these microfibrils in the development of the fibrous elements of connective tissue is still not clear. Their involvement in the biosynthesis and laying down of collagen fibres has been suggested (Gross et al., 1954; Jackson, 1954, 1956; Chapman, 1961), and Haust (1965) proposed that they were the common precursor of both collagen and elastin fibres. When stained with uranyl acetate and/or lead, the microfibrils have often been observed as a dense mantle enveloping the central, weakly staining core of the elastic fibres (Haust, 1965; Fahrenbach et al., 1966; Greenlee, Jr. et al., 1966; Hashimoto and Dibella, 1966; Greenlee, Jr. and Ross, 1967; Haust and More, 1967; Takagi and Kawase, 1967; Ross and Bornstein, 1969; Takagi, 1969a). In some cases the microfibrils have also been observed to penetrate into the amorphous core (Low, 1962; Haust et al., 1965). This relationship is especially prominent in fetal and young elastic tissue. Indeed, in the youngest of tissues only the microfibrillar elements are visible. Later the central, weakly staining core appears within the microfibrils and increases in size with development of the tissue until, at maturity, the microfibrils are seen only as a sparse mantle around this amorphous core (Fahrenbach et al., 1966; Greenlee, Jr. et al., 1966; Hashimoto and Dibella, 1966; Greenlee, Jr. and Ross, 1967; Haust and More, 1967; Ross and Bornstein, 1969).

As a result of such observations, many investigators have suggested that the microfibrils form an integral part of the developing elastic fibres, participating in some way in their biosynthesis (Jensen, 1962; Haust, 1965; Fahrenbach et al., 1966, Haust and More, 1967), perhaps as a type of nucleation centre for the formation of the elastin macromolecules (Greenlee, Jr. et al., 1966; Ross and Bornstein, 1969).

Fahrenbach et al. (1966) called the microfibrils "pre-elastin filaments", and Fyfe et al. (1968) and Takagi (1969b) refer to them as "proelastin".

Ross and Bornstein (1969), correlating electron microscopic observations with biochemical data, identified the central amorphous core as elastin by its amino acid composition and susceptibility to elastase digestion. The microfibrils, which could be removed from the central core by extraction with dithioerythritol in 5M guanidine or by treatment with chymotrypsin or trypsin, were composed of a hydroxyproline-free polar protein containing relatively large proportions of aspartic and glutamic acid residues. The microfibrils could also be extracted from the elastin core with alkali, although treatment with hyaluronidase or β-glucuronidase had no effect.

3. Crosslinking of Elastin

(a) Rubber-like Properties

The condition of rubber-like elasticity and extensibility is an unusual property, one shown by relatively few polymeric substances. In general this property is possessed by networks of long chain polymeric molecules which are randomly orientated and freely mobile with respect to one another, their mobility being limited only by the presence of a few crossbridges between polymer chains. If these crossbridges are represented by simple physical entanglements, the strained network is likely to flow with time, resulting in a permanent deformation. Thus, perfect elasticity is only possible if there are strong, stable covalent crosslinks between the chains. The number of these crosslinks per unit volume must be small and, for perfect elasticity, the distance between them should be uniform (Andersen and Weis-Fogh, 1964; Andersen, 1966).

The physical properties of elastin, studied both in the native and purified states, characterize this protein as a true elastomer (King, 1946; Lloyd and Garrod, 1948; Hoeve and Flory, 1958). Gotte et al. (1968), by means of stress-strain measurements on hot alkali-purified lagamentum nuchae, estimated the average molecular weight between crosslinks in elastin to be 4,000.

Most rubber-like polymers are self lubricating, non-hydrophilic networks. However, elastomeric proteins such as elastin and the insect protein, resilin, although non-polar relative to other proteins, still contain some polar amino acids and the peptide bond itself has polar character. Thus, in the dry state where the interactions between chains will be accentuated, these proteins are hard and brittle. Solvation of the proteins, effectively shielding these polar groups from one another, is necessary for the demonstration of their rubber-like properties.

Because of the slow or non-existent turnover of elastin, any alteractions in the protein will remain and accumulate throughout life. Many investigators have attributed the age-related changes in the physical properties of the elastic tissues to an increased density of crosslinking (King, 1946; LaBella and Lindsay, 1963; Bjorksten, 1968; Hall, 1968; Krug, 1968). Although a few widely spaced crosslinks are necessary for the rubber-like properties of the protein, subsequent continued formation of crosslinks renders the tissue less elastic.

(b) Crosslinks Derived from Lysine

The α-elastin fragments of oxalic acid-solubilized elastin were thought to contain peptides derived from the more crosslinked areas of the elastin macromolecule (Partridge and Davis, 1955). In an attempt to

characterize these crosslinks, Partridge and his colleagues (1963) degraded elastin using a series of proteolytic enzymes and selected out the larger peptides by a technique similar to gel permeation chromatography. This peptide fraction was yellow and fluorescent and contained a chromophore absorbing in the ultraviolet. Further hydrolysis of these peptides resulted in the isolation of two tetra-substituted pyridinium amino acids which were called desmosine and isodesmosine (Thomas et al., 1963).

A consideration of the structures of these two compounds suggested that they might be formed by condensation of the side chains of four lysine residues. This was confirmed by studies involving the incorporation of radioisotopically labelled lysine into developing elastin (Miller et al., 1964, 1965a; Partridge et al., 1964, 1966; Anwar and Oda, 1966, 1967). Furthermore, the increase in the proportion of desmosines in developing elastic tissue was coincident with an appropriate fall in the content of lysine in the protein (Miller et al., 1964, 1965a; LaBella and Vivian, 1967).

At approximately the same time Franzblau and his colleagues isolated N^{ϵ}-(5-amino 5-carboxypentanyl)-lysine from elastin, naming this compound "lysinonorleucine" (Franzblau et al., 1965a, 1965b). This amino acid was formed by the condensation of the side chains of two lysine residues into a Schiff base intermediate, which was subsequently reduced to the final structure (Lent and Franzblau, 1967; Franzblau et al., 1969; Lent et al., 1969).

Since that time, much effort has gone into the isolation and characterization of the various intermediates involved in the synthesis of these potentially crosslinking amino acids. Most of the studies have

involved stabilization of the intermediates by means of reduction with borohydride. In this way, Salcedo et al. (1969) demonstrated the presence in elastin of an aldol condensation product of two lysine residues. Similarly, Starcher and his colleagues (Starcher et al., 1967; Partridge, 1969) have isolated from elastin an amino acid formed from three lysine residues, which is proposed to be one of the later intermediates in the formation of the desmosines. They called this intermediate "merodesmosine".

Partridge et al. (1966) demonstrated that the process of formation of the desmosines was a slow one, requiring up to 17 days for completion. He suggested that during the period of time before the formation of the permanent chemical link the situation can be best described as a dynamic equilibrium of reversible aldols and Schiff bases (Partridge , 1969). One of the problems in an understanding of the formation of the desmosines is to explain how the exact geometric presentation of the lysine groups from different chains can be secured and maintained so that the desmosines, otherwise sterically improbable crosslinks, can be formed. Partridge has approached this problem by envisioning immature elastin as a three-dimensional crosslinked network of corpuscular or globular proteins, suggesting that the specific tertiary structure of these globular proteins is involved in the orientation of the elastin chains with respect to one another (Partridge, 1966, 1967a, 1967b, 1969).

Much of the information concerning these lysine-derived crosslinks has resulted from studies of the pathological state known as lathyrism, characterized, in part, by the fragmentation of the elastic fibres and lamellae, especially in young animals (Churchill et al., 1955; O'Dell et al., 1961; Coulson and Carnes, 1962; Simpson et al., 1962, Simpson and

Harms, 1964). This state can be brought about in animals either by feeding a variety of chemical agents known as lathyrogens (Bensusan et al., 1962) or by withholding copper from the diet. The root cause of the defect appears to be an interference with the biosynthesis of new desmosine and lysinonorleucine crosslinks without affecting the general process of protein synthesis (Miller et al., 1965b, 1967; O'Dell et al., 1965, 1966b).

Early in the history of the desmosines Partridge proposed that the probable first step in their formation was the oxidative deamination of the ϵ-amino group of the lysine side chain to an aldehyde (Partridge et al., 1964). Aldehydes have been detected in elastin (Nakao and Angrist, 1968) and have been shown to be present in smaller quantities in lathyritic elastin as compared to normal elastin (Miller and Fullmer, 1966). This oxidative deamination probably occurs through the mediation of an enzyme and, in fact, an amine oxidase capable of oxidizing protein-bound lysine residues has been reported in several tissues. This enzyme requires copper for its activity (Page and Benditt, 1967a), and is competatively inhibited by β-aminopropionitrile, one of the most potent of the lathyrogenic agents (Page and Benditt, 1967a, 1967b; Pinnel and Martin, 1968; Rucker et al., 1969; Siegel and Martin, 1970).

One of the properties of copper-deficient elastic tissue is a general increase in the quantities of proteins which can be solubilized from the tissue by various procedures (Weissman et al., 1963; 1965; Kimball et al., 1964; O'Dell et al., 1966a). Using mild treatments with salt solutions, Smith et al. (1968) and Sandberg et al. (1969a, 1969b) extracted and purified a protein from copper-deficient aortic tissue which has an amino acid composition identical to that of elastin, except for a higher

lysine content and the absence of desmosines. They proposed that this protein, which they called "proelastin", is the normal soluble precursor of elastin which has remained soluble because the copper deficiency has prevented the formation of the desmosine crosslinks.

In copper-deficient elastic tissue there is an overabundance of the microfibrillar elements associated with elastin (Keech, 1960; Waisman and Carnes, 1967). Replacement of copper in the diet is initially accompanied by a further increase in the quantity of microfibrils (Waisman et al., 1969). Although it was first suggested that these microfibrils represented the lysine-rich proelastin protein described above (Waisman and Carnes, 1967), later studies demonstrated that this was not the case (Waisman et al., 1969). These microfibrils are probably identical to those seen in the normal elastic tissue. The reason for their unusual abundance in this pathological state is not yet clear.

(c) Other Crosslinks:

Elastin, even when highly purified, retains its yellow pigmentation and brilliant blue-white fluorescence (LaBella, 1957, 1961; Partridge, 1962). Although Loomeijer (1958, 1961) and Thornhill and LaBella (1965) have reported the presence in elastin of a fatty acid or aldehyde showing a yellow-white fluorescence, most attempts to characterize either the yellow pigment (Karkela and Kulonen, 1959; LaBella, 1962) or the fluorescent material (LaBella, 1962; Thornhill and LaBella, 1965; Thornhill, personal communication) have been frustrated by the apparent instability of these components.

Any continuing crosslinking of elastin with age cannot be due to the desmosines or lysinonorleucine since the contents of these amino

acids in elastin become constant very early in life (Miller et al., 1964; LaBella et al., 1966; LaBella and Vivian, 1967). On the other hand, there is a continuous increase with age in both the yellow pigment and the blue-white fluorescent material, suggesting that these components may be involved in the age-related crosslinking of elastin (LaBella and Lindsay, 1963). Indeed, as Thornhill and LaBella (1965) have pointed out, the highly crosslinked, desmosine-rich peptide fragments isolated by Partridge and his colleagues (1963) were reported to be bright yellow in color and to possess the blue-white fluorescence characteristic of elastin, although the desmosines are neither yellow nor fluorescent. Moreover, even after extensive digestion of elastin with elastase, the yellow and fluorescent components remain non-dialyzable (LaBella, 1961; Walford et al., 1961).

LaBella and his colleagues have suggested that the spectral characteristics of these components indicate that they may be quininoid derivatives of aromatic amino acid residues in elastin (LaBella and Thornhill, 1965). There is ample precedent for the suggestion of the involvement of aromatic amino acids in crosslinking processes. Dabbous (1966) and Joseph and Chandrakasan (1967) have suggested the involvement in the crosslinking of collagen of compounds similar to the tyrosine-derived o-quinones proposed to be involved in the sclerotization and hardening of the insect cuticle (Mason, 1955; Andersen and Weis-Fogh, 1964; Pryor, 1962; Brunet, 1965, 1967). Tyrosine derivatives are known to be involved in the formation of lignin and tannin in plants (Nord and Schubert, 1962; Brown, 1964) and Kimura and Kubota (1966, 1967, 1968, 1969) have suggested that this amino acid may be involved in some way in the crosslinking of

the shark connective tissue protein, elastoidin. Andersen has isolated biphenolic and terphenolic derivatives of tyrosine from the rubber-like insect protein, resilin (Andersen, 1963; Andersen and Weis-Fogh, 1964; Andersen, 1964, 1966), and has also demonstrated the presence of another tyrosine derivative in the hinge ligament of molluscs (Andersen, 1967).

There are indications that crosslinks other than the desmosines and lysinonorleucine may be involved in the stabilization of developing elastic tissue. The necessity of providing a stable confirmation for the elastin molecules during the period of formation of the desmosine crosslinks has been discussed and similar arguments may be applied, to some extent, to the lysinonorleucine crosslinks. In this regard, Andersen (1966) pointed out that the data of Miller et al. (1964) indicated that even before the formation of desmosine crosslinks in newly synthesized elastin these chains are sufficiently firmly bound to the elastin matrix to resist extensive hot alkali extraction. Thus, besides the slowly forming, lysine-derived crosslinks, developing elastin may also contain more rapidly forming crosslinks of an unknown nature.

GROUND SUBSTANCE

In spite of the fact that the ground substance constitutes the immediate environment of most cells, very little is known, except in the most general terms, about the "complex and important" organization and functions of this material. This lack of knowledge is, at least in part, due to the complexity of the interactions among the many components of the ground substance. Thus, the manipulations required for the isolation of these various components both destroy the interactions among them and perhaps also create new, artifactual associations. We can, at present,

do little else but list the identifiable components of the ground substance, discuss some of the interactions among these components that have been reported, and speculate on the overall organization of the material in situ.

Although at least some of the mucopolysaccharide components of the ground substance were at one time though to arise from the mast cells (Asboe-Hansen, 1957), it is now generally agreed that the fibroblast is the main source of both the protein and polysaccharide components (Gersh and Catchpole, 1949; Bunting and Bunting, 1953; Jackson, 1953; Bertelsen and Jensen, 1960; Gaines, 1960; Bhatnager and Prockop, 1966). Plasma proteins have been identified in the ground substance (Humphery et al., 1957; Neuberger, 1957) and Sobel (1967) has suggested that these may have some role in the manufacture of ground substance constituents.

1. Components of the Ground Substance

Acid mucopolysaccharides, including hyaluronic acid, chondroitin sulfate A, B and C and heparin, were the earliest recognized components of the ground substance. Their occurrence and distribution in various tissues has been the subject of several reviews (Meyer et al., 1956, 1957; Muir, 1964; Spicer et al., 1967).

The protein components of the ground substance have been less well defined and, for the purposes of this discussion, will be divided into those that are readily extracted by relatively mild reagents such as neutral salt solutions and water, and those that are considerably less soluble, requiring more vigorous procedures for their extraction.

The relatively soluble protein constituents include the protein-polysaccharide complexes which have been solubilized especially from cartilaginous tissues (Meyer and Smyth, 1937; Shatton and Schubert, 1954;

Muir, 1957; Mathews and Lozaityte, 1958; Partridge et al., 1961; Eyring and Yang, 1968; Franek and Dunstone, 1968; Serafini-Fracassini, 1968; Campo et al., 1969; Serafini-Fracassini et al., 1969). The reported interactions between these protein-polysaccharide complexes and collagen suggests that they may play a role in the formation and organization of collagen fibrils in vivo (Partridge, 1948; Jackson, 1953; Courts and Giles, 1965; Disalvo and Schubert, 1966; Milch, 1966; Mathews and Decker, 1968; Wasteson and Obrink, 1968; Steven et al., 1969). Relatively soluble glycoproteins have also been extracted from connective tissues (Fishkin and Berenson, 1961; Fishkin et al., 1961; Berenson and Fishkin, 1962; Radhakrishnamurthy et al., 1964; Berenson et al., 1966), sometimes associated with these protein-polysaccharide complexes (Partridge and Davis, 1958; Partridge and Elsden, 1961; Partridge et al., 1965; Kao et al., 1968).

Other considerably less soluble glycoproteins are also present in a variety of connective tissues (Bowes et al., 1956, 1957; Andersen, 1961; Kao et al., 1962; Robert and Dische, 1963) including aortic tissue (Bertelsen, 1960; Enselme et al., 1961; Gotte et al., 1963a). Barnes (1965) and Barnes and Partridge (1968) have isolated and extensively characterized an alkali-soluble glycoprotein from human aorta which they suggest is intimately associated with the elastin matrix in the tissue. Similar glycoproteins have been extracted from human intervertebral disc (Steven et al., 1968; Pearson et al., 1969) where they have been suggested to possess rubber-like properties and exhibit a blue-white fluorescence (Moschi and Little, 1966). Timpl and his colleagues (Timpl et al., 1968, 1969) and Robert and his colleagues (Robert and Comte, 1968; Robert and Robert, 1969; Moczar and Robert, 1970) have extracted similar glycoproteins from a vari-

ety of connective tissues and suggested that they represent a previously unrecognized class of structural proteins.

2. Organization of the Ground Substance

At present little is known about the structural organization of the ground substance, although speculations have been made on the basis of the physical properties of some of the identified components (Gersh and Catchpole, 1949; Jackson, 1965; Mathews, 1967). Interactions among these components as well as with the fibrous elements of the connective tissue are known to occur but, as yet, the nature of these interactions is not well defined. Although the concept of the ground substance as a three-dimensional, heterogeneous macromolecular array whose structure is sensitive to various physiological and pathological perturbations is attractive, little is known about how the various constituents contribute to the structure, how changes in the proportions of these constituents can change the structure or how these changes in structure affect physiological or pathological processes.

DITYROSINE

In 1959 Gross and Sizer reported that the reaction of horseradish peroxidase and hydrogen peroxide with tyrosine and tyramine initially resulted in the production of the o,o'-biphenolic analogs of the compounds (designated dityrosine and dityramine, respectively). These products were analogous to those produced by the peroxidase-mediated oxidation of p-cresol previously reported by Westerfield and Lowe (1942). Further oxidation of tyrosine or tyramine resulted in the formation of terphenol derivatives and, finally, a brown amorphous pigment (Gross and Sizer, 1959). The reaction was suggested to proceed via a free radical mechanism involving the

abstraction of a hydrogen atom from the phenolic hydroxyl followed by a shift in the position of the unpaired electron and subsequent dimerization to form the biphenol (Gross and Sizer, 1959; Andersen, 1966).

In 1963 Andersen reported the presence of two brilliantly blue-white fluorescent compounds in the insect protein, resilin. This protein, found in the wing hinges of various insects, has remarkable rubber-like properties. Subsequent work identified these fluorescent compounds as dityrosine and trityrosine and suggested that they functioned as inter-chain crosslinks in the protein (Andersen and Weis-Fogh, 1964; Andersen, 1964, 1966). Traces of tetratyrosine were also reported to be present in resilin. Previous to the present investigation, this protein was the only known natural source of these unusual fluorescent amino acids.

III. IDENTIFICATION OF DITYROSINE IN ELASTIN AND AN ALKALI-SOLUBLE CONNECTIVE TISSUE PROTEIN

INTRODUCTION

Previously unrecognized amino acids have in recent years been isolated from hydrolyzates of bovine elastin. Their chemical constitutions are compatible with conclusions that two of them, desmosine and isodesmosine (Figs. 1a, 1b), result from the condensation of side chains of four lysine residues (Thomas et al., 1963), and the other, lysinonorleucine (Fig. 1c), from two lysine residues (Franzblau et al., 1965b). Studies with radioisotopic lysine have shown the gradual conversion of this amino acid into the three apparent crosslinks (Miller et al., 1964; Partridge et al., 1964), and indicate that crosslinking between peptide chains occurs in elastin for some time following the elaboration of elastic tissue.

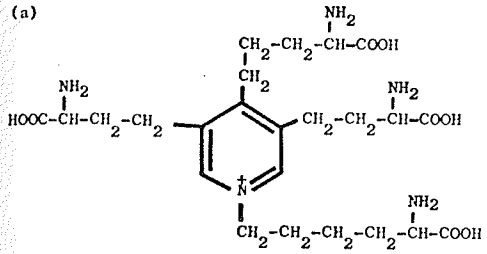
Dityrosine (Fig. 2), a strongly fluorescent compound formed by two molecules of tyrosine in o,o'-biphenol linkage, was first found in nature by Andersen (1964, 1966) in acid hydrolyzates of resilin, a rubber-like protein from the wing hinge ligaments of locusts. Andersen proposed that dityrosine is the intermolecular covalent crosslink in resilin responsible for the elastic properties of the protein.

In this section we report the presence of an unknown fluorescent substance in acid hydrolyzates of elastin, and in an alkali-soluble, hydroxyproline-free protein removed from chick aorta and bovine ligamentum nuchae during the purification procedure for elastin. The substance has fluorescence and chromatographic properties similar to dityrosine, and is derived from radioisotopic tyrosine in tissue culture.

Figure 1:

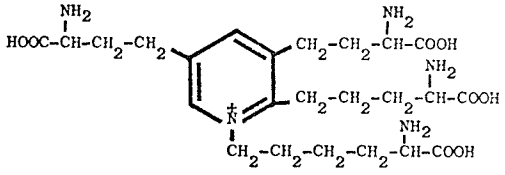
Structure of desmosine (a), isodesmosine (b) and lysinonorleucine (c).

(a)



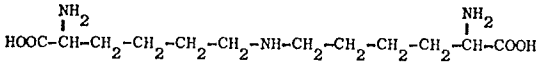
DESMOSINE

(b)



ISODESMOSINE

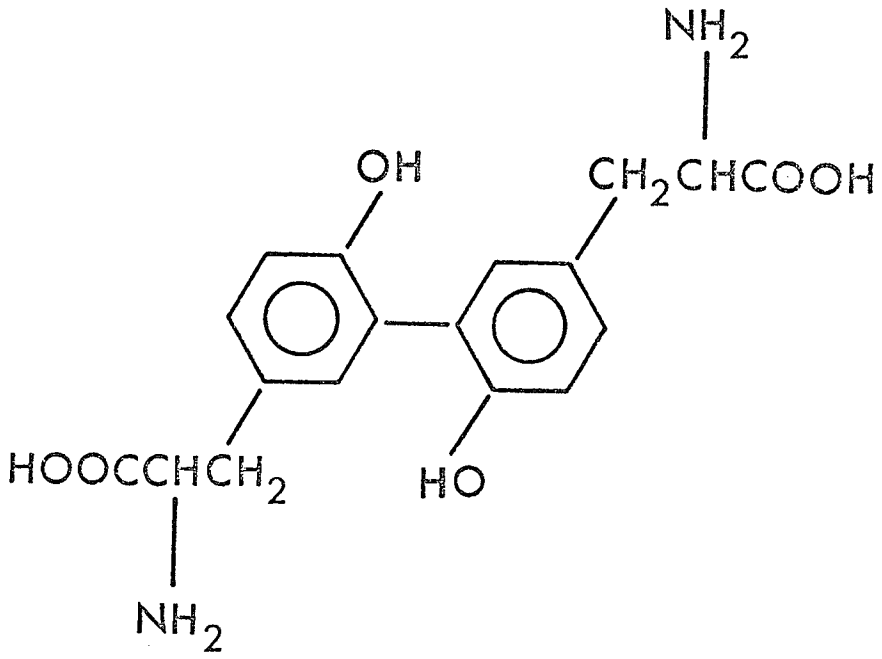
(c)



LYSINONORLEUCINE

Figure 2:

Structure of dityrosine.



Dityrosine

METHODS

Tissue Culture of Embryo Chick Aortas

Segments of thoracic aorta from 12 day chick embryos were fixed with blood clots to washed Millipore filters in test tubes containing 2 ml of Eagle's tissue culture medium (Eagle, 1959), ph 7.4, with 50 units each of penicillin-streptomycin/ml. The tubes were incubated at 37° for two days, the medium replaced with 2 ml of medium containing 1.4 μc of tyrosine-U-^{14}C (300 mc/mmole, New England Nuclear Corp.), and incubated for three more days. The tubes were then cultured in the absence of ^{14}C-tyrosine for 6 days, with a medium change after 3 days. The aortas were carefully removed from the Millipore filters, washed well with distilled water, and the alkali-soluble protein and elastin prepared by the procedures described below.

Preparation of the Alkali-Soluble Protein and Elastin

Aortas from day-old chicks, cultured chick embryo aortas or ligamentum nuchae from adult cattle were cleaned of adventitia, defatted by stirring in chloroform:methanol (3:1) for 2 hours at 20°, twice extracted for 24 hours with 0.2M NaCl at 4°, and autoclaved in water at 110° for 24 hours. The alkali-soluble protein (ALSP) was removed by three 10 minute treatments with 0.1M NaOH at 98°, and the extracts were pooled and exhaustively dialyzed at 4° against several changes of distilled water. During dialysis some of the protein precipitated in the dialysis bag, but could be redissolved at alkaline pH. The residue remaining after the above extractions was considered to be purified elastin.

Amino Acid Analysis

Proteins were hydrolyzed in 6N HCl (fluorometric grade, Harleco Division, American Hospital Supply Corporation) for 24 hours at 110° in sealed ampoules under nitrogen. Amino acid analyses were carried out on a Technicon instrument using either the 75 cm column, 5 1/2 hour gradient system (C-2 Chromobead resin) (A), or a similar system using double the normal concentration of NaCl in the final buffer (B). No corrections were made for destruction of amino acids during acid hydrolysis; methionine is reported as the sum of methionine and methionine sulfone, and cysteine was not determined in these acid hydrolyzates. The effluent stream from the column was split so that one portion of the eluant was reacted with ninhydrin and the other mixed with 2M NaOH and monitored for fluorescence at 315-405 nm using a 5 x 5 mm quartz flow cell. In the case of the radioisotopic experiments, this stream was also continuously monitored for radioactivity by means of a 1 ml Packard flow cell using an anthracene scintillator in a Packard Tricarb scintillation counter. The digital output from the scintillation counter was mathematically processed using a running average technique and plotted on the same time scale as the amino acid chromatogram.

The area of the fluorescence peak corresponding to the elution volume of authentic dityrosine was measured. Assuming a ninhydrin color equivalent for authentic dityrosine of 2.0 (relative to norleucine), each cm^2 of fluorescence was estimated to represent 1.4×10^{-4} μmoles of dityrosine.

Synthesis of Authentic Dityrosine

Authentic dityrosine was prepared by incubating a mixture of

L-tyrosine, hydrogen peroxide and horseradish peroxidase as described by Gross and Sizer (1959). The reaction mixture was fractionated on Dowex-50 with pyridine acetate buffer, tyrosine being eluted at pH 4.5 and dityrosine at pH 7.0. The purified fluorescent compound migrated as dityrosine on the paper chromatographic system of Gross and Sizer (1959).

Paper Chromatography

Protein hydrolyzates were examined by descending paper chromatography, and chromatograms were scanned for radioactivity using a Nuclear-Chicago Actigraph III instrument.

Fluorescence Measurements

Fluorescence was measured on an Aminco-Bowman spectrophotofluorometer.

RESULTS

Elastin

Only two major radioactive regions were apparent in ion exchange chromatograms of acid hydrolyzates of elastin from cultured embryo chick aortas (Fig. 3), one corresponding to tyrosine, and a smaller peak having an elution volume approximately the same as that of authentic dityrosine. Similarly, chromatography of a hydrolyzate of elastin from day-old chick aorta showed a fluorescent peak in the eluant which corresponded to the elution position of authentic dityrosine (Fig. 4). When the column temperature of the Technicon system was lowered to 40^{o} from the usual operating temperature of 60^{o}, the already strong adsorption of aromatic substances to the resin was markedly enhanced, and both authentic dityrosine and the fluorescent peak in the eluant of the elastin hydrolyzate were eluted in a larger volume, and the peak broadened considerably (Fig. 5).

Figure 3:

Ion exchange chromatography on the Technicon system (B) at 60^{o}.

(a) basic portion of a chromatogram of an elastin hydrolyzate showing the amino acid elution pattern (upper tracing) and the corresponding radioactivity of the eluant (lower tracing).

(b) elution pattern of authentic dityrosine.

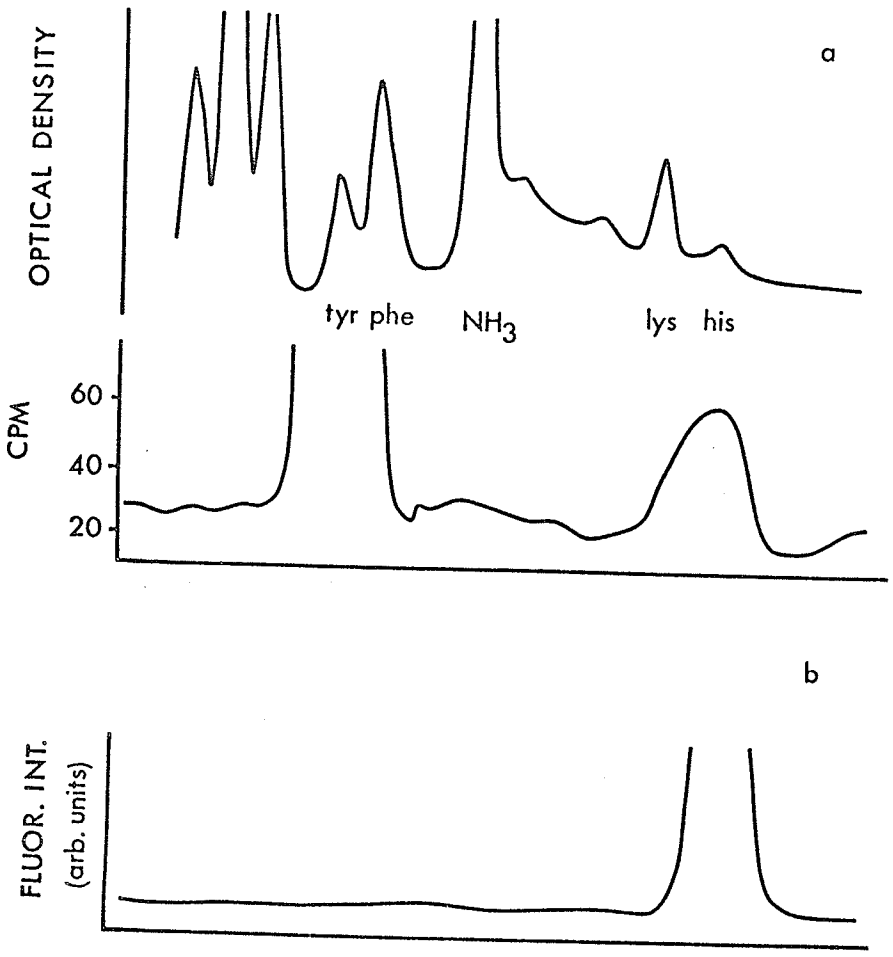

OPTICAL DENSITY
a
tyr phe
NH_3
lys his
CPM
60
40
20
b
FLUOR. INT.
(arb. units)

Figure 4:

Ion exchange chromatography on the Technicon system (A) at 60^{o}.

(a) basic portion of a chromatogram of an elastin hydrolyzate showing the amino acid elution pattern (upper tracing) and the corresponding fluorescence of the eluant (lower tracing).

(b) elution pattern of authentic dityrosine.

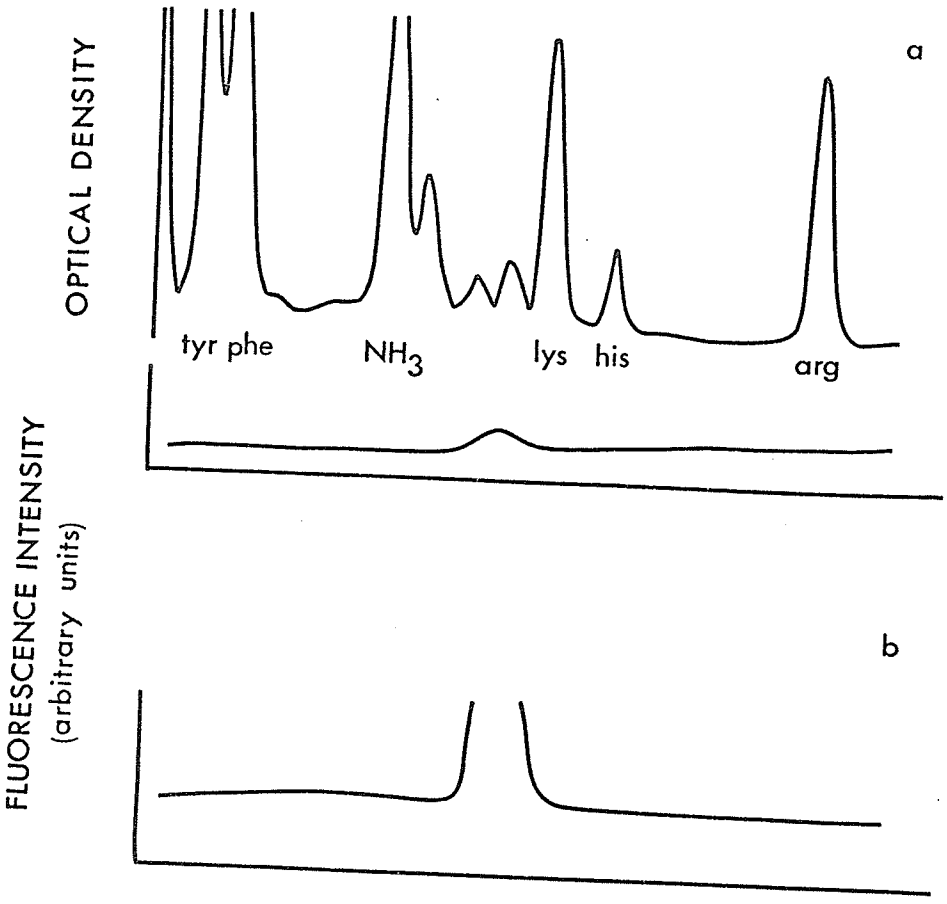
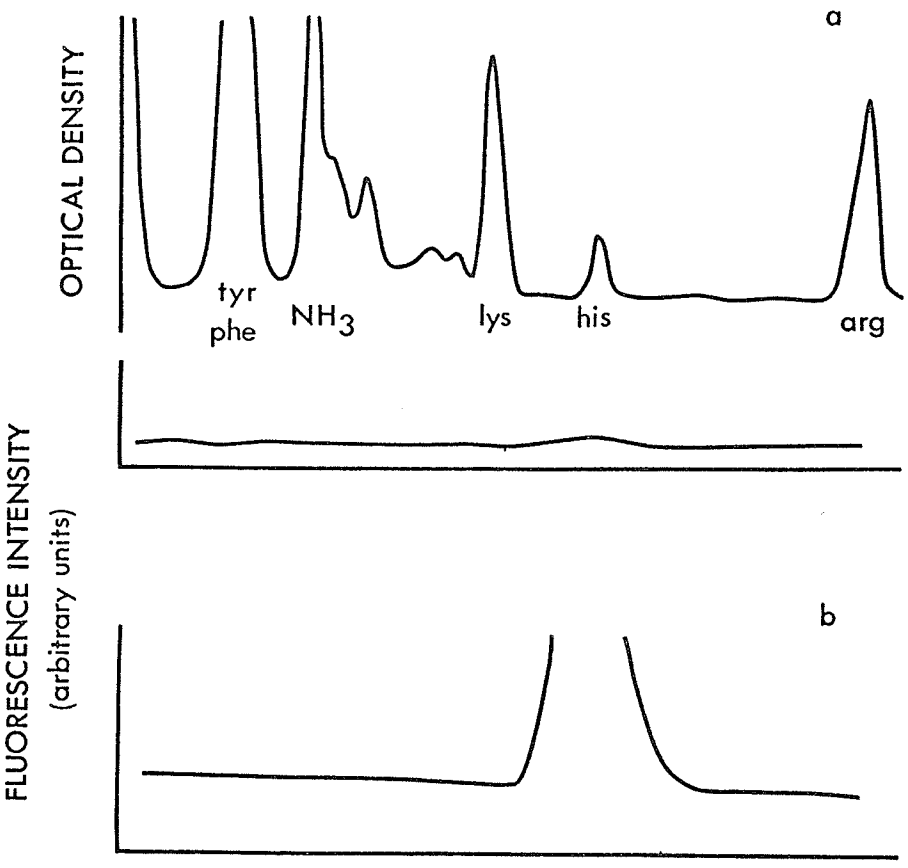

OPTICAL DENSITY
a
tyr phe
NH3
lys
his
arg
FLUORESCENCE INTENSITY
(arbitrary units)
b

Figure 5:

Ion exchange chromatography on the Technicon system (A) at 40^{o}.

(a) basic portion of a chromatogram of an elastin hydrolyzate showing the amino acid elution pattern (upper tracing) and the corresponding fluorescence of the eluant (lower tracing)

(b) elution pattern of authentic dityrosine.


a
OPTICAL DENSITY
tyr
phe
NH3
lys
his
arg
FLUORESCENCE INTENSITY
(arbitrary units)
b

Descending paper chromatography of a radioactive elastin hydrolyzate in a low polarity solvent system (Fig. 6) showed the presence of a small radioactive peak distinct from tyrosine. When fractions eluted from this chromatogram were rechromatographed in a solvent system of higher polarity, two fractions other than tyrosine were resolved, one of which was coincident with the elution position of authentic dityrosine (Fig. 7).

ALSP

Twenty-three percent, by weight, of defatted, collagen-free day-old chick aortas was solubilized by the hot alkali extraction, compared to 3% of similarly treated adult bovine ligamentum nuchae. The amino acid compositions of ALSP from aorta and ligament were very similar (Table I), suggesting a common protein in these tissues.

Ion exchange chromatography of acid hydrolyzates of ALSP from day-old chick aortas showed a fluorescent peak at the elution volume of authentic dityrosine, which, in the case of ALSP from cultured aortas, was accompanied by a radioactive peak at column temperatures of both 60^{o} (Fig. 8) and 40^{o} (Fig. 9).

Descending paper chromatography of hydrolyzates of radioisotopically labelled ALSP revealed a radioactive component which was eluted identically to authentic dityrosine in several solvent systems (Fig. 10).

Using fluorescence data for authentic dityrosine, estimates were made of the dityrosine contents of ALSP and elastin from chick aorta and bovine ligamentum nuchae (Table II).

Acid hydrolyzates of several pure proteins, including papain,

Figure 6:

Radioactive scan of a paper chromatogram of a hydrolyzate of ^{14}C-tyrosine-labelled elastin. The solvent was n-butanol: acetic acid:water (63:10:27). Standard tyrosine (arrow) was chromatographed on the same paper.

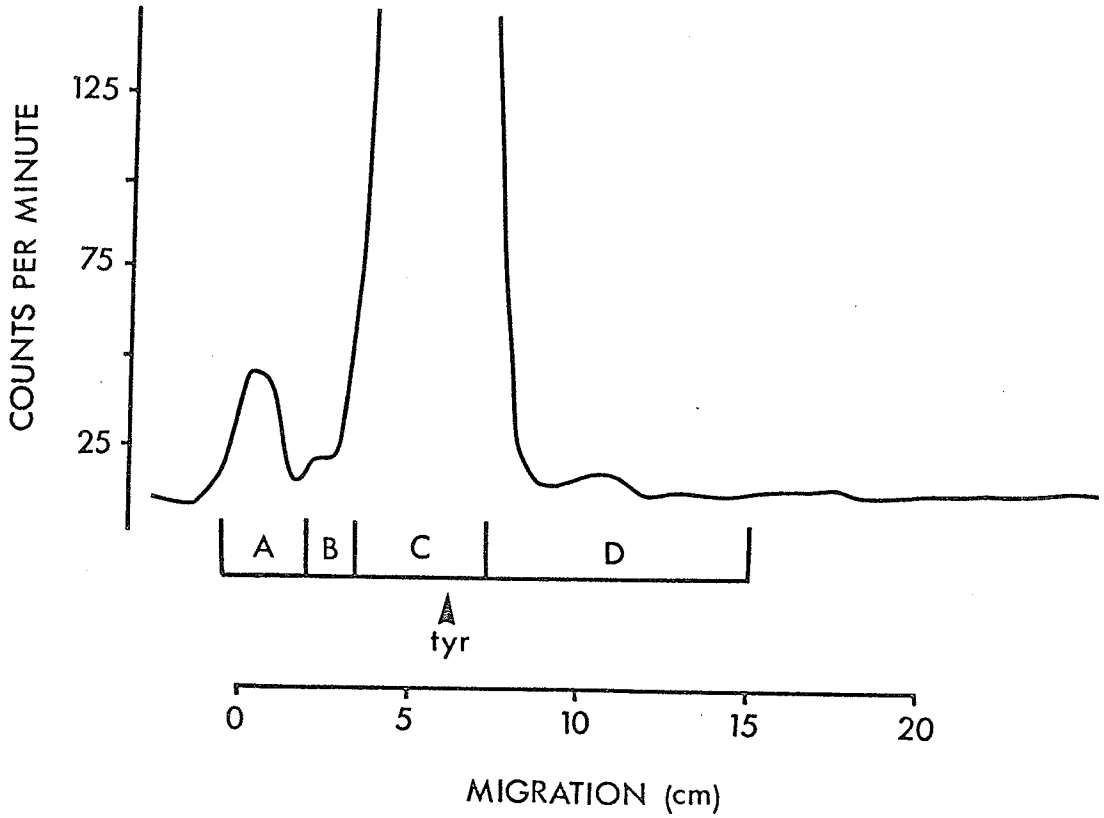

COUNTS PER MINUTE
125
75
25
A
B
C
D
tyr
0
5
10
15
20
MIGRATION (cm)

Figure 7:

Radioactive scans of paper chromatograms of fractions from Figure 6:

(a) fraction A

(b) fraction B

(c) fraction C

(d) fraction D

The solvent was n-butanol:acetic acid:water (35:30:35). Standard tyrosine and dityrosine (arrows) were chromatographed on the same paper.

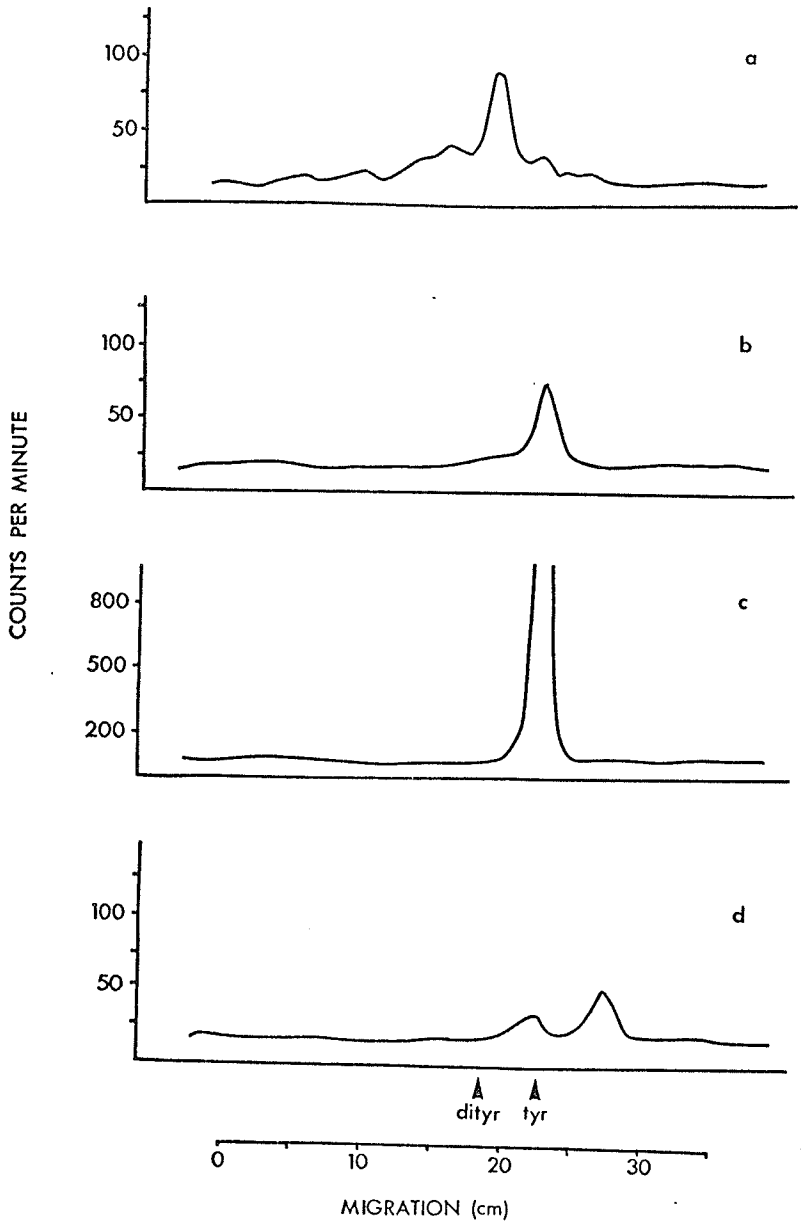

COUNTS PER MINUTE
100
50
a
100
50
b
800
500
200
c
100
50
d
dityr
tyr
0
10
20
30
MIGRATION (cm)

TABLE I

AMINO ACID COMPOSITIONS
OF ALKALI-SOLUBLE PROTEINS

	Residues/1000 total residues	
	BOVINE LIGAMENTUM NUCHAE	DAY-OLD CHICK AORTA
HPRO	0	0
ASP	102	103
THR	35	37
SER	51	60
GLU	139	125
PRO	87	61
GLY	103	115
ALA	75	85
VAL	73	69
MET*	13	12
ILEU	38	43
LEU	81	93
TYR	31	29
PHE	42	39
LYS	62	54
HIS	17	16
ARG	37	40

* sum of methionine and methionine sulfone

Figure 8:

Ion exchange chromatography on the Technicon system (A) at 60^{o}.

(a) basic portion of a chromatogram of an ALSP hydrolyzate showing the amino acid elution pattern (upper tracing), the corresponding fluorescence of the eluant (middle tracing) and the corresponding radioactivity of the eluant (lower tracing).

(b) elution pattern of authentic dityrosine.

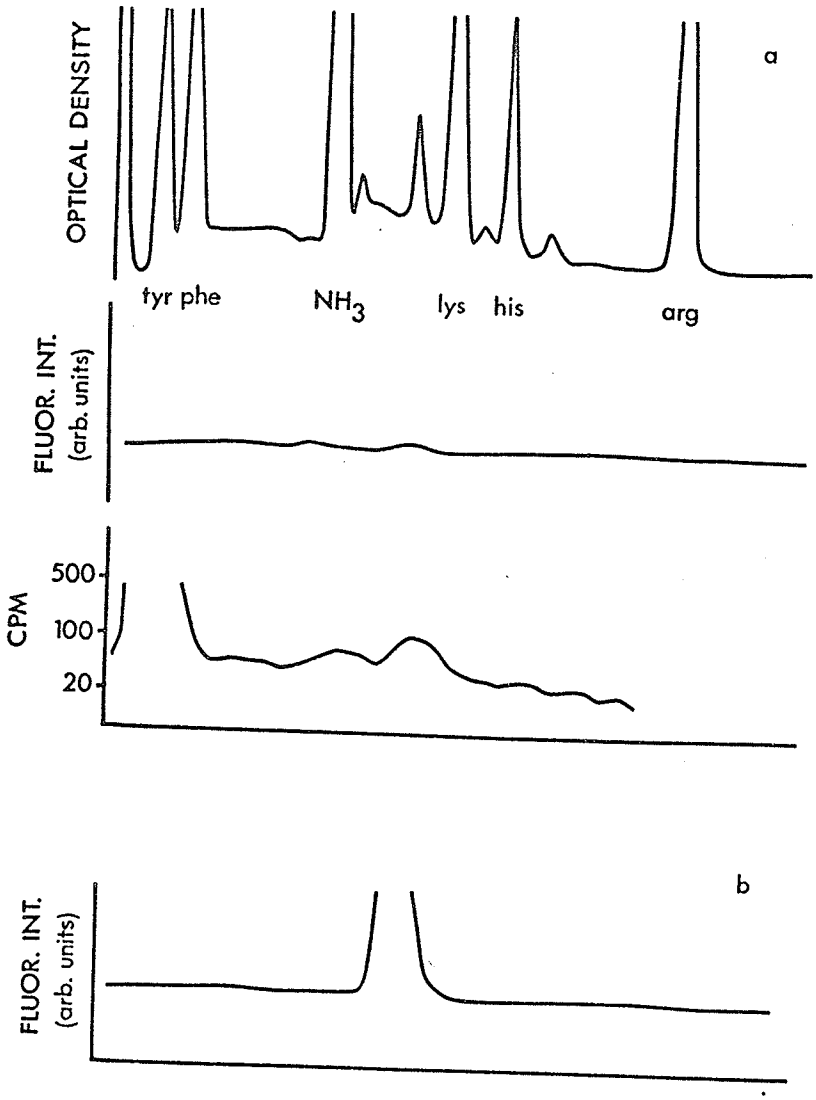

OPTICAL DENSITY
a
tyr phe
NH3
lys
his
arg
FLUOR. INT.
(arb. units)
CPM
500
100
20
b
FLUOR. INT.
(arb. units)

Figure 9:

Ion exchange chromatography on the Technicon system (A) at 40^{o}.

(a) basic portion of a chromatogram of an elastin hydrolyzate showing the amino acid elution pattern (upper tracing), the corresponding fluorescence of the eluant (middle tracing) and the corresponding radioactivity of the eluant (lower tracing).

(b) elution pattern of authentic dityrosine.

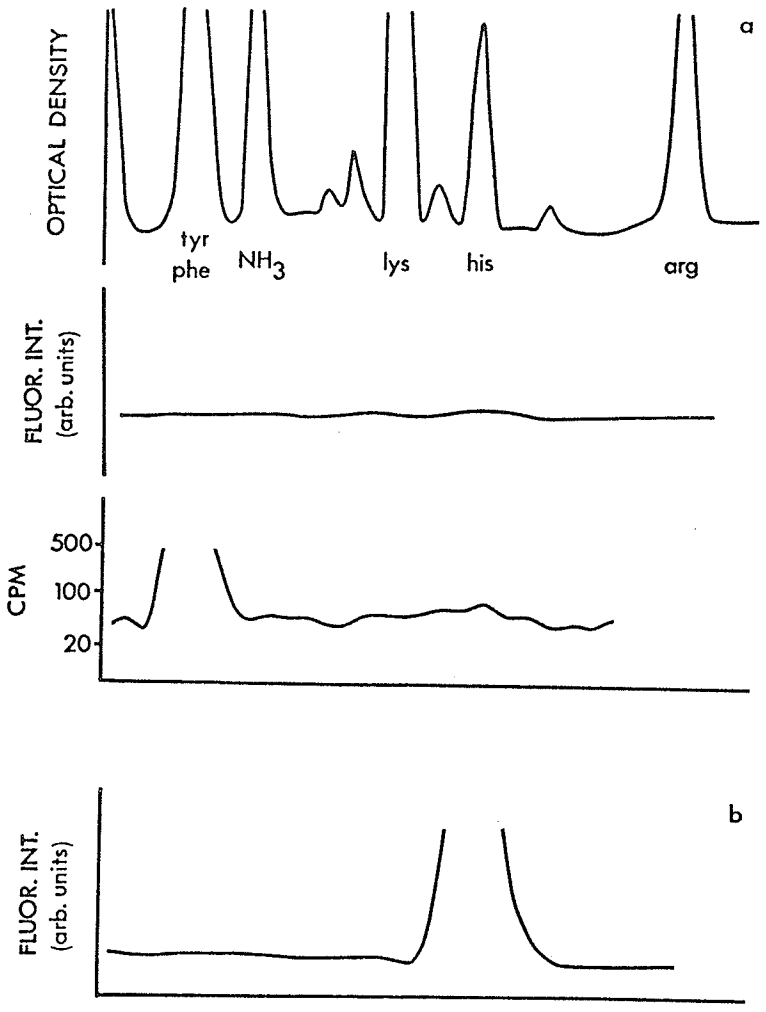

a
OPTICAL DENSITY
tyr
phe
NH3
lys
his
arg
FLUOR. INT.
(arb. units)
CPM
500
100
20
b
FLUOR. INT.
(arb. units)

Figure 10:

Radioactive scans of paper chromatograms of ^{14}C-tyrosine-labelled ALSP in four solvent systems.

(a) n-butanol:acetic acid:water (35:30:35)

(b) n-butanol:formic acid (88%):water (75:10:15)

(c) iso-propanol:ammonia:water (8:1:1)

(d) solvent (c) followed by solvent (a) (in the same direction)

Standard tyrosine and dityrosine were chromatographed on the same papers.

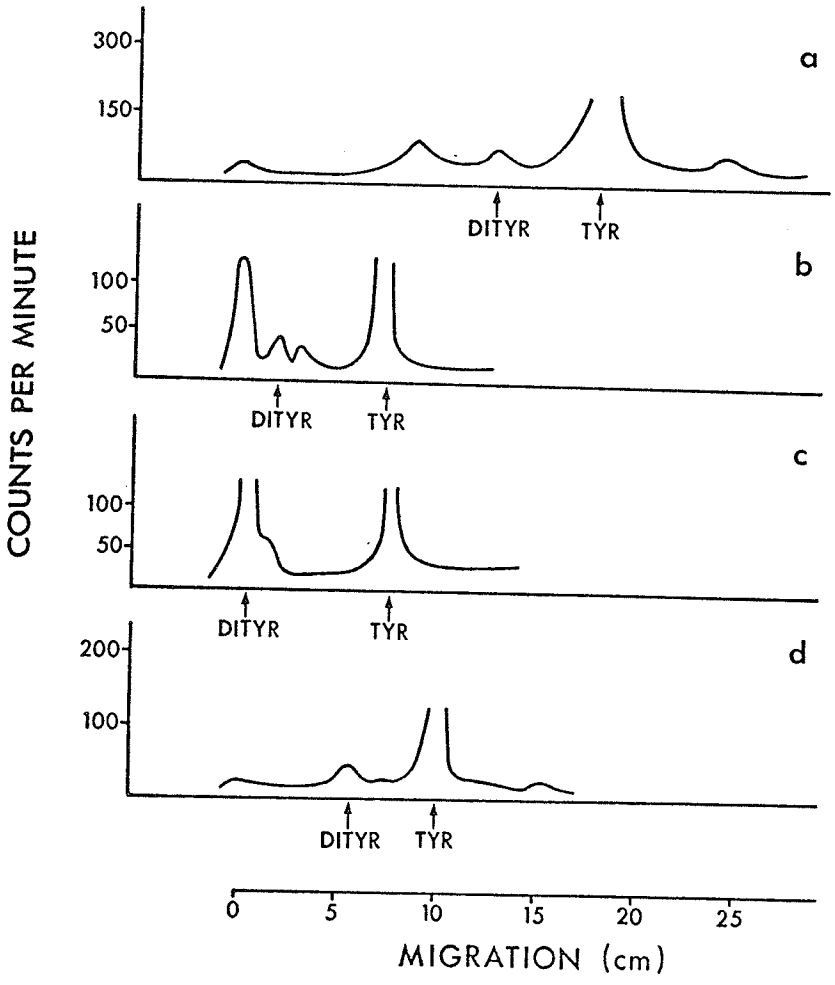

COUNTS PER MINUTE
300
150
a
DITYR
TYR
100
50
b
DITYR
TYR
100
50
c
DITYR
TYR
200
100
d
DITYR
TYR
0
5
10
15
20
25
MIGRATION (cm)

TABLE II

DITYROSINE CONTENT OF ALSP AND
ELASTIN FROM LIGAMENTUM AND AORTA

	Residues/10^5 total residues	
	BOVINE LIGAMENTUM NUCHAE	DAY-OLD CHICK AORTA
ALSP	4	10
ELASTIN	<0.5	3

poly-L-tyrosine and bovine serum albumin, as well as embryo chick blood and structural proteins such as albuminoid, elastoidin and abductin did not contain detectable amounts of dityrosine, nor was dityrosine produced by 'acid hydrolysis' of free tyrosine. Moreover, dityrosine was absent from acid hydrolyzates of ribonuclease, chymotrypsinogen A and bovine serum albumin which had previously been treated with hot alkali, under conditions similar to those employed to extract ALSP.

DISCUSSION

The unknown fluorescent substance found in elastin and ALSP behaved similarly to authentic dityrosine on paper and ion exchange chromatographic systems, and was shown to be derived from ^{14}C-tyrosine. It is doubtful that any other substance would migrate identically in the several different systems employed. Absolute proof of the structure of the unknown fluorescent substance can only be achieved, however, by its isolation in pure form (see Section IV).

Andersen (1964, 1966) has suggested that dityrosine functions as an interchain crosslink in resilin, and it is tempting to speculate that it may play a similar role in ALSP and elastin. In some samples of cultured chick aortas we have detected traces of trityrosine, a terphenolic species also reported by Andersen in resilin (Andersen, 1964, 1966), although these observations have not been pursued further. It is possible that dityrosine in these proteins is of a transient nature, and may be an intermediate stage in the formation of a polyphenolic type of crosslink. In this respect, we have noted that up to 10% of the total radioactivity in acid hydrolyzates of ALSP and elastin from embryo chick aortas labelled in culture with ^{14}C-tyrosine could be eluted from the ion exchange resin

of the Technicon system only by 0.2M NaOH. This firmly bound component was not present in hydrolyzates of embryo chick blood proteins labelled *in ovum* with ^{14}C-tyrosine, nor in hydrolyzates of ALSP and elastin labelled in culture with ^{14}C-phenylalanine or ^{3}H-histidine. Although we have not observed this firmly bound component in ALSP and elastin labelled with ^{3}H-lysine, Sandberg and Cleary (1968) recover 5-12% of the total radioactivity of hydrolyzates of elastin labelled with ^{14}C-lysine in this alkali wash. Similarly, Lent et al. (1969) report that 20% of the total radioactivity of a hydrolyzate of elastin reduced with sodium borotritide is firmly bound to the Technicon column resin. This firmly bound fraction is especially prominent in hydrolyzates of day-old chick aortas incubated for long periods in the presence of ^{14}C-tyrosine and horseradish peroxidase. Gross and Sizer (1959) reported that prolonged treatment of tyrosine or tyramine with peroxidase results in the formation of brown, polymeric pigments. Andersen (1964, 1966) reported the presence in resilin of the ter- and tetraphenolic analogs of dityrosine, neither of which could be eluted from Dowex-50 resin at room temperature, even with 0.2M NaOH (Andersen, 1963). These observations suggest the presence of a polyphenolic or quininoid component in ALSP and elastin, of which dityrosine may be a precursor.

The alkali-soluble protein which we have extracted from chick aorta and bovine ligamentum nuchae appears to be similar in amino acid composition to glycoprotein preparations extracted by a variety of methods from calf skin (Bowes et al., 1957), bovine cartilage (Partridge and Elsden, 1961), bovine ligamentum nuchae (Gotte et al., 1963a), rabbit skin (Timpl et al., 1968) and human aorta (Barnes and Partridge, 1968).

The values for the amounts of material extracted by hot alkali from aorta and ligamentum nuchae are in agreement with the reported 20% loss of weight of collagen-free human aortas (LaBella and Lindsay, 1963) and 2% loss of weight of adult bovine ligamentum nuchae (Gotte et al., 1963b; Cleary et al., 1967) on hot alkali treatment. Gotte and Serafini-Fracassini (1963) concluded from electron microscopical observations that the glycoprotein in bovine ligamentum nuchae served as an amorphous cementing material between thin elastin fibrils which comprised large elastic bundles, and they suggested that the protein was a common constituent of the ground substance of various connective tissues. The role of dityrosine in this ground substance protein will be discussed later (see Section V).

IV. ISOLATION OF DITYROSINE FROM AN ALKALI-SOLUBLE CONNECTIVE TISSUE PROTEIN

INTRODUCTION

Dityrosine, the o,o'-biphenol analog of tyrosine, was first synthesized by Gross and Sizer (1959) by the reaction of horseradish peroxidase and hydrogen peroxide with tyrosine. Later, Lehrer and Fasman (1967) reported that dityrosine could be produced by ultraviolet irradiation of poly-L-tyrosine. LaBella et al. (1968) reported that treatment of soluble collagen with peroxidase and hydrogen peroxide resulted in the appearance of dityrosine in the protein, accompanied by the rapid formation of rigid gels. We have presented evidence for the natural occurrence of a substance in elastin and in an alkali-soluble, hydroxyproline-free protein intimately associated with elastin (Section III) with fluorescence and chromatographic properties identical to authentic dityrosine. This fluorescent substance was derived from radioactive tyrosine in cultures of chick embryo aortas. This section describes the isolation of this fluorescent substance from the alkali-soluble protein of bovine ligamentum nuchae, and chemical confirmation of its identity as dityrosine.

METHODS

Extraction and Hydrolysis of the Alkali-Soluble Protein

An acetone powder of adult bovine ligamentum nuchae was extracted with chloroform:methanol (3:1) for 2 hours at room temperature, followed by four 12 hour extractions at 4^{o} with a large volume of 0.2 M NaCl. Collagen was removed from the residue by two 24 hour extractions with distilled water at 110^{o}. The collagen-free residue was extracted for three 10 minute periods with 0.1 M NaOH at 98^{o}, and the extracts were filtered, pooled and dialyzed for 72 hours at 4^{o} against several changes of distilled water. After dialysis, the contents of the bag, including

a precipitate which had formed during dialysis, were lyophilized. This lyophilized material was the alkali-soluble protein (ALSP) used in subsequent procedures.

Approximately 2 grams of ALSP was refluxed for 48 hours in 250 ml of 6N HCl (fluorometric grade, Harleco Division, American Hospital Supply Corporation). The apparatus was initially flushed with nitrogen and thereafter protected from atmospheric oxygen by a pyrogallol trap. A few crystals of stannous chloride were added to the solution to further retard oxidation. The hydrolyzate was concentrated at 50° under vacuum, dried in a vacuum dessicator over solid sodium hydroxide, dissolved in distilled water and frozen.

Isolation of a Fluorescent Compound from ALSP

A 1.5 x 40 cm column of cellulose-phosphate (Bio-Rad Laboratories) was washed extensively with 0.2 M acetic acid. Several aliquots of the ALSP hydrolyzate, each equivalent to approximately 80 mg of the original protein, were evaporated to dryness, redissolved in a small volume of 0.2 M acetic acid (final pH 2.5), applied to the column and eluted with a continuous gradient of 0-0.8M NaCl in 0.2 M acetic acid. Fractions were collected and monitored for conductivity and fluorescence. Between separations, the column was washed with 1 M NH_4OH and re-equilibrated with 0.2 M acetic acid. Fractions corresponding to the elution volume of authentic dityrosine were pooled, evaporated to dryness at 50° under vacuum, redissolved in a small volume of 0.2 M acetic acid and applied to a 2 x 40 cm column of Bio-Gel P-2 (200-400 mesh, Bio-Rad Laboratories) which had been washed thoroughly with 0.2 M acetic acid. The column was eluted with 0.2 M acetic acid and fractions were collected and monitored for

conductivity and fluorescence. Fractions corresponding to the elution volume of authentic dityrosine were pooled, evaporated to dryness at 50° under vacuum, dissolved in a small volume of distilled water and frozen. This material was the source of the isolated fluorescent compound used in subsequent procedures.

Amino Acid Analysis

Proteins were hydrolyzed in 6N HCl (fluorometric grade, Harleco Division, American Hospital Supply Corporation) for 24 hours at 110° in sealed ampoules under nitrogen. Amino acid analyses were carried out on a Technicon instrument using the 75 cm column, 5 1/2 hour gradient system (C-2 Chromobead resin). One-half of the eluant from the column was reacted with ninhydrin and the other was mixed with 2.0 N NaOH and monitored for fluorescence at 315-405 nm.

Enzymic Synthesis of Authentic Dityrosine

Authentic dityrosine was synthesized by the action of horseradish peroxidase and hydrogen peroxide on tyrosine (Gross and Sizer, 1959), and purified by the methods described above for the isolation of the fluorescent compound from ALSP. The synthetic compound migrated appropriately on the paper chromatographic system of Gross and Sizer (1959).

Optical Measurements

Fluorescence was measured on an Aminco-Bowman spectrophotofluorometer. A 5 x 5 mm quartz flow cell was used for continuous monitoring of fluorescence during amino acid separation. Ultraviolet absorption was measured on a Beckman DK-2 spectrophotometer.

RESULTS

The separation pattern of an acid hydrolyzate of ALSP on

cellulose-phosphate (Fig. 11) shows a fluorescent peak corresponding to the elution volume of authentic dityrosine. This peak was further resolved into two major fluorescent peaks on Bio-Gel P-2 (Fig. 12), one of which was again coincident with the elution volume of authentic dityrosine, and the other eluted at a much larger volume.

Chromatography on the normal Technicon system of the fluorescent compound from the Bio-Gel column corresponding to authentic dityrosine (Fig. 13) showed it to have an elution volume identical to that of authentic dityrosine and to the fluorescent peak, present in hydrolyzates of ALSP, which we had previously tentatively identified as dityrosine (Section III). Because of the strong adsorption of aromatic substances to the resin used in the Technicon system, lowering of the column temperature to 40^{o} from the usual operating temperature of 60^{o} delayed the elution of dityrosine and markedly broadened the peak. When chromatographed at 40^{o} in this system, the isolated fluorescent compound was again eluted in the same volume as both authentic dityrosine and the fluorescent substance in the hydrolyzate of ALSP (Fig. 14).

The component eluted from Bio-Gel P-2 (Fig. 12) at approximately 240 ml was strongly fluorescent at 280-450 nm and could not be eluted from the Technicon column at 60^{o}.

The isolated fluorescent compound migrated as authentic dityrosine on paper chromatography in two solvent systems (Fig. 15).

The pH dependence of fluorescence activation of authentic dityrosine and of the isolated fluorescent compound is shown in Figure 16. These curves are characteristic of o,o'-biphenols, (Andersen, 1966) and identical to those published by Andersen (1966) for dityrosine. Similarly,

Figure 11:

Fractionation of an ALSP hydrolyzate on cellulose-phosphate. The elution patterns of tyrosine (_____ . _____, fluorescence at 280-310 nm), authentic dityrosine (------, fluorescence at 285-405 nm) and the ALSP hydrolyzate (_____, fluorescence at 285-405 nm) are shown. The NaCl gradient is indicated by the changing conductivity of the eluant (.....).

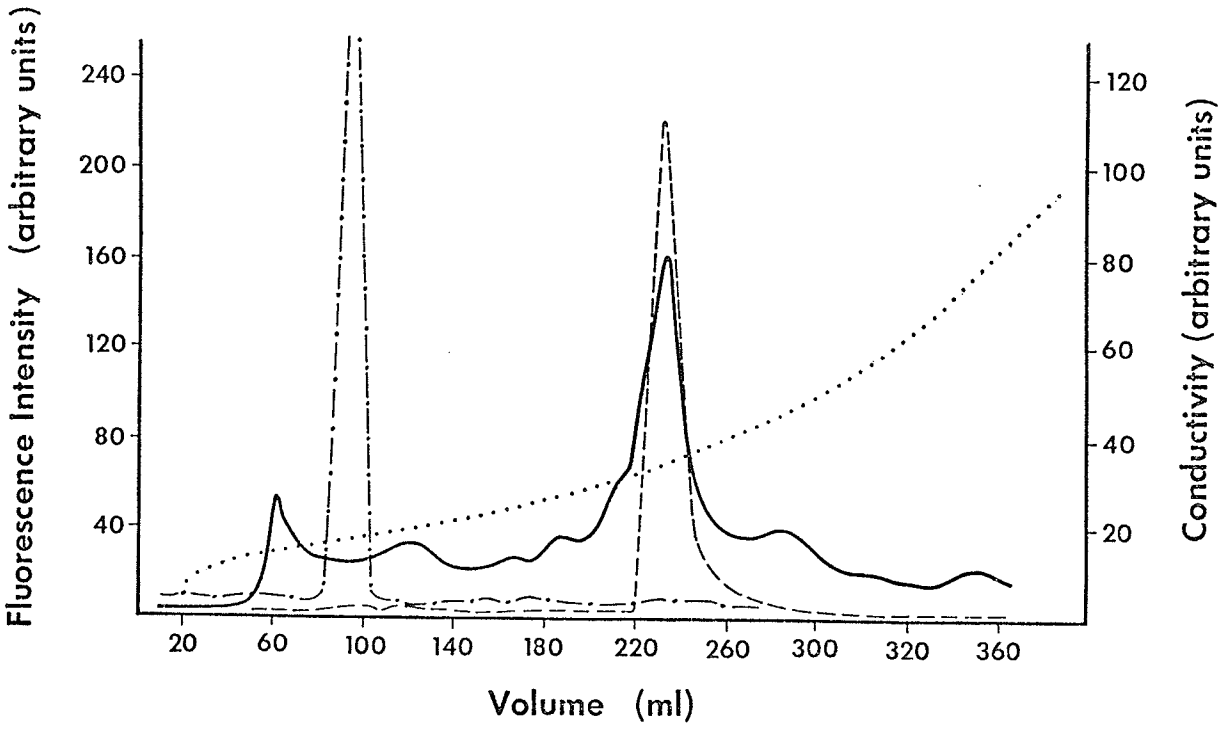

Fluorescence Intensity (arbitrary units)
240
200
160
120
80
40
120
100
80
60
40
20
Conductivity (arbitrary units)
20
60
100
140
180
220
260
300
320
360
Volume (ml)

Figure 12:

Chromatography on Bio-Gel P-2 of the pooled fluorescent fractions from cellulose-phosphate corresponding to authentic dityrosine. The elution patterns of authentic dityrosine (------, fluorescence at 285-405 nm), the pooled fluorescent fractions (_____, fluorescence at 285-405 nm), and NaCl (.....) are shown. The arrow indicates the void volume of the column.

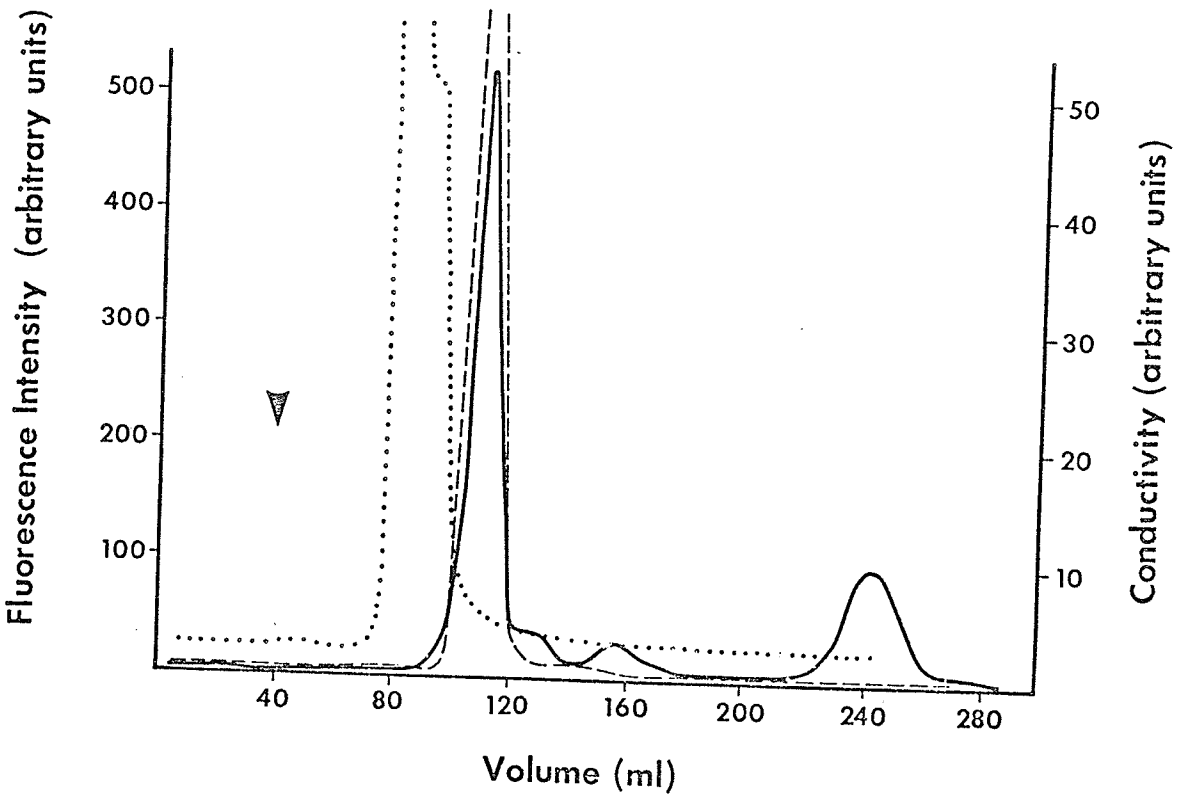

Fluorescence Intensity (arbitrary units)
500
400
300
200
100
40
80
120
160
200
240
280
Volume (ml)
50
40
30
20
10
Conductivity (arbitrary units)

Figure 13:

Ion exchange chromatography on the Technicon system at 60°.

A: basic portion of a chromatogram of an ALSP hydrolyzate showing the amino acid elution pattern (upper tracing) and the corresponding fluorescence of the eluant (lower tracing).

B: elution pattern of the isolated fluorescent substance.

C: elution pattern of authentic dityrosine.

Fluorescence was measured at 315-405 nm.

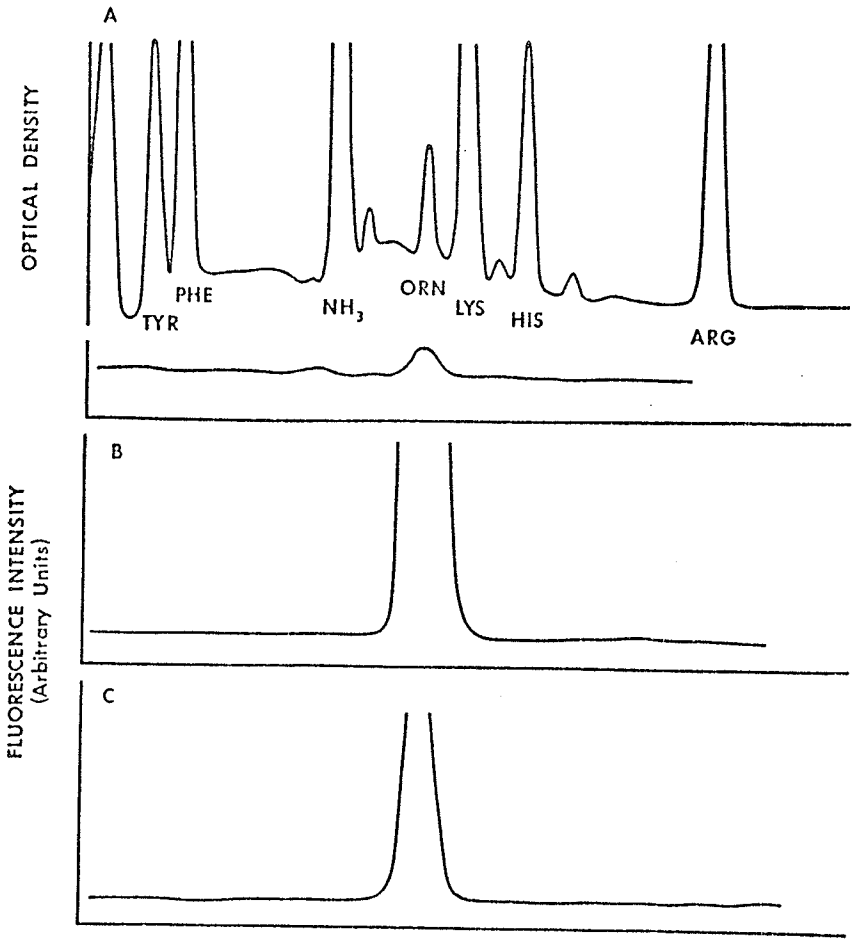

A
OPTICAL DENSITY
TYR
PHE
NH3
ORN
LYS
HIS
ARG
B
C
FLUORESCENCE INTENSITY
(Arbitrary Units)

Figure 14:

Ion exchange chromatography on the Technicon system at 40°.

A: basic portion of a chromatogram of an ALSP hydrolyzate showing the amino acid elution pattern (upper tracing) and the corresponding fluorescence of the eluant (lower tracing).

B: elution pattern of the isolated fluorescent substance.

C: elution pattern of authentic dityrosine.

Fluorescence was measured at 315-405 nm.

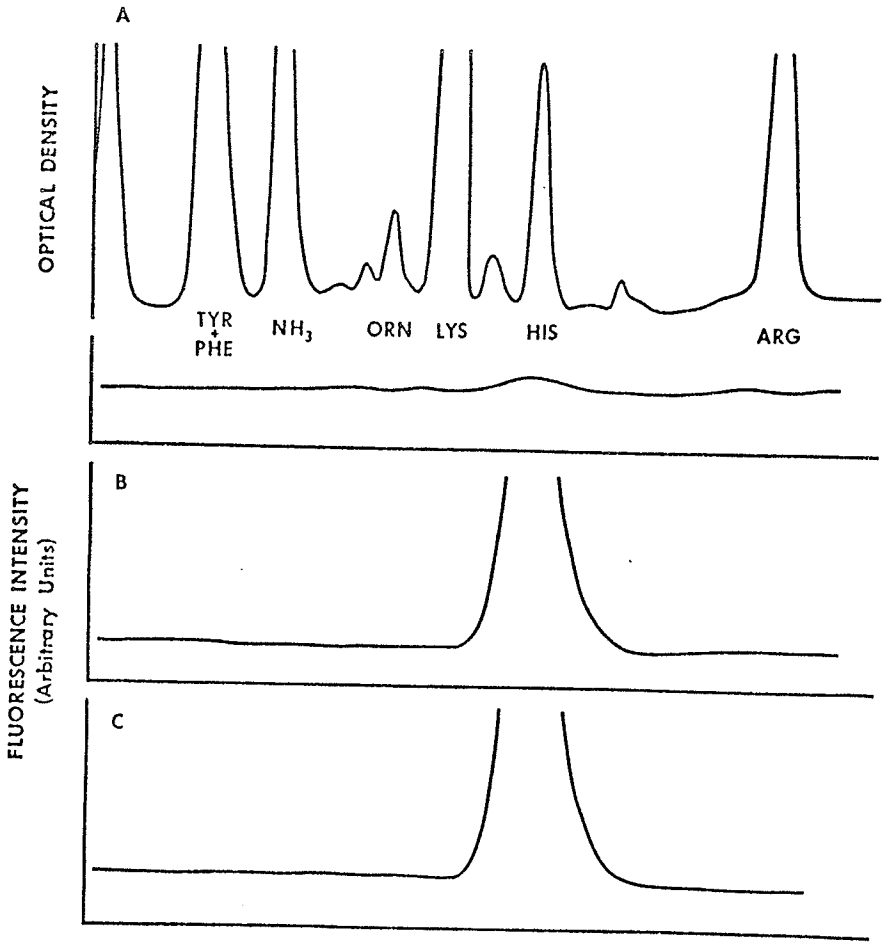

A
OPTICAL DENSITY
TYR
+
PHE
NH3
ORN
LYS
HIS
ARG
B
C
FLUORESCENCE INTENSITY
(Arbitrary Units)

Figure 15:

Descending paper chromatography of authentic dityrosine and the isolated fluorescent substance. An amino acid standard (STD), authentic dityrosine (DT), and the isolated fluorescent substance (I) were chromatographed in

A: n-butanol:acetic acid:water (55:15:30), and

B: n-butanol:acetic acid:water (35:30:35). Solid outlines indicate ninhydrin-positive spots. Dashed outlines indicate spots which are both ninhydrin-positive and fluorescent under ultraviolet irradiation.

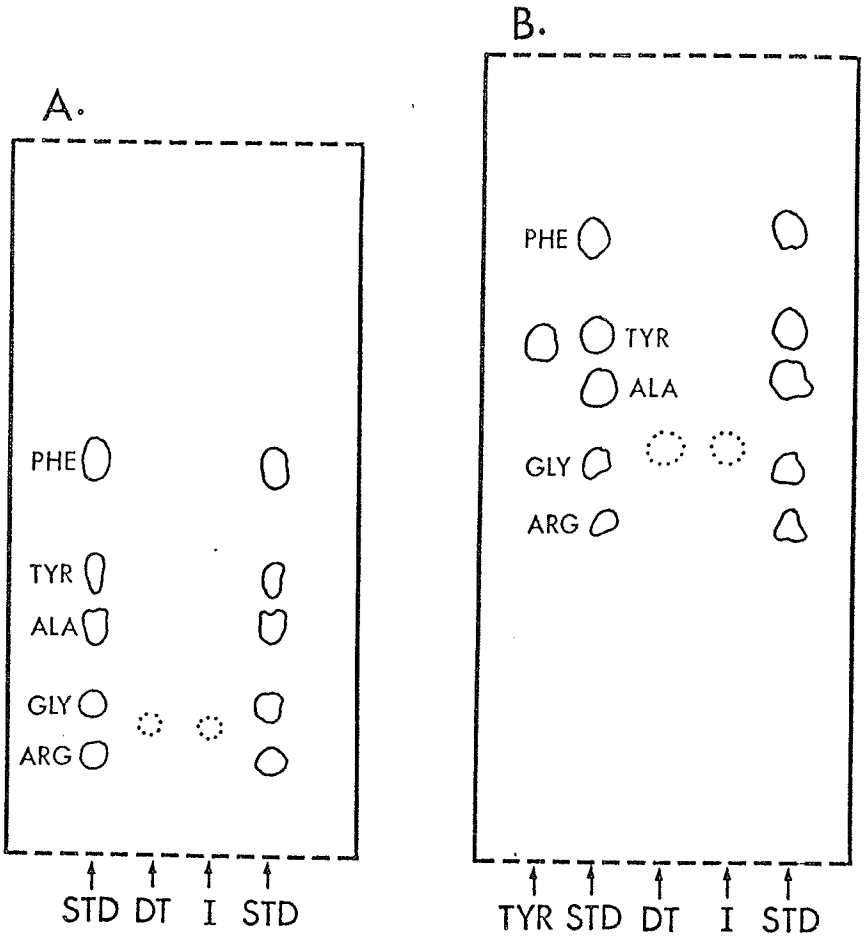
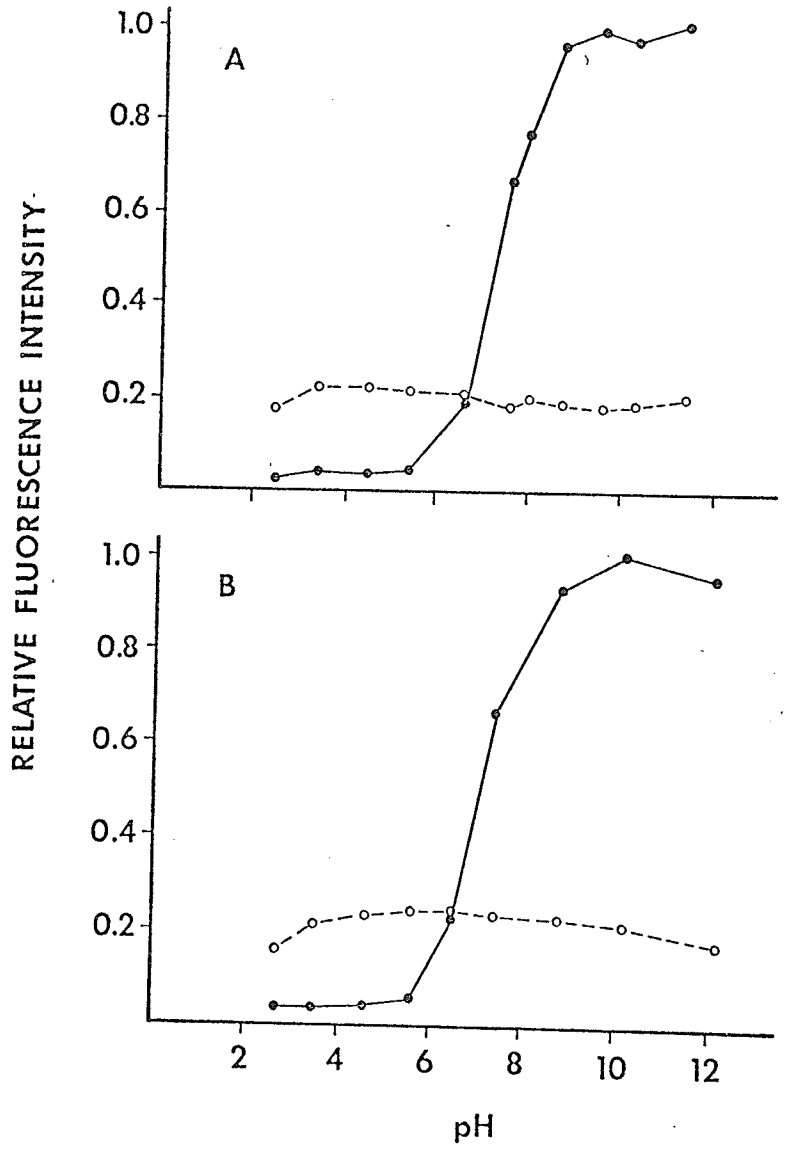

A.
PHE
TYR
ALA
GLY
ARG
STD DT I STD
B.
PHE
TYR
ALA
GLY
ARG
TYR STD DT I STD

Figure 16:

Dependency of fluorescence intensity on pH and activation wavelength. Relative intensity of fluorescence at 405 nm as a function of pH when activated at 285 nm (------) and 315 nm (_____) for authentic dityrosine (A) and the isolated fluorescent substance (B).


RELATIVE FLUORESCENCE INTENSITY
1.0
0.8
0.6
0.4
0.2
A
B
2
4
6
8
10
12
pH

the ultraviolet absorption spectra of authentic dityrosine and the isolated fluorescent compound (Fig. 17) are identical to one another and to those published by Andersen (1966) and Lehrer and Fasman (1967).

DISCUSSION

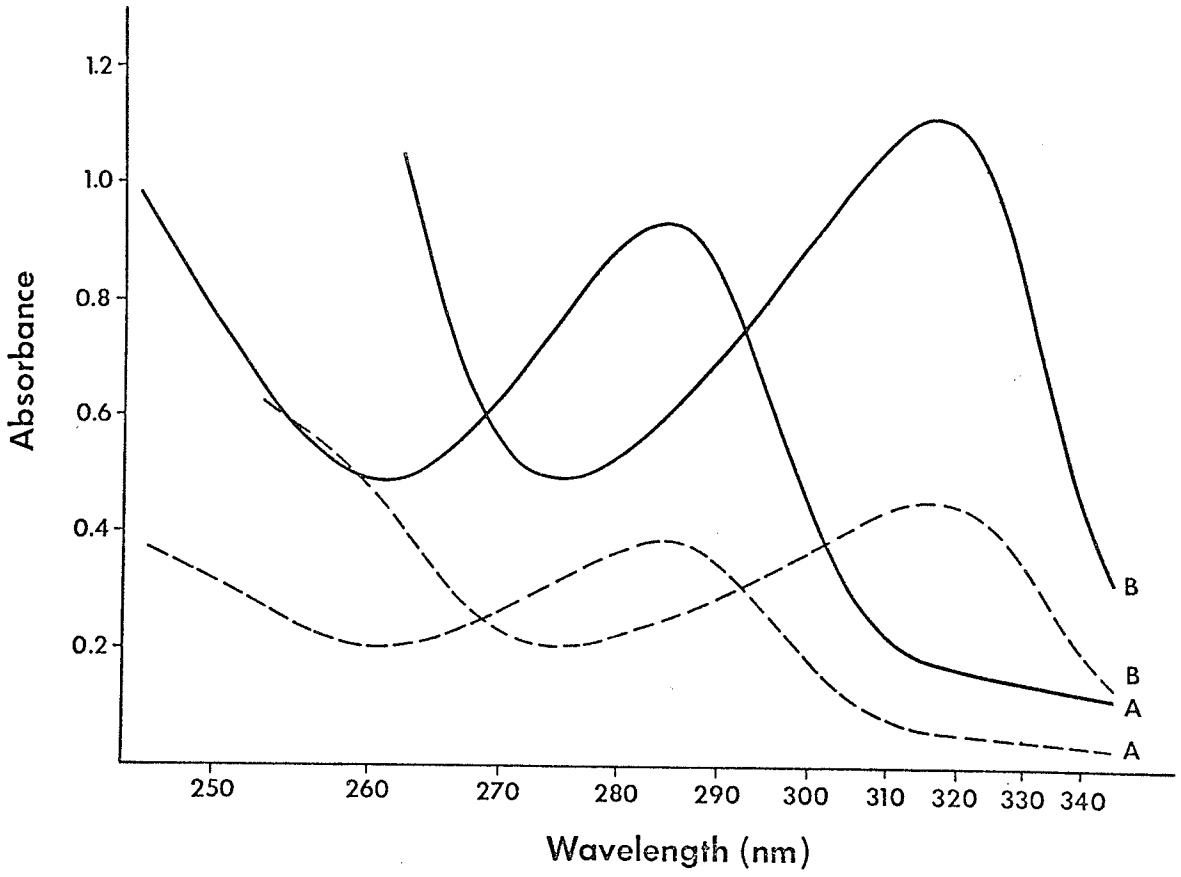
On the basis of the evidence presented, the fluorescent compound isolated from ALSP from bovine ligamentum nuchae is undoubtedly dityrosine, substantiating our previous proposal of the presence of this unusual substance in ALSP (Section III). Although, in the present study, ALSP from bovine ligamentum nuchae was specifically used for the isolation of dityrosine, it is reasonable to conclude that the analogous fluorescent compound previously reported in ALSP and elastin from day-old chick aortas (Section III) is also dityrosine. To our knowledge, these are the only mammalian proteins in which dityrosine has been shown to occur naturally.

The relatively large amount of ornithine present in acid hydrolyzates of ALSP and eluted at the same volume as dityrosine in the Technicon system at 60^{o} is probably due to the breakdown of arginine during the strongly alkaline conditions used to extract ALSP. However, Steven and Jackson (1968), noting the presence of large quantities of ornithine in elastin hydrolyzates, have suggested the possibility of a labile complex in elastin, yielding ornithine on acid hydrolysis.

Although the identity of the fluorescent component eluted late from the Bio-Gel column has not been established, its large elution volume in this system, and failure to be eluted from the Technicon system suggests a highly aromatic character. Whether this as yet unknown component occurs naturally in ALSP or is an artifact of acid hydrolysis, as is suggested by recent work of Ledvina and LaBella (in press), remains to be determined.

Figure 17:

Ultraviolet absorption spectra as a function of pH. Absorption spectra of authentic dityrosine (_____) and the isolated fluorescent substance (------) at pH 2.6 (A) and pH 12.2 (B).


Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
250
260
270
280
290
300
310
320
330
340
Wavelength (nm)
B
B
A
A

V. EVIDENCE FOR A CROSSLINKING ROLE OF DITYROSINE IN AN ALKALI-SOLUBLE CONNECTIVE TISSUE PROTEIN

INTRODUCTION

Protein-polysaccharide complexes have been identified in all types of connective tissue and studied in detail in cartilaginous tissues (Shatton and Schubert, 1954; Anderson, 1961; Partridge et al., 1961; Partridge and Elsden, 1961; Eyring and Yang, 1968; Kao et al., 1968; Serafini-Fracassini, 1968). Hydroxyproline-free glycoproteins in cartilage (Partridge et al., 1967; Kao et al., 1968) form part of a macromolecular aggregate believed to be involved in the maintenance of the overall structural integrity of the ground substance (Jackson, 1965; Mathews, 1967). Similar glycoproteins, present in intervertebral disc (Moschi and Little, 1966; Pearson et al., 1969), exhibit blue-white fluorescence on ultraviolet irradiation and may possess rubber-like properties (Moschi and Little, 1966).

Glycoproteins have been solubilized from aorta of several species by mild procedures such as extraction with water (Berenson and Fishkin, 1962; Radhakrishnamurthy et al., 1964; Berenson et al., 1966). Barnes and Partridge (1968) purified and extensively characterized a glycoprotein removed from human aorta by extraction with cold alkali. Timpl et al. (1968), Robert and Comte (1968), and Robert and Robert (1969) extracted glycoproteins from several connective tissues with urea or cold alkali, and suggested that they represent a previously unrecognized class of structural proteins. Both Barnes and Partridge (1968) and Timpl et al. (1968) reported that the extracted proteins exist in varying states of aggregation. It is striking that all of these glycoproteins, extracted from diverse tissues by varying procedures, have notably similar amino acid compositions.

Gotte et al. (1963a) and Gotte and Serafini-Fracassini (1963), correlating biochemical data with electron microscopical observations, showed that a glycoprotein of this general composition forms an amorphous sheath around elastin fibrils of bovine ligamentum nuchae. Extensive extraction with 2M NaCl partially solubilized the protein, but its complete removal required treatment with hot alkali. Recently, Ross and Bornstein (1969) isolated the microfibrillar protein which is morphologically in close association with elastin fibres, especially in developing tissues (Haust et al., 1965; Fahrenbach et al., 1966; Greenlee, Jr. et al., 1966; Greenlee, Jr. and Ross, 1967), and showed its amino acid composition to be very similar to that of the family of proteins described above.

In a previous section (Section III) we have described the extraction of a hydroxyproline-free, alkali-soluble protein (ALSP) from collagen-free chick aorta and bovine ligamentum nuchae having an amino acid composition similar to that of the glycoproteins isolated by others. Furthermore, we demonstrated that this protein contains dityrosine, (Sections III, IV), the o,o'-biphenol analog of tyrosine previously identified only in the rubber-like insect protein, resilin, where it is proposed to function as an interchain crosslink (Andersen, 1964, 1966). This section deals with the characterization of ALSP and, in particular, the elucidation of the role of dityrosine in this protein.

METHODS

Extraction Procedures

Aortas from day-old chicks were cleaned of blood and adventitia and extracted for 2 hours at room temperature with chloroform:methanol

(3:1), followed by two extractions of 24 hours each with 0.2M NaCl at 4°. The residue was autoclaved in distilled water at 110° for 24 hours. This collagen-free tissue was used as a starting material for subsequent procedures.

Alkali-soluble protein (ALSP) was removed from the collagen-free aortas by three extractions of 10 minutes each with 0.1 M NaOH at 98°. In some cases, as indicated, a fourth 10 minute extraction was made. The extracts were pooled, dialyzed extensively at 4° against distilled water and lyophilized. During dialysis a precipitate formed in the bag which could be redissolved by the addition of a few drops of alkali.

Alternatively, collagen-free aortas were stirred in 0.05 M dithiothreitol (Cleland's Reagent) in 5 M guanidine, 0.1% EDTA and 0.1M tris buffer, pH 8.5, for 24 hours at 37° under an atmosphere of nitrogen. This procedure was similar to that of Ross and Bornstein (1969). The extract was filtered, dialyzed extensively at 4° against distilled water and lyophilized. The residue was washed thoroughly with distilled water and dried in air.

Column Chromatography

Ion exchange chromatography of ALSP was carried out on 1 x 20 cm column of DEAE-cellulose (Bio-Rad Laboratories) which had been washed with 0.1M HCl and 0.1M NaOH and equilibrated with 0.005M tris buffer, pH 7.1, containing 0.001M EDTA. The sample of ALSP was dissolved in 0.1M NaOH and dialyzed extensively against the buffer. The clear retentate was filtered, applied to the column and eluted with a 0-1M KCl gradient in the tris buffer. After the gradient was exhausted, the

column was washed with 0.1M NaOH. The eluant from the column, including the alkali wash, was monitored for fluorescence and fractions were collected.

Gel permeation chromatography was carried out on a 1.5 x 65 cm column of Sephadex G-200. Protein samples were dissolved in a small volume of pyridine-collidine-acetic acid buffer, pH 8.1 (0.1M in both pyridine and collidine), filtered, applied to the column and eluted with the same buffer.

Treatment of ALSP with Peroxidase

Approximately 30 mg of ALSP was dissolved in 40 ml of citrate-phosphate (McIlvaine) buffer, pH 7.4, to which was added 500 μg of horseradish peroxidase (type VI, RZ approximately 3.0, 295 purpurogallin units per mg, Sigma Chemical Company) and 40 μl of 30% hydrogen peroxide. The solution was incubated at 37^{o} for 90 minutes, dialyzed extensively at 4^{o} against several changes of distilled water and lyophilized.

Enzymic Hydrolysis of ALSP

Approximately 40 mg of ALSP was dissolved in 20 ml of the pyridine-collidine-acetic acid buffer, containing 0.005M $CaCl_2$, and 2 mg of pronase (streptomyces griseus protease, B grade, 45 proteolytic units per mg, Calbiochem.). The solution was incubated at 37^{o} for 48 hours, an additional 1 mg of pronase added, and incubation continued for another 24 hours. After incubation, the solution was immersed in a boiling water bath for 20 minutes and evaporated to dryness at 60^{o} under vacuum. The residue was dissolved in 20 ml of the original buffer containing 0.005M $MnCl_2$ and 500 μg of leucine aminopeptidase (type III-CP, Sigma Chemical Company), and the mixture incubated at 40^{o} for 24 hours. An

aliquot of the enzymic hydrolyzate was evaporated to dryness at 60° under vacuum, and the residue dissolved in a small volume of 0.2M acetic acid and chromatographed on a 2 x 40 cm column of Bio-Gel P-2 (200-400 mesh, Bio-Rad Laboratories) in 0.2M acetic acid. The eluant from the column was monitored for ultraviolet absorption at 280 nm and fractions were collected.

Amino Acid Analysis

Proteins for amino acid analysis were hydrolyzed in 6N HCl (fluorometric grade, Harleco Division, American Hospital Supply Corporation) for 24 hours at 110° in sealed ampoules under nitrogen. Amino acid analyses were carried out on a Technicon instrument using the 75 cm column, 5 1/2 hour gradient system (C-2 Chromobead resin). One half of the effluent from the column was reacted with ninhydrin, and the other was mixed with 2N NaOH and monitored for fluorescence.

The area of the fluorescence peak corresponding to the elution volume of dityrosine was measured. Authentic dityrosine was prepared by the method of Gross and Sizer (1959), and purified as described elsewhere (Section IV). Assuming a ninhydrin color equivalent of 2.0 (relative to norleucine), each cm^2 of fluorescence was estimated to represent 1.4×10^{-4} micromoles of dityrosine.

Optical Measurements

Continuous monitoring of column eluants for ultraviolet absorption was carried out using an ISCO UA-2 Ultraviolet Analyzer, while ultraviolet absorption spectra were obtained on a Beckman DK-2 spectrophotometer. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer.

Protein Determinations

Protein was determined by the Lowry method (Lowry et al., 1951) using a bovine serum albumin standard.

RESULTS

Extraction of ALSP

The composition of ALSP from day-old chick aortas has been presented elsewhere (Section III). When collagen-free aortas were extracted with hot alkali for four 10 minute periods, the quantity of protein in the extracts fell exponentially (Fig. 18). However, the amino acid compositions of the four extracts were essentially identical. Because only approximately 2% of extractable protein appeared in the fourth extract, extraction for three 10 minute periods was adopted as a routine procedure. The dialyzate of the pooled hot alkali extracts contained approximately 3% of the total material extracted, and no dityrosine. The precipitate which formed in the bag towards the completion of dialysis, when separated from the water soluble supernatant by low speed centrifugation, contained approximately 80% of the total protein extracted. The amino acid compositions of the precipitate and supernatant were essentially identical. However, the dityrosine content of the precipitated protein was 2-3 times that of the soluble protein (Table III).

Ion Exchange Chromatography of ALSP

The elution pattern of ALSP from DEAE-cellulose is shown in Figure 19. Only 55% of the total protein was recovered in the eluant, including that in the alkali wash. The amino acid compositions and dityrosine contents of the pooled fractions C and D, as indicated in Figure 19, are shown in Table IV. Fractions A and B, although fluorescent,

Figure 18:

Amount of protein solubilized from collagen-free aortas by successive 10 minute treatments with hot 0.1 M NaOH.

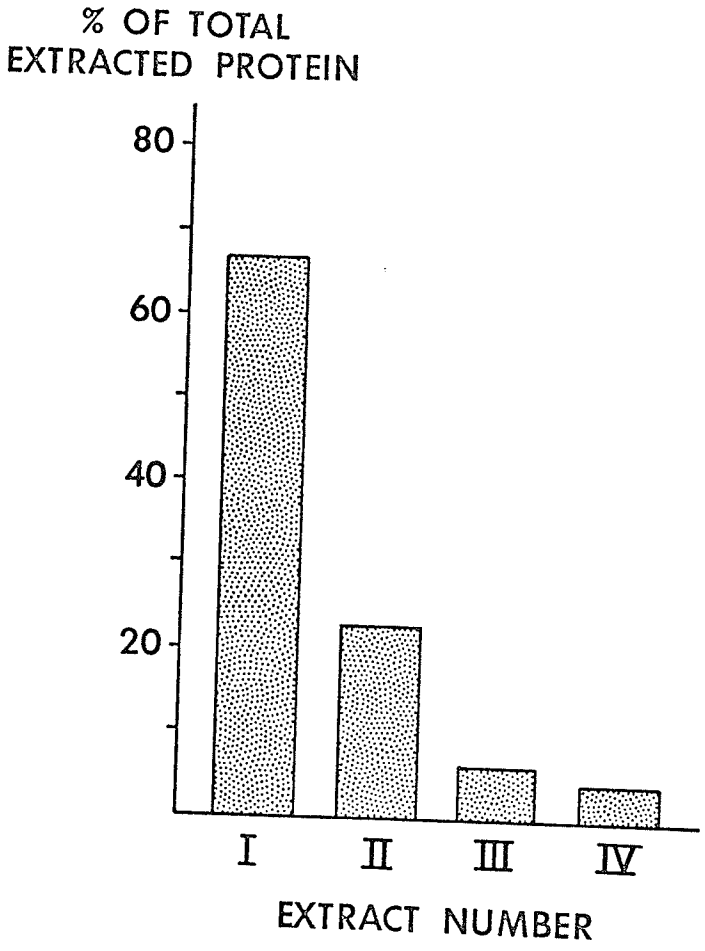

% OF TOTAL
EXTRACTED PROTEIN
80
60
40
20
I
II
III
IV
EXTRACT NUMBER

TABLE III

AMINO ACID COMPOSITION OF
WATER-SOLUBLE AND WATER-INSOLUBLE
COMPONENTS OF ALSP

	residues per 1000 total residues	
	water soluble	water insoluble
HPRO	0	0
ASP	97	83
THR	46	48
SER	51	49
GLO	136	127
PRO	72	55
GLY	124	86
ALA	88	83
VAL	72	69
MET	22	15
ILEU	49	53
LEU	74	94
TYR	28	33
PHE	37	45
LYS	44	69
HIS	15	22
ARG	38	64
DITYR(a)	0.031	0.079

(a) Determined from fluorescence measurements.

Figure 19:

Ion exchange chromatography of ALSP on DEAE-cellulose. Protein was eluted by a 0-1 M KCl gradient in 0.005 M tris buffer, pH 7.1, containing 0.001 M EDTA. The arrow indicates initiation of column washing with 0.1 M NaOH. The amount of protein recovered in each fraction is indicated.

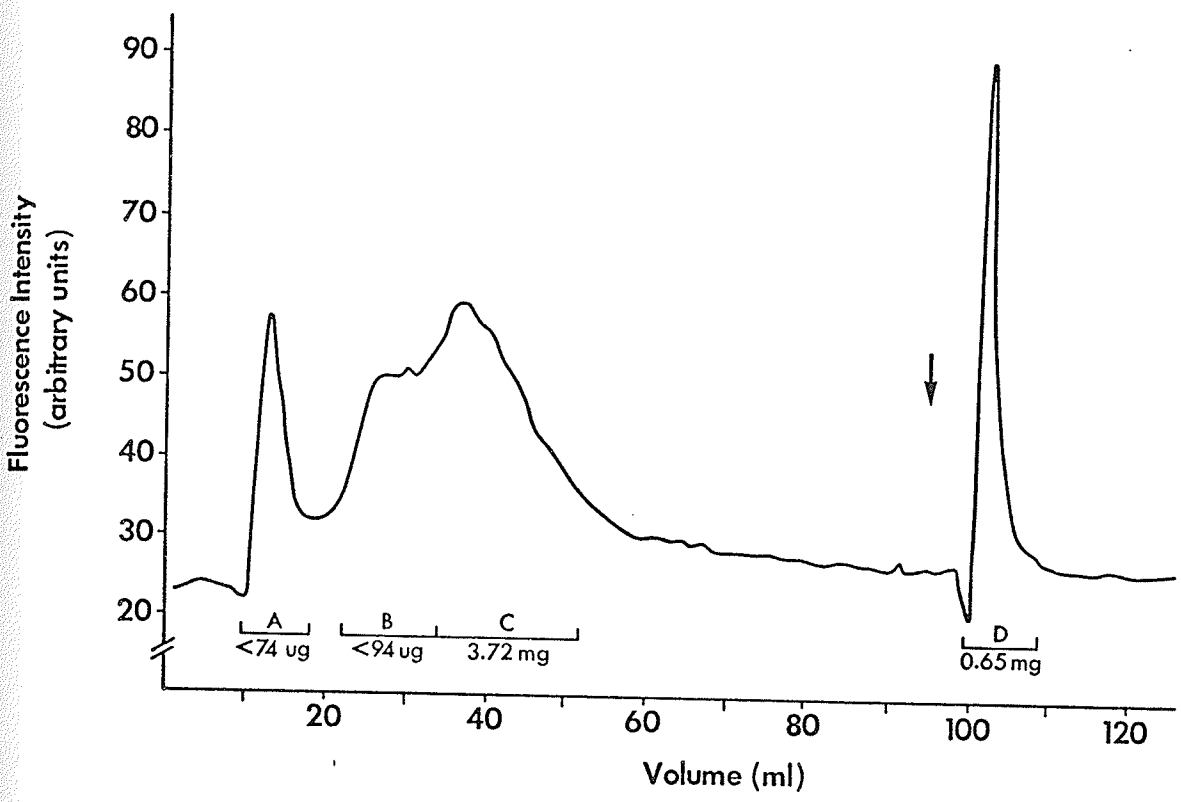

Fluorescence Intensity
(arbitrary units)
90
80
70
60
50
40
30
20
A
<74 ug
B
<94 ug
C
3.72 mg
D
0.65 mg
20
40
60
80
100
120
Volume (ml)

TABLE IV

AMINO ACID COMPOSITION OF
FRACTIONS OBTAINED BY CHROMATOGRAPHY
OF ALSP ON DEAE-CELLULOSE

residues per 1000 total residues

	C	D
HPRO	0	0
ASP	92	89
THR	36	54
SER	66	74
GLU	128	113
PRO	93	72
GLY	86	88
ALA	78	87
VAL	62	74
MET	20	10
ILEU	47	48
LEU	84	89
TYR	29	24
PHE	33	35
LYS	66	69
HIS	19	20
ARG	40	44
DITYR[a]	0.024	0.042

(a) Determined from fluorescence measurements.

contained little protein and no detectable dityrosine.

Comparison of Extraction Procedures

The amino acid compositions and dityrosine contents of the proteins solubilized from collagen-free aortas by hot alkali and dithiothreitol-guanidine (DTT-G) are shown in Table V. DTT-G extracted only 50% of the protein solubilized by hot alkali. When the residue after DTT-G treatment was further extracted with hot alkali, the balance of the protein was solubilized. Conversely, after hot alkali treatment, DTT-G failed to extract additional protein.

Gel Permeation Chromatography of ALSP

The elution pattern of ALSP from Sephadex G-200 is shown in Figure 20a. The pattern was evidently not due to a readily reversible aggregation phenomenon since, upon rechromatography, proteins in regions A, B and C of the chromatogram were eluted only in their respective elution volumes (Figs. 20b, 20c, 20d). Fractions were pooled as indicated in Figure 20a, and the amino acid compositions and dityrosine contents of the proteins in these fractions are shown in Table VI.

Treatment of ALSP with Peroxidase

Treatment of ALSP with horseradish peroxidase and hydrogen peroxide resulted in an increase in the amount of dityrosine in the protein, although there was no significant alteration in the proportions of amino acids, including tyrosine (Table VIIa). The elution patterns from Sephadex G-200 of peroxidase-treated ALSP and the non-treated protein are compared in Figure 21. Only 80% of this peroxidase-treated protein could be dissolved in the pyridine-collidine-acetic acid buffer; the pattern shown in Figure 21b is that of the soluble portion only.

TABLE V

AMINO ACID COMPOSITIONS OF PROTEINS EXTRACTED BY HOT ALKALI, DTT-G OR HOT ALKALI AFTER DTT-G

	residues per 1000 total residues		
	hot alkali	DTT-G	hot alkali after DTT-G
HPRO	0	0	0
ASP	102	87	96
THR	44	56	47
SER	54	72	51
GLU	129	120	127
PRO	60	52	51
GLY	98	101	102
ALA	84	85	92
VAL	70	65	71
MET	16	20	24
ILEU	45	48	49
LEU	87	88	98
TYR	26	24	27
PHE	37	34	39
LYS	61	66	61
HIS	19	18	17
ARG	43	62	47
DITYR[(a)]	0.101	0.071	0.139

(a) Determined from fluorescence measurements.

Figure 20:

Gel permeation chromatography of ALSP on Sephadex G-200 in pyridine-collidine-acetic acid buffer, pH 8.1.

(a) ALSP

(b) fraction A from (a)

(c) fraction B from (a)

(d) fraction C from (a)

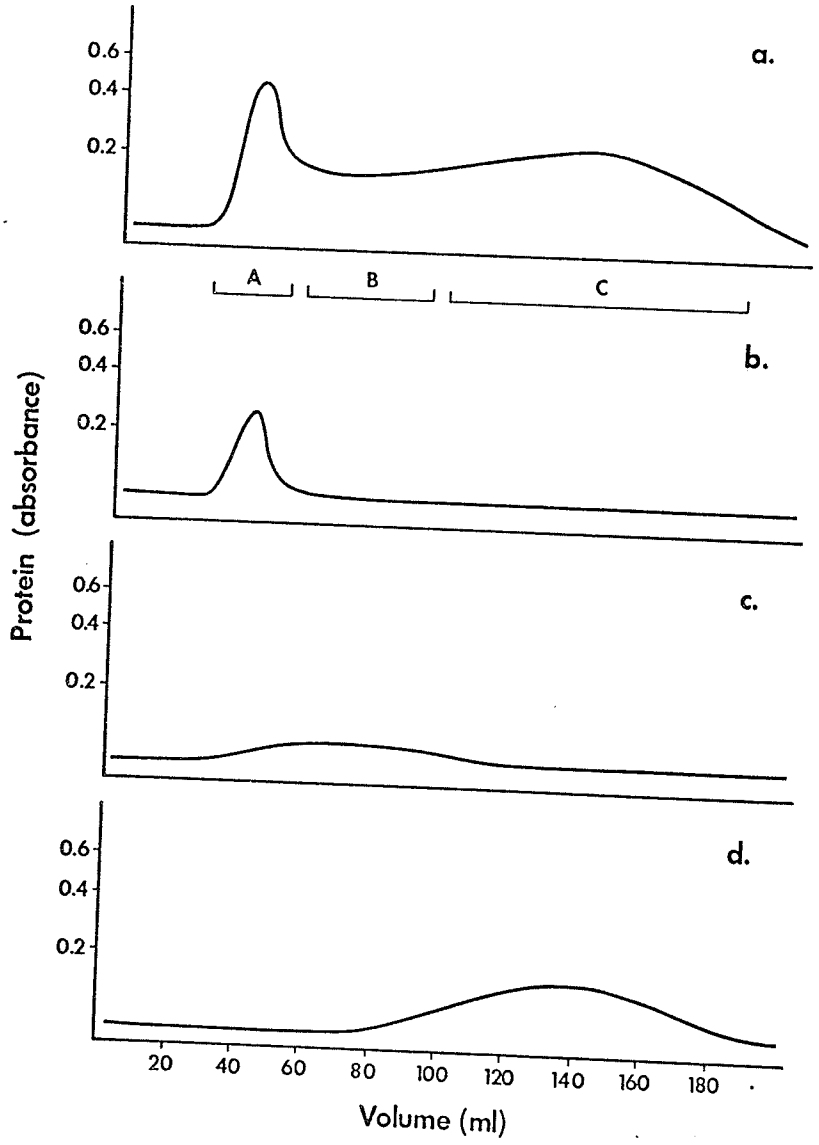

a.
b.
c.
d.
0.6
0.4
0.2
A
B
C
Protein (absorbance)
20
40
60
80
100
120
140
160
180
Volume (ml)

TABLE VI

AMINO ACID COMPOSITIONS OF ALSP
AND OF FRACTIONS OBTAINED BY
CHROMATOGRAPHY OF ALSP ON SEPHADEX G-200

	residues per 1000 total residues			
	total protein	A	B	C
HPRO	0	0	0	0
ASP	102	94	106	109
THR	44	67	51	47
SER	54	84	57	54
GLU	129	95	132	136
PRO	60	46	73	70
GLY	98	92	89	97
ALA	84	92	85	82
VAL	70	85	75	77
MET	20	17	11	15
ILEU	45	47	48	48
LEU	87	85	83	83
TYR	26	20	24	28
PHE	37	35	37	37
LYS	61	74	65	53
HIS	19	14	18	16
ARG	43	37	43	42
DITYR[(a)]	0.065	0.091	0.084	0.041

(a) Determined from fluorescence measurements.

TABLE VII

AMINO ACID COMPOSITIONS OF UNTREATED ALSP, PEROXIDASE-TREATED ALSP AND FRACTIONS OBTAINED BY CHROMATOGRAPHY OF PEROXIDASE-TREATED ALSP ON SEPHADEX G-200

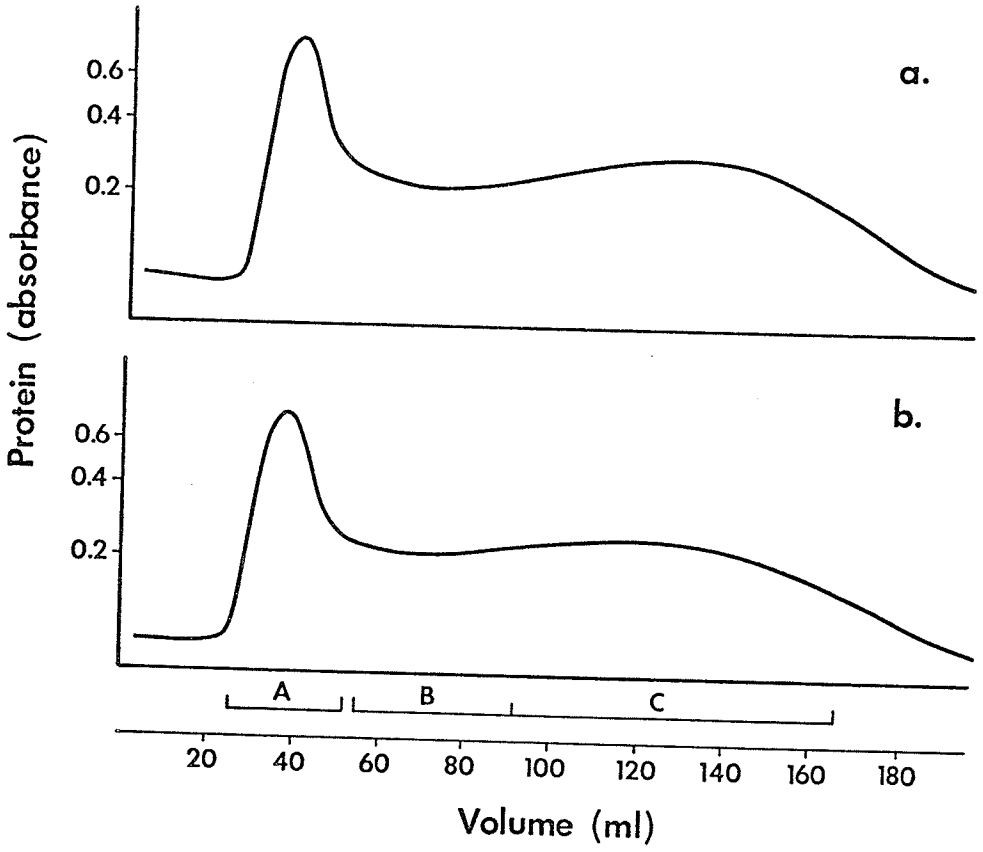
(a)	residues per 1000 total residues	
	untreated ALSP	peroxidase-treated ALSP
HPRO	0	0
ASP	91	96
THR	47	47
SER	53	57
GLU	128	128
PRO	57	53
GLY	107	101
ALA	88	90
VAL	69	77
MET	15	17
ILEU	47	51
LEU	90	90
TYR	26	25
PHE	40	41
LYS	60	60
HIS	22	16
ARG	48	52
DITYR(*)	0.084	0.126

(b)	residues per 1000 total residues			
	insoluble component	A	B	C
HPRO	0	0	0	0
ASP	77	91	93	99
THR	47	46	49	51
SER	52	49	47	52
GLU	110	133	132	136
PRO	61	46	47	48
GLY	97	104	90	105
ALA	83	83	75	88
VAL	75	70	67	69
MET	16	22	32	23
ILEU	53	49	48	47
LEU	103	87	109	88
TYR	30	27	30	33
PHE	52	39	43	45
LYS	67	73	66	51
HIS	19	16	18	16
ARG	54	55	52	43
DITYR(*)	0.202	0.169	0.144	0.099

(*) Determined from fluorescence measurements.

Figure 21:

Gel permeation chromatography of untreated (a) and peroxidase-treated ALSP (b) on Sephadex G-200 in pyridine-collidine-acetic acid buffer, pH 8.1.


Protein (absorbance)
0.6
0.4
0.2
a.
0.6
0.4
0.2
b.
A
B
C
20
40
60
80
100
120
140
160
180
Volume (ml)

The amino acid compositions and dityrosine contents of this insoluble component and the pooled fractions of the eluant of the soluble portion of the peroxidase-treated ALSP are shown in Table VIIb.

Enzymic Hydrolysis of ALSP

The extent of hydrolysis of ALSP by pronase, as indicated by the release of ninhydrin-positive groups from the protein, was 40%. Further hydrolysis by leucine aminopeptidase could not be measured in this way, since the enzyme solution contained interfering $(NH_4)_2SO_4$. Table VIIIa shows the amino acid composition and dityrosine content of an acid hydrolyzate of ALSP used as substrate for the enzymes, and the free amino acids and dityrosine released by the enzymic hydrolysis. The percent yield of each of the amino acids by enzyme hydrolysis as compared to acid hydrolysis is also shown. The amino acid compositions of the pooled fractions of the eluant of the enzyme hydrolyzate from Bio-Gel P-2 (Fig. 22), A, B and C, are shown in Table VIIIb. Fraction D contained only free tyrosine and dityrosine, and fraction E contained only tryptophan. The large ultraviolet absorbing peak corresponding to fraction C in Figure 22 is probably due to the presence of traces of pyridine and collidine in the hydrolyzate.

DISCUSSION

Extraction and Polydispersity of ALSP

The pattern of extraction of ALSP (Fig. 18), the similarity of amino acid compositions of the protein in successive alkali extracts, and the very small amount of dialyzable ninhydrin-positive material present in the pooled alkali extracts indicate the removal of a distinct protein species by this treatment.

TABLE VIII

AMINO ACID COMPOSITIONS OF ACID OR ENZYME HYDROLYZATES OF ALSP AND OF FRACTIONS OBTAINED BY CHROMATOGRAPHY OF THE ENZYME HYDROLYZATE ON BIO-GEL P-2

(a) residues per 1000 total residues

	acid hydrolyzed ALSP	enzyme hydrolyzed ALSP (‡‡)	% yield
HPRO	0	0	-
ASP	86	20	8.8
THR	41	48	39.4
SER	42	44	33.1
GLU	125	56	13.4
PRO	68	9	5.0
GLY	119	105	31.9
ALA	91	116	43.5
VAL	79	124	31.9
MET	19	25	43.5
ILEU	46	80	53.5
LEU	93	151	39.1
TYR	26	44	59.9
PHE	41	48	63.3
LYS	53	54	53.8
HIS	16	21	46.3
ARG	40	41	39.4
DITYR (*)	0.106	0.025	6.3

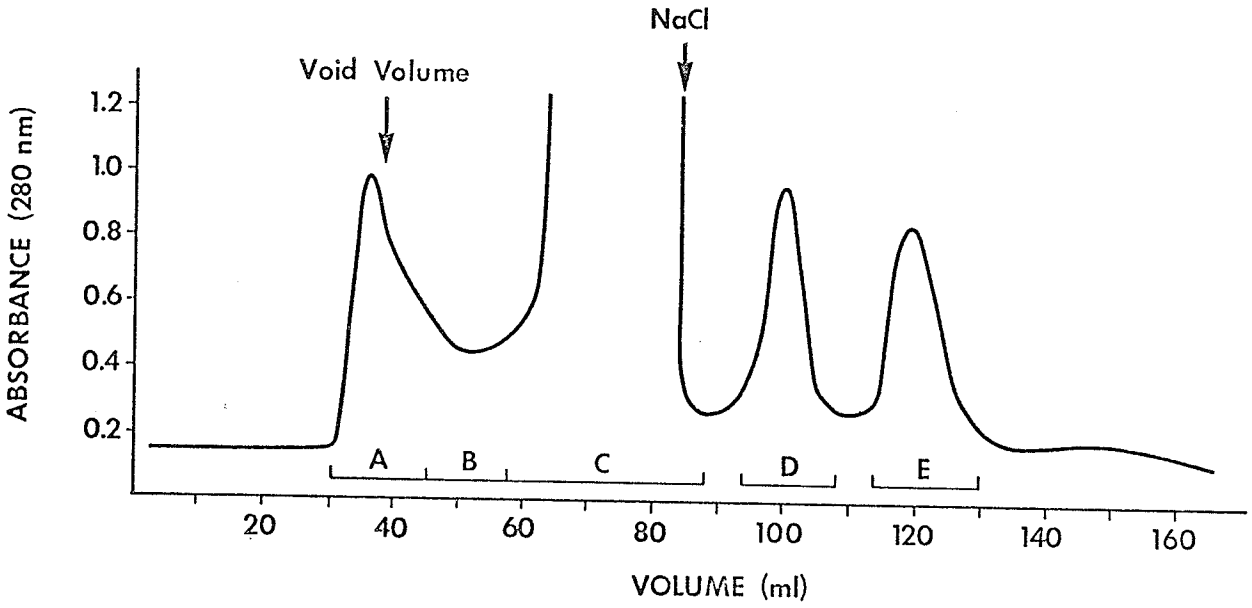
(b) residues per 1000 total residues

	A (**)	B (**)	C
HPRO	0	0	0
ASP	102	95	23
THR	44	38	62
SER	40	44	52
GLU	156	139	65
PRO	62	71	N.D. (***)
GLY	120	117	119
ALA	87	85	121
VAL	59	58	127
MET	10	31	31
ILEU	36	33	84
LEU	59	60	169
TYR	13	11	0 (‡)
PHE	27	25	38
LYS	76	114	26
HIS	22	21	16
ARG	43	37	37
DITYR (*)	0.300	0.175	0 (‡)

(*) Determined from fluorescence measurements.
(**) Acid hydrolyzed.
(***) Not detected.
(‡) Free dityrosine and tyrosine are eluted in fraction D.
(‡‡) Free amino acids.

Figure 22:

Gel permeation chromatography of an enzyme hydrolyzate of ALSP on Bio-Gel P-2 in 0.2 M acetic acid. The void volume of the column and the elution volume of NaCl are indicated.


NaCl
Void Volume
ABSORBANCE (280 nm)
1.2
1.0
0.8
0.6
0.4
0.2
A
B
C
D
E
20
40
60
80
100
120
140
160
VOLUME (ml)

Extraction with hot alkali appears to be the more efficient of the extraction methods employed, since this treatment solubilizes protein from tissue previously extracted with dithiothreitol and guanidine, whereas the converse is not true. The amino acid compositions of the proteins solubilized by hot alkali, DTT-G or hot alkali after previous extraction with DTT-G are virtually identical, suggesting that DTT-G removes a more readily solubilized form of the same protein. The composition reported here for the protein extracted by DTT-G is similar, but not identical, to that published by Ross and Bornstein (1969) for microfibrillar protein, although the discrepancies in reported compositions may be attributed to differences in procedures for prior removal of collagen and other more soluble proteins.

ALSP appears to belong to the large family of glycoproteins which have been extracted from connective tissues by various workers. Because the compositions of these proteins from various sources are so similar, it is likely that the diverse methods of extraction remove protein in different states of aggregation, the larger aggregates being solubilized by only the more vigorous procedures. In this regard, we have noted the presence of proteins other than collagen in NaCl and water extracts of day-old chick aortas, and these extracts may contain even less aggregated forms of the same protein.

It is also possible that ALSP may comprise two or more proteins of similar amino acid composition. We have found that the amino acid composition of ALSP, especially the proportions of the acidic and neutral amino acids, is more variable from batch to batch than would be expected from the precision of the amino acid analyses or from any

possible variations in the preparation of the protein. Differences in the proportions of the postulated proteins present in the aggregates could account for the varying compositions of our extracts, as well as for the small differences in composition reported for other preparations of similar proteins.

Barnes and Partridge (1968) found that 80-85% of a similar protein extracted with cold alkali precipitated on extensive dialysis against water, and noted the similar amino acid compositions of the water-soluble and water-insoluble fractions. Although both fractions showed the same two components by ultracentrifugal analysis, the larger, faster sedimenting component was predominant in the water-insoluble fraction, whereas the smaller, slower sedimenting component was predominant in the water-soluble fraction. They concluded that the water-insoluble fraction contained a greater proportion of a more aggregated form of the protein.

The identities of the fluorescent materials in fractions A and B eluted from the DEAE-cellulose column are unknown, although they are apparently not proteinaceous and do not contain dityrosine. Although fractions C and D have similar amino acid compositions, the latter is eluted only during column washing with alkali. The tenacity of fraction D suggests that it is an aggregated form of the same protein found in fraction C. Barnes and Partridge (1968) also noted poor recovery of their protein from DEAE-cellulose and attributed this to "an irreversible adsorption of material of very high molecular weight".

The elution pattern of ALSP from Sephadex G-200 and the similarity of amino acid composition of protein from the three areas of

the chromatogram supports the conclusion that the protein exists in varying degrees of aggregation. Timpl et al. (1968) reported that 80-90% of a similar polydisperse protein, obtained from connective tissues by extraction with urea or cold alkali, was excluded on Sephadex G-200. The various aggregated forms of the protein are apparently not in ready equilibrium with one another, since redistribution of the protein of a given size range in the fractionated extract did not occur upon rechromatography.

Treatment with peroxidase appears to promote aggregation of the protein as determined by gel permeation chromatography. The insoluble portion of the peroxidase-treated ALSP presumably represents a highly aggregated state.

Role of Dityrosine in ALSP

The dityrosine content of ALSP is apparently directly related to the size and solubility of the protein aggregates in the extracts. In our investigations, the water-insoluble precipitate formed during dialysis, in which there appears to be a preponderance of the more aggregated forms of the protein (Barnes and Partridge, 1968), contains 2-3 times more dityrosine than the water-soluble protein. Similarly, the more soluble protein extracted with DTT-G contains less dityrosine than does protein extracted by hot alkali from the same collagen-free tissue. At the same time, dityrosine is concentrated in the protein which is insoluble in DTT-G but subsequently extracted by hot alkali. Gel permeation chromatography clearly indicates that the dityrosine content is directly related to the molecular size of the protein fractions. Similarly, the dityrosine content of protein which is eluted from DEAE-

cellulose only by alkali is greater than that of the presumably less aggregated form eluted by the salt gradient. Moreover, introduction of additional dityrosine into ALSP by treatment with peroxidase is associated with increased aggregation of the protein.

Gross and Sizer (1959) were unable to detect dityrosine in bovine fibrinogen, insulin or pepsin treated with peroxidase. On the other hand, Andersen (1966) treated silk fibroin, a relatively tyrosine-rich protein, with peroxidase, and reported dityrosine formation accompanied by gelation of the protein. The fact that dityrosine can be readily produced in ALSP suggests that at least some of the tyrosine residues in the protein are sterically well situated for this type of coupling.

Extensive enzymic hydrolysis of ALSP indicated that dityrosine is concentrated in areas of the protein that are relatively inaccessible to the enzymes. At the same time, the fact that a small amount of dityrosine is released in the free form by enzymic hydrolysis argues against the artifactual production of dityrosine by acid hydrolysis. We consider the observed relationship between dityrosine content and the size and solubility of the protein aggregate, together with the relative concentration of dityrosine in the enzyme-resistant core of the protein, to be strong evidence that this fluorescent amino acid functions as an interchain crosslink which contributes to the aggregation of the protein.

Aspartic acid, glutamic acid and proline are not readily released from ALSP by enzymic hydrolysis, although bonds containing these amino acids are not particularly resistant, at least to pronase (Nomoto et al., 1960). These findings might initially suggest that the β and

γ carboxyls of the acidic amino acids occur in the amide form in the protein. However, asparagine and glutamine would probably have been deaminated during the hot alkali treatment and, in any case, are not seen in chromatograms of the free amino acids released by enzymic hydrolysis. Furthermore, because ALSP is least soluble at pH 3-4 (Barnes and Partridge, 1968; Keeley, unpublished observations) and a glycoprotein of similar composition extracted from connective tissue with water is reported to have an isolelectric pH of 3.8 (Fishkin and Berenson, 1961), most of the β and γ carboxyls of these amino acids must be in the free form to account for the acidity of the protein. Therefore, these three amino acids may be specifically located near the site of the proposed dityrosine crosslink, and are thus relatively inaccessible to the enzymes.

Although the quantities of dityrosine present seem small, LaBella, et al., (1968) demonstrated that the introduction into soluble collagen of only one residue of dityrosine per 5000 total amino acid residues was apparently sufficient to promote the formation of a rigid, insoluble gel. The relatively low content of the proposed crosslink suggests that dityrosine is perhaps not the only crosslink responsible for the maintenance of aggregation in the protein. That disulfide bonds are involved is evidenced by the fact that at least some of the protein is solubilized by dithiothreitol and guanidine. Barnes and Partridge (1968) reported the presence of cysteine in the protein which they isolated, and we have detected cysteine and cysteic acid in acid hydrolyzates of ALSP. Interchain glycosidic linkages, some of which may be broken by hot alkali, may also be involved. As the protein aggregate becomes larger, physical entanglement of peptide chains may contribute

to further aggregation and insolubility. It is also possible, as suggested earlier (Section III), that dityrosine represents an intermediate stage in the synthesis of a more extensive polyphenolic or quininoid crosslink.

Barnes and Partridge (1968) reported the presence of tryptophan in a similar protein which they extracted with cold alkali. Although this amino acid is destroyed by acid hydrolysis, its occurrence as a free amino acid in enzyme hydrolyzates indicates its presence in ALSP.

The role played by ALSP in the larger protein-polysaccharide complex responsible for the structural integrity of the ground substance of connective tissue is not clear. Its apparent similarity in composition to the microfibrillar protein associated with developing elastin (Ross and Bornstein, 1969), as well as evidence from our laboratory (see Section VI) suggests that it may be involved more directly in the biosynthesis of other connective tissue protein, specifically elastin.

VI. AMINO ACID COMPOSITION OF ELASTIN IN THE DEVELOPING CHICK AORTA. ROLE OF AN ALKALI-SOLUBLE CONNECTIVE TISSUE PROTEIN IN THE BIOSYNTHESIS OF ELASTIN

INTRODUCTION

Previous work has demonstrated the presence of a polar, alkali-soluble, hydroxyproline-free protein in close association with elastin in chick aorta and bovine ligamentum nuchae (Section III). This protein was unusual in that it contained dityrosine, a fluorescent, biphenolic compound which may be a crosslink in insect resilin (Andersen, 1966) and in an alkali-soluble connective tissue protein of developing chick aortic elastin (Section V). Proteins of similar amino acid composition have been extracted from several connective tissues by other workers (Berenson and Fishkin, 1962; Gotte et al., 1963a; Cleary et al., 1967; Barnes and Partridge, 1968; Ross and Bornstein, 1969), and have been proposed by some to represent a distinct type of structural protein (Robert and Comte, 1968; Timpl et al., 1968). We have noted that amino acid compositions of elastin from tissues of fetal and newborn animals contain greater proportions of polar amino acids than elastin from mature animals. Others have observed the unusual composition of developing elastin, attributing it to contamination by a polar protein which tenaciously resists conventional purification procedures (Gotte et al., 1963b; Cleary et al., 1965; 1967; Steven and Jackson, 1968). Recent morphological studies on developing elastic tissues have drawn attention to microfibrillar structures which appear to be continuous with the matrix of newly formed elastin (Haust et al., 1965; Greenlee et al., 1966; Fahrenbach et al., 1966; Takagi and Kawase, 1967; Ross and Bornstein, 1969). The anatomical relationship of the microfibrils to elastic fibres suggests that the former play a role in the formation of the elastin macromolecule. Furthermore, histological and biochemical

properties of the microfibrils differ from those of elastin obtained from adult tissues, but are similar to those of the polar proteins mentioned above. This section describes the results of attempts to characterize the proteins in intimate association with elastic fibres and to elucidate their relationship to the elastin macromolecule, especially in developing elastic tissue.

METHODS

Thoracic aortas from chick embryos and chicks of various ages were cleaned of fat, adventitia and blood and extracted with chloroform: methanol (3:1) for 2 hours at room temperature. The defatted tissue was twice extracted for 24 hours with 0.2 M NaCl at 4^{o}, and autoclaved in water at 110^{o} for 24 hours. The collagen-free tissue was then extracted for 10 minutes three times with 0.1 M NaOH at 98^{o}, and the residue washed with distilled water and air dried. The pooled alkali extracts were exhaustively dialyzed against distilled water and the retentate freeze-dried. The final product will be referred to as "alkali-soluble protein" (ALSP). The residue after the hot alkali extractions was taken as elastin.

Proteins were hydrolyzed in 6 N HCl for 24 hours at 110^{o} in sealed ampoules under N_2, and the hydrolyzates evaporated to dryness and reconstituted in 0.1 N HCl. Amino acid analyses were carried out on a Technicon instrument using the 75 cm column, 5 1/2 hour gradient system (C-2 Chromobead resin).

To quantitate ALSP, collagen-free tissue was dried for 12 hours over P_2O_5 in a vacuum dessicator and weighed before and after alkali extraction. Protein was determined by the Lowry method (Lowry et al., 1951).

RESULTS AND DISCUSSION

ALSP Content of Chick Aortas as a Function of Age

The amount of ALSP obtained from autoclaved aortas as a function of age is seen in Figure 23. The yield is largest in very young tissue, and falls to a constant amount by about 10 days after hatching. Similar results have been reported by Cleary (Cleary et al., 1965), who found that pre-natal bovine ligamentum nuchae elastin has a considerable non-collagen proteinaceous contamination which decreases in amount with increasing fetal age.

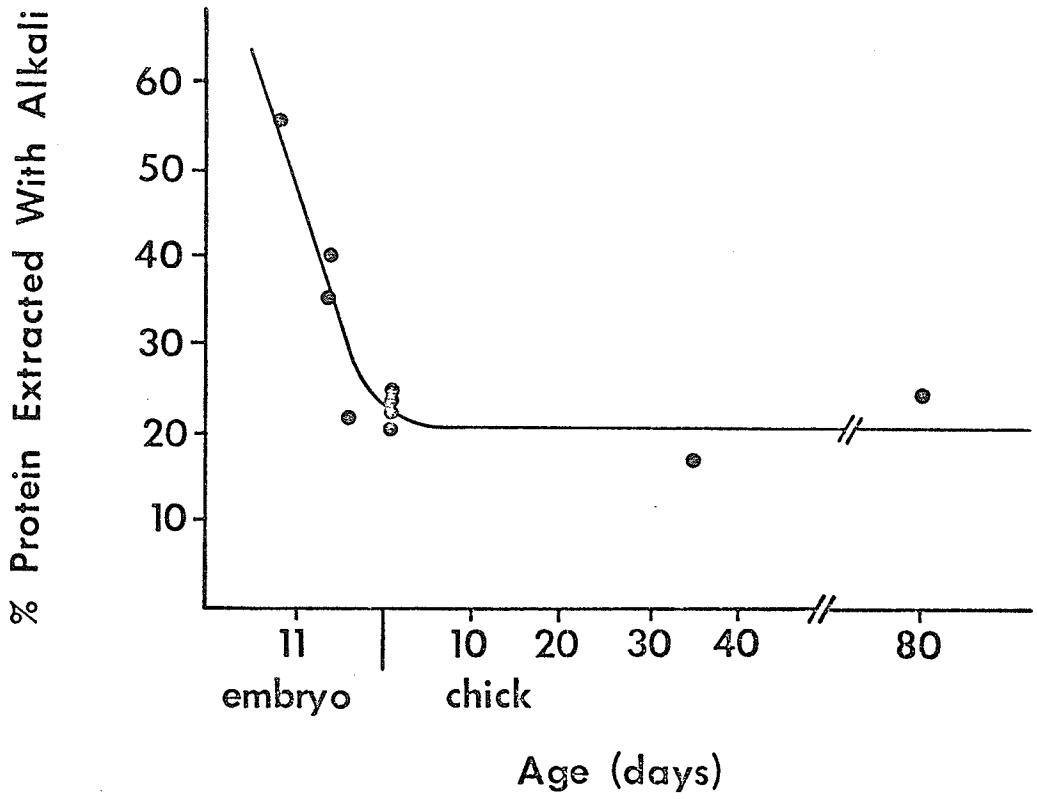
The presence of a relatively insoluble, non-collagenous protein in several connective tissues has been suggested by many authors on the basis of: (a) the increased proportion of polar amino acids in elastins purified by water autoclaving as compared to hot alkali treatment, and (b) the loss in weight upon extraction of collagen-free elastins with hot alkali (Gotte et al., 1963a, 1963b, 1965; Farrar et al., 1965; Fitzpatrick and Hospelhorn, 1965a; Cleary et al., 1967).

Similar proteins have been extracted from collagen-free connective tissues by urea, cold alkali, and hot alkali (Robert et al., 1965; Barnes and Partridge, 1968; Robert and Comte, 1968; Timpl et al., 1968), and have been considered by Robert and Comte (1968) and Timpl (1968) to represent a previously unrecognized class of structural proteins. Barnes and Partridge (1968) isolated a similar protein from bovine aorta and characterized it as an aggregated glycoprotein with a minimum molecular weight of 80,000.

Our finding that the amount of protein extracted becomes constant soon after hatching is consistent with previous results from this

Figure 23:

Amount of ALSP extracted from autoclaved chick aortas. The total quantity of protein removed by the three ten minute extractions with 0.1 M NaOH is expressed as a percentage of the weight of the collagen-free tissue before extraction.


% Protein Extracted With Alkali
60
50
40
30
20
10
11
10
20
30
40
80
embryo
chick
Age (days)

laboratory on human aortas, which showed no significant differences in the proportion of alkali-soluble material from individuals of ages 1-80 years (LaBella and Lindsay, 1963).

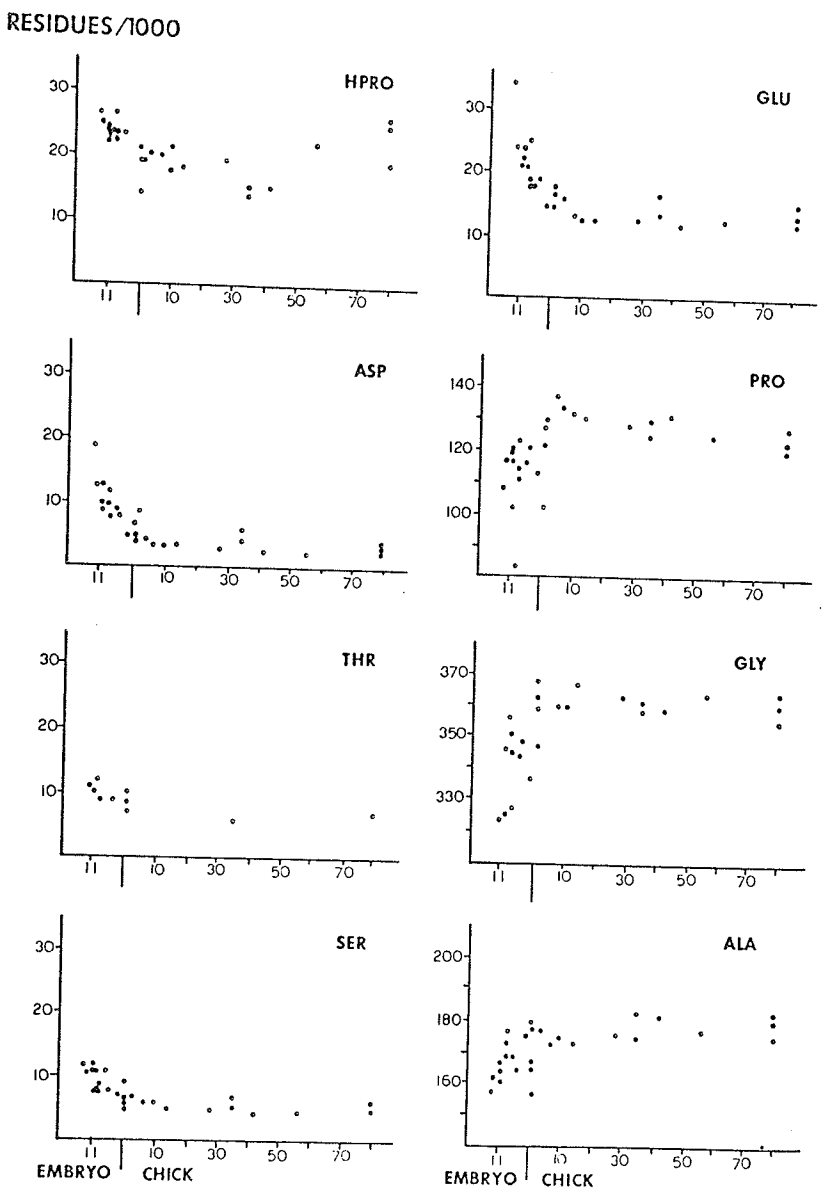
Amino Acid Composition of Alkali-Purified Elastin as a Function of Age

Results of amino acid analysis of alkali-purified elastin indicated age-related differences in amino acid composition, especially in the proportion of the polar amino acids (Figs. 24 and 25). In general, there is a rapid change in composition of embryonic elastin, with a constant composition reached in the 10 day old chick. A straight line was fitted by the method of least squares to the points for each amino acid in elastin as a function of age, and the slope of this line was tested for a significant difference from zero. A significant change with age was found for all amino acids except hydroxyproline and valine. The apparently anomalous behavior of hydroxyproline may be due to interference by methionine sulfone (see below). Because the composition of alkali-purified elastin is constant in chicks over 10 days of age, the amino acid data for elastin from these older animals were averaged to yield the composition of "mature elastin". This mean composition is given in Table IX and is in good agreement with that of non-embryonic chick elastin reported by Miller et al. (1964).

Amino acid data for ALSP were similarly treated, and no significant changes with age were found. Therefore, its mean composition was estimated by averaging the data from embryos and chicks of all ages. The resulting amino acid composition for ALSP is given in Table IX and is in close agreement with our previous results for ALSP from aortas of day-old chicks (Sections III, V).

Figures 24 and 25:

Changes in the proportions of the amino acids present in alkali-purified chick aortic elastin as a function of age.


RESIDUES/1000
HPRO
GLU
ASP
PRO
THR
GLY
SER
ALA
EMBRYO
CHICK
AGE (days)

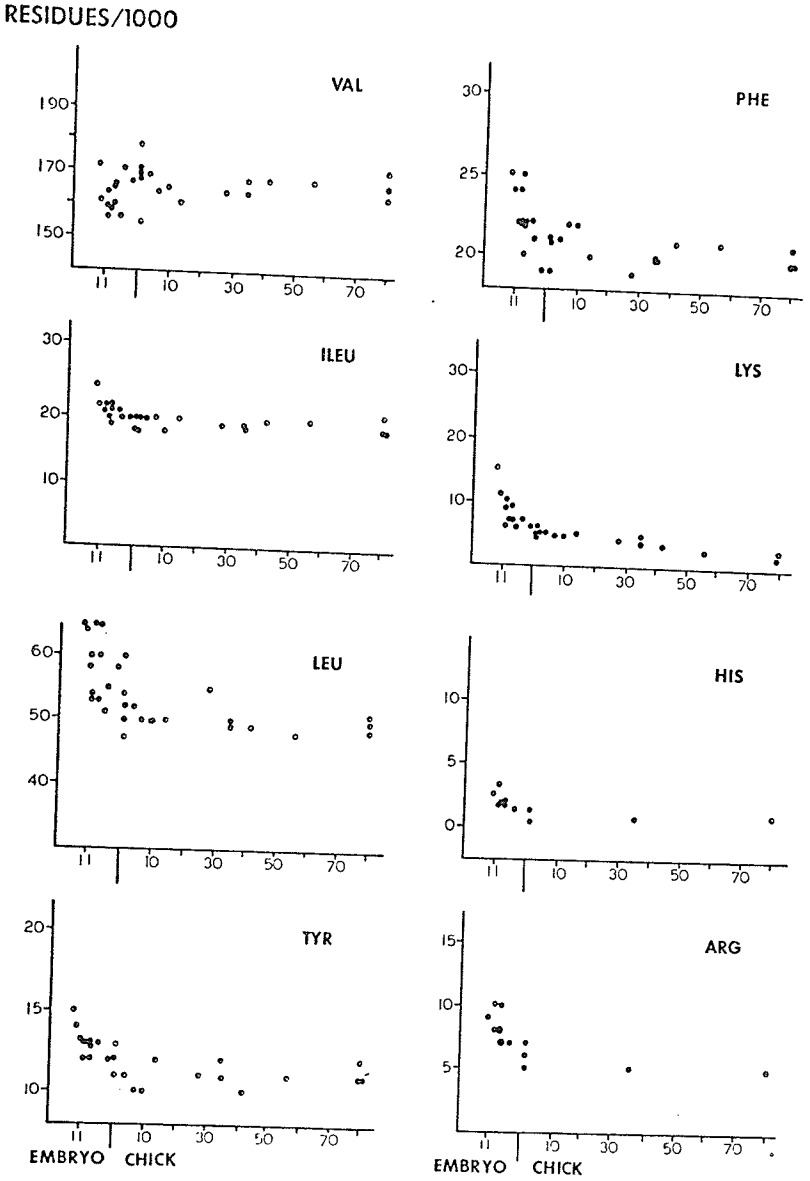

RESIDUES/1000
VAL
190
170
150
PHE
30
25
20
ILEU
30
20
10
LYS
30
20
10
LEU
60
50
40
HIS
10
5
0
TYR
20
15
10
ARG
15
10
5
11
10
30
50
70
EMBRYO
CHICK
AGE (days)

TABLE IX

MEAN AMINO ACID COMPOSITION OF MATURE ELASTIN AND ALSP

	Residues/1000 total residues	
	ALSP	Elastin
HPRO	0	19
ASP	102	4
THR	44	7
SER	54	5
GLU	129	13
PRO	60	127
GLY	98	361
ALA	84	177
VAL	70	166
MET*	18	0
ILEU	45	19
LEU	87	50
TYR	26	11
PHE	37	20
LYS	61	3
HIS	19	1
ARG	43	5

* estimated as methionine plus methionine sulphone

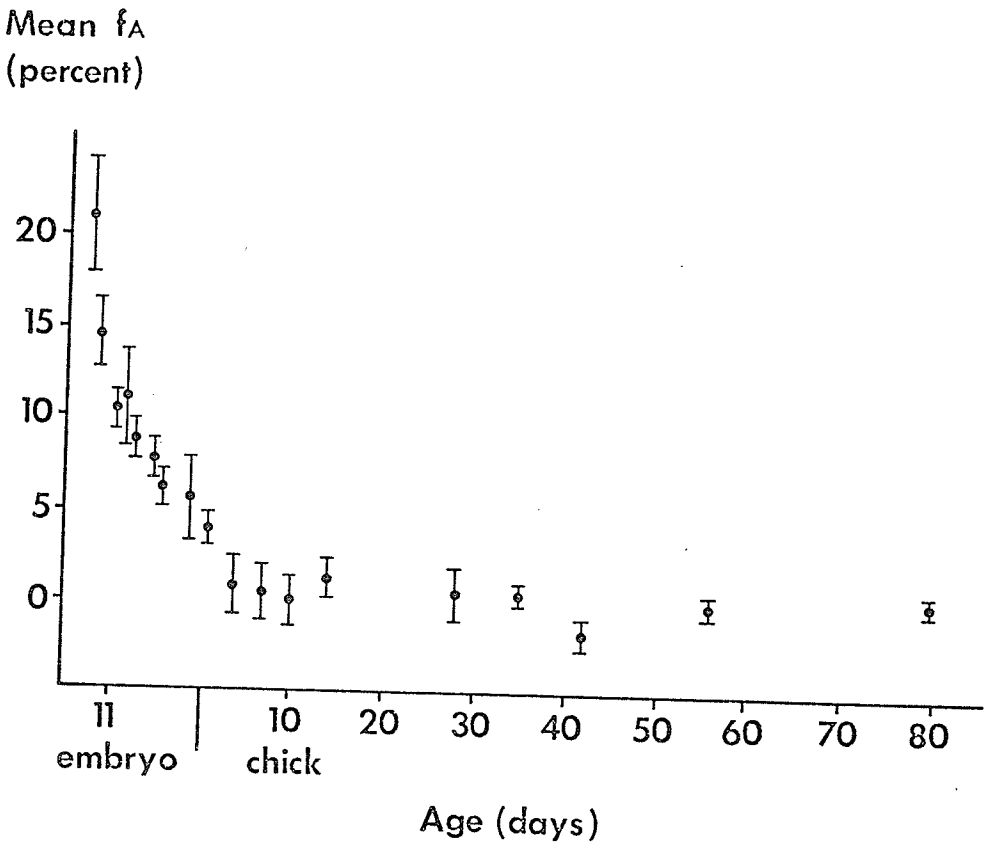
Characterization of the Polar "Contaminant" of Young Elastins

In the case of all the amino acids except hydroxyproline and valine, the proportion of any given amino acid present on the young, alkali-purified elastin is intermediate to that in "mature" elastin and in ALSP. This is consistent with the interpretation that a protein of composition similar to ALSP remains in the younger elastins, either as an insoluble contaminant or as a part of the newly synthesized elastin molecule itself. If this interpretation is correct, the amount of each amino acid in alkali-extracted elastin at any given age is the sum of contributions from mature elastin and ALSP. From the amino acid compositions of mature elastin and ALSP (Table IX), using the relationship given in Figure 26, the relative contributions of the two proteins to the net composition of the elastin can be calculated. For example, to produce a net content of 23 residues of glutamic acid per 1000 total residues (as is found for 10 day embryo elastin) the relative contributions must be $\frac{23-13}{129-13} \times 100\% = 8.6\%$ ALSP (which contains 129 glutamic acid residues per 1000) and 91.4% mature elastin (which contains 13 glutamic acid residues per 1000). This calculation was made for each amino acid (except hydroxyproline) in a given age sample, and the mean and standard error of the values determined. The mean contribution of ALSP to the composition of elastin as a function of age is seen in Figure 26. The contribution is evidently considerable in very young tissue and falls to a small proportion in the 10 day-old chick, as would be expected if the change in composition with age of the young elastin is indeed a reflection of a decreasing ALSP character of the protein.

Because hydroxyproline and methionine sulphone (the oxidation

Figure 26:

Calculation of the ALSP content of the apparent mixture of ALSP and mature elastin to account for differences in amino acid compositions of elastins from tissues of various ages. The percentage of ALSP in the mixture is calculated according to the following relationship: if 'AB' is a mixture of protein 'A' and protein 'B', then $f_A R_A + (1-f_A)R_B = R_{AB}$, where f_A = fraction of 'A' in the mixture, R_A = content of a given amino acid in protein 'A' (expressed as residues per 1000), R_B = content of that same amino acid in protein 'B', R_{AB} = content of that same amino acid in the mixture 'AB'. In this case 'A' represents ALSP, 'B' represents mature elastin, and 'AB' represents the apparent mixture. A value of f_A is calculated for each amino acid in an elastin sample of a given age, and the mean and standard error of the values are plotted.


Mean f_A
(percent)
20
15
10
5
0
11
embryo
10
chick
20
30
40
50
60
70
80
Age (days)

product formed during acid hydrolysis of methionine) are eluted in the same position in our amino acid chromatographic system, and since ALSP contains considerable amounts of methionine, hydroxyproline quantities in younger elastin samples are probably overestimated, thus accounting for the apparently anomalous curve for hydroxyproline in Figure 24.

Unusual amino acid compositions of purified fetal elastins have been reported by others. Cleary et al. (1965, 1967) point out that, whereas autoclaving with water was adequate to yield an elastin with a typical amino acid composition from adult bovine ligamentum nuchae, purification of fetal ligament even with hot alkali leaves a protein with a more polar character than mature ligamentum nuchae. Steven and Jackson (1968) made similar observations in bovine fetal ligamentum nuchae and aorta. Gotte et al. (1963b) reported that this "contaminant" is not removed from bovine ear cartilage elastin before the elastin itself is solubilized by the hot alkali.

These results and our own findings on chick aorta elastin are in contrast to those of Miller et al. (1964), who reported no change in the composition of alkali-treated chick elastin among aortas from 12 day chick embryos to year-old chickens.

The results shown in Figure 26 indicate that the composition of the alkali-resistant elastin at any age can be successfully accounted for as a mixture of "mature" elastin and ALSP, with the proportion of ALSP in the mixture decreasing with increasing age of the tissue. These results might be most readily explained by postulating incomplete removal of the ALSP by the extraction procedure. In fact, the increased content of polar amino acids in fetal elastins has been generally

attributed to a "persistent contamination" by a non-collagenous protein. However, in the alkali extraction procedure virtually all of the extractable protein is removed in the first two 10 minute treatments. The third extraction removes very little protein (Section V), in spite of the fact that the composition of the residue after these extractions still indicates the presence of ALSP in considerable quantities. Therefore, we conclude that the ALSP and the alkali-resistant, non-elastin protein, although they have the same amino acid composition, differ significantly in solubility. It is not clear whether the alkali-insoluble, ALSP-like protein is a more aggregated, and therefore less soluble form of ALSP (Section V) simply mixed with the elastin or, alternatively, if this protein is insoluble because of some interaction with the insoluble elastin matrix. The fact that the alkali-insoluble, polar protein is limited to fetal and young tissues (except for ear cartilage), the "contamination" being most persistent in the youngest of tissues, may suggest the latter alternative, although by no means rules out the former.

Morphological evidence relating to these alternatives may be seen during the development of elastin fibres in bovine ligamentum nuchae (Greenlee, Jr. et al., 1966; Fahrenbach et al., 1966), rat flexor digital tendon (Greenlee, Jr. et al., 1966; Greenlee, Jr. and Ross, 1967), human aorta (Haust et al., 1965) and chick aorta (Takagi and Kawase, 1967; Takagi, 1969a, 1969b). Very young elastin fibres consist mainly of bundles of fine (100Å) microfibrils which stain deeply with uranyl acetate and lead. As the tissue matures, a central weakly staining core appears and increases in proportion with age until the microfibrils are seen only as a sparse mantle around the amorphous core.

Ross and Bornstein (1969) observed this sequence of development in bovine fetal ligamentum nuchae and characterized the central amorphous core as elastin on the basis of its amino acid composition and susceptibility to elastase. The microfibrils,which could be removed by proteolytic enzymes or extracted with dithioerythritol in 5 M guanidine, had an amino acid composition very different from that of elastin and similar to that of our ALSP. They also note that alkali-treated elastin "contains largely the amorphous component". Fyfe et al., (1968) who observed microfibrils in developing mouse aorta suggest that they have some precursor function in the biosynthesis of elastin and refers to them as "proelastin".

Proposed Model of Elastin Biosynthesis

Thus, we have demonstrated the presence of a polar protein mixed with elastin in fetal and young tissues and resisting hot alkali extraction. This protein seems to have the same composition as ALSP, which is removed by hot alkali extraction, the two proteins differing only in solubility. The quantity of this firmly bound protein is greatest in the youngest tissue, and decreases with increasing age until about 10 days after hatching, when all of the protein is extractable with alkali. The ALSP (both soluble and insoluble) seems to be identical to the microfibrillar protein of Ross and Bornstein (1969), shown to be in intimate association with developing elastic tissue, and whose presence precedes the appearance of elastin.

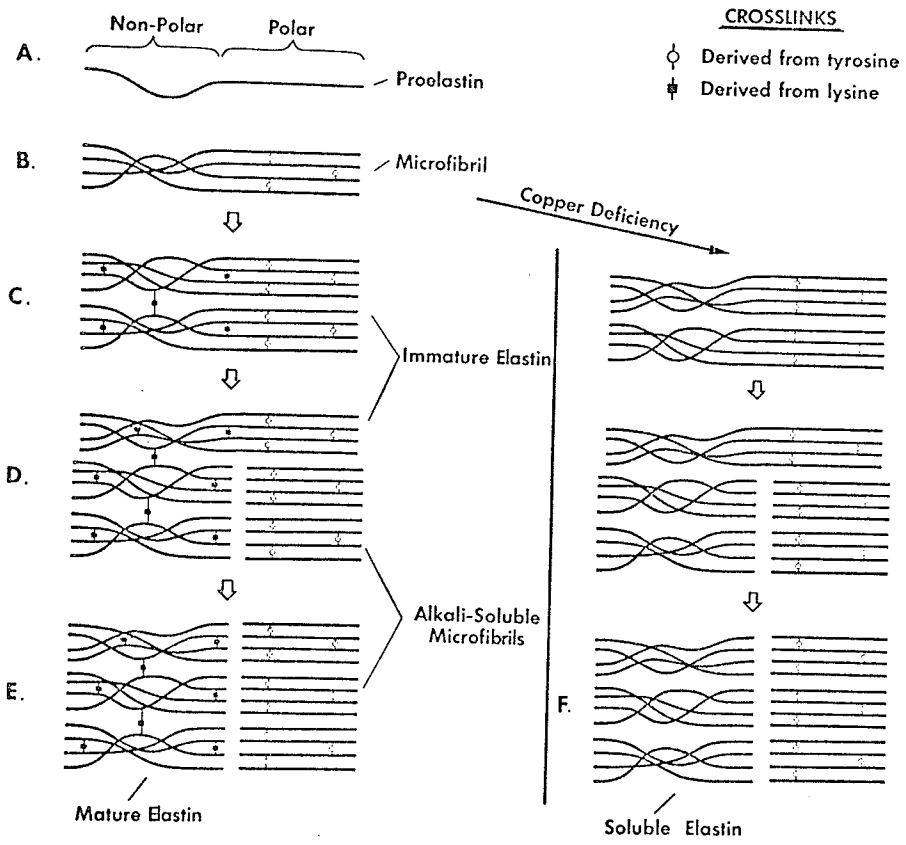
If we accept the alternative that the ALSP-like protein is insoluble duc to some interaction with the insoluble elastin matrix, the possible types of interaction are limited. It is clear that hydrogen

bonds are not involved, since these should have been broken by the autoclaving procedure by which collagen was removed, or by the hot alkali treatment. Moreover, although Ross and Bornstein (1969) extracted their microfibrillar material using dithioerythritol in 5M guanidine, we have found that this procedure results in an incomplete extraction of the microfibrillar protein relative to hot alkali treatment (Section V). Thus disulfide bonds are probably not involved and, in any case, elastin is accepted to contain few, if any, sulfur-containing amino acid residues. Steven and Jackson (1968) were able to render the alkali-insoluble polar contaminant protein of fetal elastic tissue susceptible to hot alkali extraction by prior treatment with a crude bacterial α-amylase preparation. This would suggest a glycosidic linkage. However, the bonds involved in glycosidic linkages would probably have been broken by the hot alkali extraction procedure. Moreover, Ross and Bornstein (1969) were able to remove the microfibrillar protein with trypsin or chymotrypsin, but not with hyaluronidase or β-glucuronidase, suggesting a peptide linkage between the proteins. Steven and Jackson's results may have been due to the presence of proteolytic activity in their α-amylase preparation.

On the basis of the above arguments, we would present the following novel hypothesis for the role of this polar microfibrillar connective tissue protein in the biosynthesis of elastin (Fig. 27). The proelastin is elaborated from the fibroblast as a soluble protein, regions of which have distinct elastin-like and ALSP (microfibrillar)-like compositions (Fig. 27A). The proelastin chains associate by non-covalent interactions to form aggregates which are subsequently cross-linked covalently (Fig. 27B). The crosslinks would include dityrosine,

Figure 27:

A proposed model of elastin biosynthesis. It should be noted that the groupings of the proelastin chains and the number of chains indicated to be involved in the formation of a single lysine-derived crosslink are for convenience of illustration only.


Non-Polar
Polar
A.
Proolastin
B.
Microfibril
CROSSLINKS
Derived from tyrosine
Derived from lysine
Copper Deficiency
C.
Immature Elastin
D.
Alkali-Soluble Microfibrils
E.
F.
Mature Elastin
Soluble Elastin

which we have shown to be present in ALSP (Sections III, IV, V), and possibly disulphide and glycosidic linkages, since ALSP is known to include sulphur-containing amino acids, and ALSP-like proteins isolated by others have been shown to be associated with considerable amounts of glycosides (Barnes and Partridge, 1968; Robert and Comte, 1968; Timpl et al., 1968). The elastin-like portions of the molecule are thus held in the appropriate steric arrangement to promote the formation of the desmosine crosslinks in that area of the molecule (Fig. 27C). As Partridge has pointed out (Partridge, 1969), complete formation of the desmosine molecule may require up to 17 days, during which time the bonds formed are labile and reversible. We suggest that a stable configuration of the peptide chains could be brought about by the sulfhydryl, glycosidic and dityrosine bridges in the polar portion of the proelastin molecule. Strong interactions between chains in the non-polar elastin moiety are more difficult to envisage, since, because of the rubber-like properties of elastin, it is clear that there can be little interaction between the chains, except at the points of crosslinking. Without a conformational stabilization of this kind it is difficult to explain how the desmosines, otherwise a sterically improbable crosslink, could be formed.

Elastin is known to be resistant to proteolytic enzymes in general. However, the ALSP-like portions of the molecule could presumably be attacked by these enzymes. In fact, Ross and Bornstein (1969) were able to cleave and digest the microfibrils from the amorphous elastin core with chymotrypsin and trypsin. Steven and Jackson (1968), who observed alkali-resistant polar contamination in bovine fetal ligamentum nuchae and aorta, stated that this contaminant could be rendered

susceptible to hot alkali extraction by prior treatment with crude bacterial α-amylase. This effect may well have been due to the action of contaminating proteolytic enzymes rather than the hydrolytic activity of the α-amylase itself. Thus, the model proposes that, with time, the polar area of the molecule is hydrolyzed from the desmosine-crosslinked, elastin-like core, releasing ALSP (Fig. 27D).

The solubility of the ALSP moiety is probably dependent on its state of aggregation, which is determined by the content of dityrosine and other crosslinks. Proteins similar to ALSP have been suggested to exist in the form of large aggregates (Barnes and Partridge, 1968; Timpl et al., 1968; Section V) and the different means by which these proteins are extracted from elastic tissue may be a reflection of their states of aggregation. Because of their polymeric structure, however, all forms of the protein, whether extracted with urea, dithioerythritol, cold alkali or hot alkali would have similar amino acid compositions.

In younger tissue, in which the synthesis of new elastin is relatively rapid, the proportion of immature elastin molecules (i.e. those from which the polar moiety has not yet been removed) will be relatively large, accounting for the ALSP-like character of young elastin (Fig. 27D). Conversely, in older tissues where elastin synthesis is very slow, the proportion of new, immature elastin molecules present will be very small (Fig. 27E).

With time, other crosslinks may be formed in the elastin macromolecules, including perhaps those of aromatic origin postulated to account for the increase in fluorescence of elastin with age (LaBella and Lindsay, 1963). The peptide chains in the regions of dityrosine

crosslinks in the microfibrils may be resistant to the extracellular proteolytic enzymes and thus remain in the tissues. These crosslinked peptides may eventually be bound back into the elastin matrix, accounting for the increase in polar amino acid content reported for alkali-purified elastins from very old individuals (Lansing et al., 1951; Fitzpatrick and Hospelhorne, 1965b).

If the formation of the desmosine crosslinks were inhibited by a copper deficiency or lathyritic state, cleavage of the polar portions of the immature elastin molecules may occur before the desmosines have formed, leaving a soluble, elastin-like protein with a high lysine content and no desmosines (Fig. 27E). A protein of this type has been reported in NaCl extracts of aortas from copper-deficient swine (Smith et al., 1968; Sandberg et al., 1969b).

Fundamental to the model is the concept of the structure of the proelastin molecules. In a recent study of the elastase-solubilized protein fragments from alkali-purified bovine ligamentum nuchae elastin, Keller et al. (1969) found small quantities of a protein having a markedly higher content of polar amino acids compared to undigested elastin. According to our model, elastase digests of younger material should be a much better source of these polar fragments.

In spite of its unconventional nature, we find the model attractive in that it allows an explanation of known but previously uncorrelated data from other laboratories as well as our own concerning the morphological and biochemical development of elastin. For this reason, it may prove to be a useful tool in the study of the biosynthesis of elastin.

VII. SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

(1). Dityrosine, the o,o'-biphenol analog of tyrosine, was identified in elastin from day-old chick aortas, and in an alkali-soluble, hydroxyproline-free protein (ALSP) from day-old chick aortas and adult bovine ligamentum nuchae. Its presence as a free amino acid in enzymic hydrolyzates of ALSP, and absence from acid hydrolyzates of several other proteins, some of which had been subjected to the strongly alkaline conditions used to extract ALSP, indicated that dityrosine in ALSP and elastin was not an artifact of isolation or hydrolysis procedures.

(2). ALSP from day-old chick aortas, like glycoproteins of similar amino acid composition extracted by others from a variety of connective tissues, appeared to exist in various states of aggregation. The dityrosine content of these aggregates was directly related to their size and insolubility. Introduction of additional dityrosine into ALSP resulted in an increase in insolubility and aggregation of the protein. Extensive enzymic hydrolysis of ALSP indicated that dityrosine was concentrated in the enzyme resistant portion of the protein. On the basis of this evidence, it was concluded that dityrosine was an interchain crosslink in ALSP, contributing to the aggregation of the protein. Other crosslinks may also be present in ALSP.

(3). Elastin purified from aortas of fetal and young chicks of various ages by hot alkali treatment had an amino acid composition considerably more polar than that of elastin from adult tissues. The younger the tissue, the greater the polarity of the elastin. The amino acid compositions of these alkali-purified young elastins was found to be consistent with that of a mixture of "mature" elastin and ALSP, the

proportion of ALSP in the mixture decreasing with increasing age. One explanation for the persistence of this polar contaminant in elastins from young tissues may be that it is involved in the biosynthesis of elastin and, as such, is insoluble at this time due to an interaction with the insoluble elastin matrix. A hypothesis is presented for the role of ALSP in the biosynthesis of elastin which accounts both for the present results, and those reported by others.

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