AN ACIDIC PROTEIN FROM UTERUS: CHARACTERIZATION AND RELATIONSHIP TO ELASTIN

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ABSTRACT

Alkali-purified elastin from uterus was found to be intimately associated with an acidic protein, similar in amino acid composition to protein fractions isolated from other tissues by several workers. Studies on the incorporation of glycine-C^{14} and tyrosine-H^3 into both soluble and insoluble forms of the acidic protein in rat uterus *in vivo* suggested that the acidic protein is easily extracted when newly synthesized and becomes progressively less soluble with time. The most highly aggregated form of the acidic protein is relatively insoluble and thus, is difficult to separate from elastin. Attempts to purify elastin from bovine uterus by treatment with hot alkali resulted in considerable solubilization of both elastin and acidic protein. Milder methods of extraction yielded polar protein residues unless hydrolytic procedures, such as 0.2N NaOH treatment or digestion with trypsin, were used. In contrast to the findings of other workers, extraction with mercaptoethanol-

urea failed to remove all acidic protein. The procedure of Timpl and coworkers [Biochim.Biophys.Acta 194, 112 (1969)] left a residue from cat uterus which contained considerable acidic protein.

The concentration of dityrosine, an o,o'-biphenol analogue of tyrosine, has been found previously in this laboratory to correlate with the degree of aggregation in alkali-soluble fractions of embryo chick aorta. The same correlation appears to exist in uterus, but dityrosine, by itself, cannot account for the aggregation of acidic protein to its insoluble form. The concentration of an unknown fluorescent compound (A/F:360/>415 nm) correlates well with the decreasing solubility of acidic protein in rat uterus, but both its identity and relationship to dityrosine are unknown.

Protein fragments were isolated by ion-exchange chromatography of an acid partial hydrolyzate of the alkali-insoluble protein from human uterus. Several fractions were obtained with amino acid compositions intermediate to those of elastin and acidic protein, suggesting a covalent linkage between the two protein species. The method of Sandberg and associates [Biochim.Biophys.Acta 236, 542 (1971)] was used in

an attempt to isolate a soluble precursor of elastin from uterus of pregnant cow. The crude material extracted was resolved into three components by gel filtration chromatography. One of these components had an amino acid composition intermediate to those of elastin and acidic protein and was shown to be homogeneous by polyacrylamide gel electrophoresis. In rat uterus, electron microscopic and biochemical evidence is presented for active fibrogenesis, particularly in pregnancy. Evidence was obtained for both aggregation and covalent bonding to elastin as mechanisms for the observed insolubility of a fraction of acidic protein in uterus. The relative importance of these processes is unknown. The functional significance of acidic protein is not well understood, but uterus contains an unusually high proportion of the protein in both soluble and insoluble forms.

To Darlene

and Ursula

and Mint

and Algernon

and Marble Angel

and Hatchet

and Red-eared Turt

and Big Turt

and Long-lost-Loch-Loach

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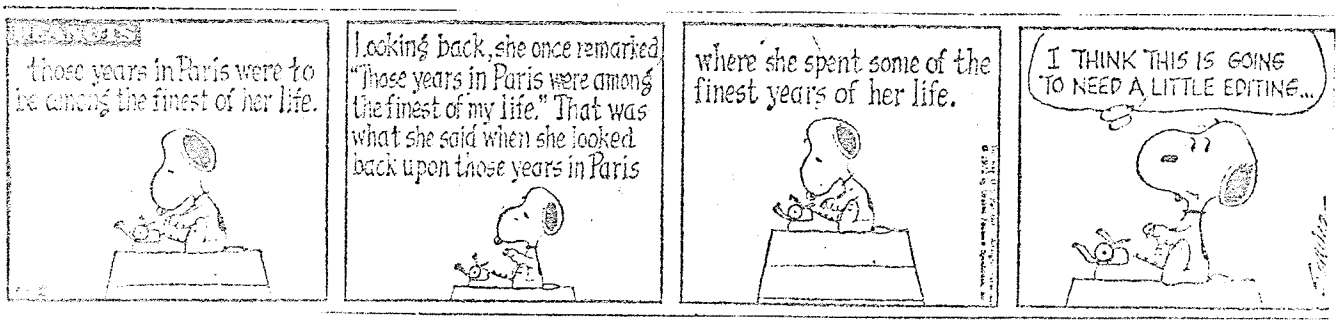

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LIST OF FIGURES

LIST OF TABLES

I. GENERAL INTRODUCTION

"It is sufficient to know, that all the Parts of the Body are made up of Threads, or Fibres, of which there be different Kinds; for there are some soft, flexible, and a little elastick; and these are either hollow, like small Pipes, or spongeous, and full of little Cells, as the nervous and fleshy Fibres; others there are more solid and flexible, but with a strong Elasticity or Spring, as the membranous and cartilaginous Fibres; and a third Sort are hard and inflexible, as the Fibres of the Bones. Now of all these, some are very sensible, and others are destitute of all Sense; some so very small as not to be easily perceived; and others, on the contrary, so big as to be plainly seen. And most of them, when examined with a Microscope, appear to be composed of still smaller Fibres.

Now these Fibres do first constitute the Substance of the Bones, Cartilages, Ligaments, Membranes, Nerves, Veins, Arteries and Muscles. And again, by the various Texture, and different Combination of some or all these Parts, the more compound Organs are framed; such as the Lungs, Stomach, Liver, Legs and Arms, the Sum of all which make up the Body."

from The Anatomy of the Human Body
by J. Keill (1698)
as quoted in Robb-Smith (1954)

ELASTIN AND ELASTIC FIBERS

Morphology

By light microscopy, elastin has been recognized for a long time as being a yellow, fibrous protein embedded in an amorphous, intercellular matrix. The staining reactions available for the histological study of elastin have been examined extensively and good reviews of the subject are available (Dempsey and Lansing, 1954; Hall, 1959; Partridge, 1962; Ayer, 1964; Gillman et al., 1955). Because of the lack of ionic side chains in elastin, many of the staining reactions are probably due to hydrogen bonding (Weiss, 1954; Gillman et al., 1954), or to staining of some other structures such as carbohydrate (many workers, including Gillman et al., 1955; Hall, 1957; Walford et al., 1961; Albert and Fleischer, 1970), or lipid (Lansing et al., 1952; Lansing, 1955; LaBella, 1957, 1958). If this is so, elastin stains would be somewhat non-specific, and tissue differences might be expected to produce variations in the staining reactions. Such variations are, in fact, seen in different tissues of the body (e.g. Gillman et al., 1957), in edematous connective tissues (Ayer, 1964), after chemical or enzymatic procedures which damage the elastic fiber or associated structures (Fullmer and Lillie, 1957; Gillman et al., 1955, 1957, Saxl,

1957) and in other situations of rapid growth and metabolism of elastin, such as in uterus during pregnancy (Iversen and Christensen, 1963) and postpartum involution (Maher, 1959), or in fetal elastic tissue (Jensen and Bertelsen, 1961; Wirtschafter et al., 1967). That elastic tissue is closely associated with mucopolysaccharide has been noted by many workers (e.g. Wislocki et al., 1947; Taylor, 1953; Bertelsen and Jensen, 1960; Jensen and Bertelsen, 1961; Paule, 1963; Yu and Lai, 1970). Although Paule (1963) suggested that mucopolysaccharides were necessary for the formation of the elastic fiber, the mechanism by which this could occur is not known.

The elastic tissues of the body have several kinds of arrangements from laceworks of fine, branching fibrils in the elastic cartilage of the external ear and epiglottis, to fenestrated sheets in the walls of blood vessels and bronchiolar tree, to thick, ribbon-like fibers in ligaments (Partridge, 1962; Hall, 1971). These different morphologic forms of elastin appear to differ in the closeness of their association with the collagen and ground substance which are present, but it is still debatable whether there are differences in chemical constitution of elastin at different

anatomical sites (Partridge, 1962).

Biochemistry

Elastin is a scleroprotein and is insoluble under any conditions which do not hydrolyze peptide bonds. "Purification" of elastin thus depends upon the removal of all other proteins. Because of the high elastin content of ligamentum nuchae and the relative ease with which the other materials can be removed, this tissue has provided the standard of elastin composition. The amino acid composition of elastin is unusual and characteristic; two-thirds of the total residues are proline, glycine and alanine, while polar residues contribute very little to the composition. Elastin contains no histidine or cysteine, and only traces of tryptophan and hydroxylysine. Hydroxyproline is a constituent of elastin (Bentley and Hanson, 1969; Partridge, 1970), although its actual contribution to the overall composition may depend on random hydroxylation of proline (Sandberg et al., 1971b). The mucopolysaccharide content of elastin is very low (Partridge et al., 1955; Loeven, 1960; Czerkawski, 1962) and it may contain some lipid material (Lansing et al., 1952; LaBella, 1957, 1958; Loomeijer, 1958, 1961; etc.), but the importance of these components is difficult to assess.

In the case of elastin, the amino acid composition of the 'pure' protein is intimately connected with the purification procedure. Autoclaving in water (Lowry et al., 1941; Neuman and Logan, 1950; Partridge et al., 1955; Partridge, 1962) has been used to remove insoluble collagen and other proteins from the elastin of bovine ligamentum nuchae. However, in other tissues, this procedure does not always remove all contaminating proteins. Treatment with hot, dilute alkali for 45 minutes was used by Lansing and coworkers (1951) in equine ligamentum nuchae, and by various workers in other tissues as well (Partridge, 1962; FitzPatrick and Hospelhorn, 1962; Gotte et al., 1963a; Anwar, 1966, LaBella and Vivian, 1967). In bovine aorta and ear cartilage, for example, these methods were compared with the result from bovine ligamentum nuchae (Gotte et al., 1963a). Although either method gave typical elastin composition in ligamentum nuchae, autoclaved elastin was always more polar than alkali-treated elastin in the other tissues. Prolonged extraction in dilute salt solutions (to remove collagen and ground substance) after digestion with proteolytic enzymes has been used by various workers (FitzPatrick and Hospelhorn, 1960; Hospelhorn and FitzPatrick, 1961; Czerkawski, 1962; Steven, 1964; Fitz-

Patrick and Hospelhorn, 1965a; Steven and Jackson, 1968). Again, although the same elastin composition was obtained from ligamentum nuchae no matter which procedure was used, in lung or aorta, digestion with proteolytic enzymes yielded a residue with a composition more polar than that typical of elastin. This could be attributed to the presence of a non-elastin, non-collagen protein which is more closely associated with elastin in these tissues than in ligamentum nuchae. The existance of such a matrix material in association with elastin had been inferred earlier on the basis of microscopical studies on enzyme-digested connective tissue (Day, 1947; Gross, 1949; Hall et al., 1952; Lansing et al., 1952; Hall et al., 1955). In most cases, this matrix material can be removed from the enzyme-resistant residues by treatment with hot, dilute alkali (Gotte et al., 1963b; Clearly et al., 1966; Steven and Jackson, 1968). The only exception known in mature elastic tissue is the elastic cartilage of the external ear, where both elastin and the contaminating protein are solubilized by hot alkali (Gotte et al., 1963a; Anwar, 1966). Many workers, however, have reported difficulty in removing all of the polar protein associated with elastin in fetal and old elastic tissues.

Elastin from fetal ligamentum nuchae, unlike the mature form, cannot be purified adequately by autoclaving in water. Hot alkali extraction is required (Cleary et al., 1966, 1967). Even after this treatment, some non-collagenous, polar contaminant remains in most fetal tissues (Cleary et al., 1967; Keeley, 1970; Keeley and LaBella, 1972). Good agreement with the composition of elastin from ligamentum nuchae could be obtained by digestion with α-amylase prior to hot alkali extraction in fetal bovine aorta and ligamentum nuchae (Steven and Jackson, 1968). Elastin from young animals has also been reported to be quite susceptible to solubilization in hot alkali (LaBella et al., 1966; Cleary et al., 1967). The possible significance of the polar protein in fetal elastic tissue will be discussed later.

Several workers have also noted an increased acidic character in elastin from old tissues (Lansing et al., 1951; Hall, 1955; Lansing, 1955; FitzPatrick and Hospelhorn, 1965b; Gotte et al., 1965; LaBella et al., 1966; Hall, 1968) and have interpreted this as indicating the presence of another protein ('pseudoelastin' (Hall, 1968, 1971)) intimately associated with aging elastin. Since mature elastin is unreactive metabolically (Slack, 1954; Kao et al., 1961a, 1961b;

Walford et al., 1964), it is subject to chemical changes which in other proteins would be ameliorated by normal metabolic turnover. Thus, with age, elastin probably becomes increasingly crosslinked (Bjorksten, 1968), both within itself (LaBella and Lindsay, 1963; LaBella and Thornhill, 1965; LaBella, 1971) and to other molecules (Hall, 1955; Carpenter, 1965; LaBella et al., 1966; Carpenter, Carpenter and Loynd, 1967; Carpenter, 1969). The resulting accretion complexes (LaBella, 1972) would provide a nucleus for further deposition of material and eventually result in masking of the original physical and chemical characteristics of the elastin molecule.

Biosynthesis

It is now generally accepted that fibroblasts (Branwood, 1963; Ross, 1968) or smooth muscle cells are responsible for the synthesis of elastin (Karrer, 1960; Karrer and Cox, 1961; Haust et al., 1965; Greenlee et al., 1966, Ross and Klebanoff, 1967; Hashimoto and DiBella, 1967; Takagi and Kawase, 1967; Ross, 1968; Takagi, 1969a; Ross, 1971; Ross and Klebanoff, 1971) as well as collagen (Gross et al., 1955; Branwood, 1963; Ross and Benditt, 1965; Ross, 1968) and probably, ground substance (Gersh and Catchpole, 1949; Robb-Smith, 1954; Kennedy, 1960; Branwood, 1963; Ross, 1968).

The biogenesis of elastin has been studied microscopically in rapidly growing tissues such as: aorta of newborn rat (Paule, 1963), fetal and newborn human (Jensen and Bertelsen, 1961; Haust et al., 1965), chick embryo (Karrer, 1960; Takagi and Kawase, 1967; Basom, 1968; Takagi, 1969a; Kadar et al., 1971), and mouse fetus (Fyfe et al., 1968); ligamentum nuchae of calf fetus (Fahrenbach et al., 1966; Greenlee et al., 1966; Wirtschafter et al., 1967); flexor digital tendon of fetal, newborn and weanling rats (Greenlee et al., 1966; Greenlee and Ross, 1967); healing wounds (Kadar et al., 1969; Williams, 1970a, 1970b).

Common to all electron-microscopic descriptions of elastin fibrogenesis is the deposition in the extracellular space of fine fibrils about 100Å in diameter. These have been termed 'microfibrils' (Low, 1962; Haust, 1965), and are distinct from other fibrous elements of connective tissue. They can be distinguished from the fine, non-banded fibrils associated with early collagen formation (Karrer, 1960; Chapman, 1961; Peach et al., 1961; Ross and Benditt, 1961; Fernando and Movat, 1963; Ross and Benditt, 1964) by the fact that the microfibrils appear to be tubular in cross-section with an electron-lucent core (Low, 1962; Fahrenbach

et al., 1966; Takagi and Kawase, 1967; Takagi, 1969a) and occasionally appear beaded or vesicular in longitudinal section (Haust, 1965; Fahrenbach et al., 1966, Greenlee et al., 1966; Haust and More, 1967; Takagi, 1969a). The microfibrils can be distinguished from the other constituents of the extracellular matrix by their reactions with heavy metal stains (Greenlee et al., 1966; Takagi and Kawase, 1967). They stain deeply with uranyl acetate and/or lead citrate, but only weakly, if at all, with phosphotungstic acid; elastin stains well with phosphotungstic acid but not with uranyl acetate or lead citrate, and collagen stains well with both phosphotungstic acid and uranyl acetate but not with lead citrate.

After the deposition of the microfibrils in the extracellular space, an amorphous core with the staining properties of elastin appears within the microfibrillar mass (Haust et al., 1965; Fahrenbach et al., 1966; Greenlee et al., 1966; Hashimoto and DiBella, 1967). As the elastic tissue matures, this amorphous core grows in size until, in the mature tissue, only a sparse mantle of microfibrils remains around the periphery (Fahrenbach et al., 1966; Greenlee et al., 1966; Hashimoto and DiBella, 1967; Greenlee and Ross, 1967; Haust

and More, 1967; Ross and Bornstein, 1969). It has been suggested that these microfibrils play a role in the orientation and orginization of the developing elastin fiber, either as an integral part of the soluble elastin precursor (Jensen, 1962; Bierring and Kobayasi, 1963; Takagi, 1969a; Keeley, 1970; LaBella, 1971) or as a nucleation center for the laying down of the elastin fibrils (Fahrenbach et al., 1966; Greenlee et al., 1966; Ross and Bornstein, 1969, 1970, 1971). Recent biochemical studies have shown that microfibrils can be extracted from ligamentum nuchae by dithioerythritol in 5M guanidine (Ross and Bornstein, 1969), mercaptoethanol in 8M urea (Robert et al., 1971), or by treatment with trypsin or chymotrypsin (Ross and Bornstein, 1969; Robert et al., 1971; cf. Bodley and Wood, 1972). The protein isolated contains no hydroxyproline and has considerable acidic character (Ross and Bornstein, 1969; Takagi, 1969b; Robert et al., 1971). That microfibrils contain carbohydrate as well has been suggested by various workers (O'Connell and Low, 1970; Yu and Lai, 1970; Robert et al., 1971). The composition of the microfibrillar protein is compared to a number of ground substance proteins in Table I (p. 18).

O'Dell and coworkers (1961) reported that young chicks,

raised on a copper-deficient diet, develop vascular lesions of the major arteries, the most conspicuous of which is a defect in elastic membranes. It is now known that the amine oxidase responsible for the first step in the formation of the desmosine crosslinks in elastin is copper-dependent (Miller et al., 1965; Kim and Hill, 1966; Chou et al., 1968). The role of copper in connective tissue biochemistry has been reviewed by Carnes (1971). The elastin isolated from copper-deficient aortas thus shows an increased content of lysine and no desmosines (Starcher et al., 1964; Miller et al., 1965; O'Dell et al., 1966). Inhibition of crosslinking permitted the isolation of a soluble precursor of elastin from these tissues (Smith et al., 1968; Sandberg et al., 1969a, 1969b, 1971b). Sandberg and coworkers have called this protein "tropoelastin" and have begun to characterize it and determine its amino acid sequence (Sandberg et al., 1969b, 1971a). Microfibrils, probably identical to the microfibrils seen in developing elastic tissue, are seen in greater quantity in copper-deficient aortas (Waisman and Carnes, 1967; Waisman et al., 1969). There is also a large increase in the content of a non-collagen, non-elastin protein extracted from copper-deficient aortas in alkali (Waisman and Carnes, 1967).

Keeley (Keeley, 1970; LaBella, 1971; Keeley and LaBella, 1972), based on studies of elastin in the aortas of young chicks, suggested that elastin was synthesized as a more polar precursor, analogous to procollagen (Speakman, 1971). He was also able to explain the observations in copper-deficient tissue on this basis (Keeley, 1970; LaBella, 1971).

GROUND SUBSTANCE

The term "ground substance" is a transliteration of the German term "Grundsubstanz" which actually means "fundamental substance". This originally conveyed the belief held by microscopists in the latter half of the nineteenth century, that all cells were derived from this common pool of amorphous building material (Klemperer, 1953/54; Jackson and Bentley, 1968).

It is now recognized that the ground substance is not a substance in the chemical sense, but rather a complex mixture of many components, the characteristics of which are different in different tissues. Moreover, the exact nature of the interactions among the components and the physiological significance these interactions are not fully understood. All we can do for the present is to identify some of the components and suggest possible ways in which they can effect the structural integrity of the tissue (Gersh and Catchpole, 1960; Jackson and Bentley, 1968; Schubert and Hamerman, 1968).

Constituents

The major, and best-defined of the constituents of ground substance are the acid mucopolysaccharides (Gersh and Catchpole, 1949; Meyer et al., 1956). The extensive research

into the nature of these components, their metabolism and function in connective tissue have been the subjects of many reviews (Meyer, 1945; Meyer et al., 1956, 1957; Dorfman, 1962; Hoffman and Meyer, 1962; Dorfman, 1964; Brimacombe and Webber, 1964; Muir, 1964; Schiller, 1966; Mathews, 1967; Spicer et al., 1967; Meyer, 1970). The proteoglycans (protein-polysaccharide complexes) of cartilage, particularly, have been studied in detail (Shatton and Schubert, 1954; Muir, 1957; Mathews and Lozaityte, 1958; Partridge et al., 1961; Eyring and Yang, 1968; Serafini-Fracassini, 1968; Campo et al., 1969; Sajdera and Hascall, 1969; Kao et al., 1970; Rosenberg et al., 1970; Hascall and Sajdera, 1970; Mashburn and Hoffman, 1971). It is believed that these large complex protein-polysaccharides may play a role in the macromolecular structure and organization of collagen fibers in various tissues (Partridge, 1948; Weiss, 1956; Mathews, 1965; Disalvo and Schubert, 1966; Toole and Lowther, 1967; Fitton Jackson, 1968; Jackson and Bentley, 1968; Farrell and Hart, 1969; Nadol et al., 1969; Myers et al., 1969; Steven et al., 1969; Nemeth-Csoka, 1970; Pease and Bouteille, 1971).

Although it has been recognized for some time that connective tissues also contain glycoproteins which are distinct from the mucopolysaccharides (Meyer, 1938; Dische

et al., 1958), they are much less well characterized (for reviews on glycoproteins see: Dische et al., 1958; Spiro, 1963; Gottschalk, 1966; Schmid, 1968; Spiro, 1970). Some relatively soluble glycoproteins have been implicated in the aggregation of collagen fibrils and the proteoglycan complexes at the molecular level (Bowes et al., 1955, 1956; Mathews and Lozaityte, 1958; Fishkin and Berenson, 1961; Moczar et al., 1967; Hascall and Sajdera, 1969; Katzman and Jeanloz, 1970; Robert et al., 1970; Rajamaki and Kulonen, 1971). Less soluble glycoproteins have been isolated from many sources, such as ligamentum nuchae (Gotte et al., 1963b; Cleary et al., 1967; Keeley et al., 1969; Ross and Bornstein, 1969; Robert et al., 1971), aorta (Barnes, 1965; Barnes and Partridge, 1968; Robert and Comte, 1968; Keeley et al., 1969; Takagi, 1969b; Moczar and Robert, 1970), skin (Bowes et al., 1956; Timpl et al., 1968, 1969; Wolff et al., 1971), cartilage (Anderson, 1961; Timpl et al., 1968, 1969; Kao et al., 1971; Wolff et al., 1971), intervertebral disc (Steven et al., 1968; Pearson et al., 1969), lens (Lerman, 1969), amyloid fibrils (Cohen, 1966), and basement membrane (Kefalides, 1971). There is a remarkable similarity in amino acid composition among all these proteins isolated by various

different methods (some are given in Table I). Recent workers have attributed a structural function to these proteins (Timpl et al., 1968, 1969; Robert et al., 1965; Moczar and Robert, 1970). Some workers (Partridge, 1962; Keeley, 1970; Partridge, 1970; LaBella, 1971; Keeley and LaBella, 1972) have attributed the polar character of fetal elastin to an intimate relationship between elastin and such ground substance proteins.

TABLE I

AMINO ACID COMPOSITIONS OF GLYCOPROTEINS FROM VARIOUS TISSUES
(residues/1000 total amino acid residues)

	CORE PROTEIN OF PROTEO-GLYCAN	UREA-SOLUBLE GLYCO-PROTEIN[2]	UREA-SOLUBLE ACIDIC PROTEIN[3]	COLD ALKALI-SOLUBLE ACIDIC PROTEIN[3]	HOT ALKALI-SOLUBLE ACIDIC PROTEIN[4]	MICRO-FIBRILLAR PROTEIN[5]	GLYCOPROTEIN FROM AMYLOID FIBRILS[6]
HPRO	0	0	0	0	0	0	0
ASP	109	93	93	97	103	114	95
THR	65	59	51	54	37	56	43
SER	97	57	55	55	60	62	64
GLU	111	111	156	160	125	114	108
PRO	78	64	41	35	61	64	54
GLY	125	101	83	66	115	110	100
ALA	72	90	89	88	85	65	88
VAL	53	78	55	55	69	56	57
CYS/2	n.r.	25	28	4	n.r.	48	19
MET	19	tr	29	31	12	16	23
ILE	38	46	55	62	43	48	41
LEU	73	84	98	100	93	67	81
TYR	31	33	29	34	29	36	35
PHE	28	35	34	42	39	38	45
LYS	41	60	58	53	54	45	59
HIS	16	22	16	16	16	15	19
ARG	33	45	31	31	40	45	59

n.r. = not reported tr = trace amounts

1 human costal cartilage (Steven et al.,1969)
2 equine aorta (Moczar and Robert, 1970)
3 rat skin (Timpl et al., 1969)
4 chick aorta (Keeley, 1970)
5 bovine ligamentum nuchae (Ross and Bornstein, 1969)
6 Cohen (1966)

CONNECTIVE TISSUE OF THE UTERUS

"There is no organ in the human body which undergoes such a veritable storm of tissue change as does the uterus after pregnancy. As a direct consequence, it is equally true that there is perhaps no organ in the body which affords such opportunity for the study of tissue change."

J.R. Goodall (1909)

Morphology

The body of the uterus in most animals is bicornuate, that is, it is divided into two horn-like projections just above the cervix; in man it is a single chamber. Each horn is attached to the wall of the abdomen by a sheet of connective tissue which houses the nerve and blood supplies to the uterus. The wall of the uterus has three layers (Bloom and Fawcett, 1962): the outer, serous membrane of the peritoneum; the middle, smooth muscle layer (myometrium); and the inner, mucous membrane layer (endometrium). The inner two are the major layers and are now considered in more detail.

The myometrium consists of zones of smooth muscle bundles running in different directions and separated by connective tissue layers containing isolated smooth muscle cells.

These zones are not sharply defined in human uterus, since the muscle bundles run between zones as well. The myometrium contains considerable collagen and elastin between the muscle bundles, and elastin in the walls of the blood vessels that permeate this layer.

The endometrium consists of a basement membrane, a single layer of columnar epithelium, and a zone of variable thickness containing the uterine glands and a rich arterial supply and capillary network embedded in connective tissue material (stroma). Elastin appears to be absent, except in the walls of the arterioles.

Connective tissue (including fibroblasts, mast cells etc.) accounts for about 50% of the total volume of the uterine wall (Harkness and Harkness, 1959a; Harkness, 1964).

Collagen

Biochemical estimates of the amount of collagen in the body of the uterus have given values of 2.8% (Harkness and Harkness, 1954) or 3.8% (Needham and Cawkwell, 1957) of wet weight and 30% of total protein (Montfort and Perez-Tamayo, 1961) in the rat at estrus; 2.2% of wet weight, 10.8% of dry weight (Cretius, 1959, as quoted by Harkness, 1964), and 37% (Kao et al., 1967) or 38% (Montfort and Perez-Tamayo, 1961)

of total protein in non-pregnant human. Although there is some disagreement as to whether there is a significant variation in total collagen content of the rat uterus during the estrus cycle (Harkness et al., 1957; cf. Morgan, 1963b; Smith and Kaltreider, 1963), it is agreed that collagen concentration is lowest in estrus due to the large increase in wet weight of the uterus at this time. Although collagen represents a large proportion of the total protein in uterus, only about 5% of total collagen is in a salt- or acid-soluble form (Kao et al., 1967). Nevertheless, even the insoluble collagen of uterus turns over rapidly (Kao et al., 1961a, 1961b).

To a large extent, interest in the uterus has centered on the extensive changes which occur during pregnancy and post-partum involution, or under the influence of hormones administered exogenously. In pregnancy, or under the influence of estrogen, there is considerable enlargement of rough endoplasmic reticulum and the Golgi apparatus of smooth muscle cells (Mark, 1956; Shoenberg, 1958; Laguens and Lagrutta, 1964; Ross and Klebanoff, 1967; Silva, 1967; Bergman, 1968; Bo et al., 1968; Dessouky, 1968; Friederici and DeCloux, 1968), suggesting active synthesis and secretion. There is also a gradual increase in the number of specialized junctional

complexes between smooth muscle cells (Laguens and Lagrutta, 1964; Ross and Klebanoff, 1967; Silva, 1967; Bergman, 1968; Althoff and Albert, 1970) which could facilitate unification of uterine contraction during parturition.

Collagen in uterus undergoes a 6- to 8-fold increase during pregnancy (Harkness and Harkness, 1954, 1956; Maibenco, 1960; Montfort and Perez-Tamayo, 1961; Morrione and Seifter, 1962; Woessner and Brewer, 1963) but this is probably accompanied by considerable remodelling, since dense collagen bundles appear to be replaced by argyrophilic fibrils which are characteristic of new collagen (Bassett, 1958; Fainstat, 1963, 1964, 1965), and there is an increase of hydroxyproline in the urine during pregnancy (Klein, 1964; Klein and Yen, 1970). However, this new collagen does not appear to be unusually soluble (Morrione and Seifter, 1962; Woessner, 1962; Woessner and Brewer, 1963; Paavola et al., 1964). The doubling of collagen content in the first half of pregnancy is likely hormonally controlled, since the empty horn of a unilaterally ovarectomized uterus (Harkness and Harkness, 1956; Harkness and Moralee, 1956; Cullen and Harkness, 1964) or the uterus treated with estrogen or estrogen and relaxin (Cullen and Harkness, 1960; Morgan, 1963a; Smith and Kaltreider, 1963;

Kao et al., 1964a, 1964b; Wood, 1964; Friederici and DeCloux, 1968) will exhibit this increase. The largest increase, however, occurs in the second half of pregnancy and this appears to be related to the volume of the horn occupied by the fetuses (Harkness and Harkness, 1954, 1956; Cullen and Harkness, 1959; Csapo et al., 1965; Takacs and Verzar, 1968).

Loss of collagen from the uterus during post-partum involution has been demonstrated both microscopically (Craig, 1956; Maibenco, 1960; Montfort and Perez-Tamayo, 1961; Fainstat, 1963, 1964; Graves et al., 1967) and biochemically in rat (Harkness and Harkness, 1954; Harkness and Moralee, 1956; Montfort and Perez-Tamayo, 1961; Woessner, 1962; Grant, 1965) and human (Montfort and Perez-Tamayo, 1961; Morrione and Seifter, 1962; Woessner and Brewer, 1963) uterus. The kinetics of collagen loss from uterus during post-partum involution were examined in detail by Woessner (Woessner, 1962; Woessner and Brewer, 1963; Woessner, 1965a, 1968), who proposed a pathway for the degradation of collagen (Woessner, 1962) which involved initial solubilization of collagen fibers, followed by thermal denaturation and degradation by proteolytic enzymes. Early attempts to isolate collagenase from the involuting uterus were unsuccessful (Woessner, 1962; Morrione and Seifter,

1962; Schaub, 1963; Woessner, 1965a) and attention was turned to an acid catheptic activity which could be demonstrated in uterus homogenates (Woessner and Brewer, 1960; Woessner, 1962; Woessner and Brewer, 1963; Schaub, 1964; Woessner, 1965a, 1965b, 1965c). Recently, this cathepsin has been purified and characterized (Woessner and Shamberger, 1971) as a cathepsin D, known to be a lysosomal enzyme in other tissues (de Duve et al., 1962). Collagen has been detected inside phagocytic cells (Luse and Hutton, 1964; Brandes and Anton, 1969; Parakkal, 1969) which are prominent in the uterus during post-partum involution (Deno, 1936; Dawson, 1946; Lobel and Deane, 1962; Morrione and Seifter, 1962; Goodall, 1965; Anton et al., 1969; Brandes and Anton, 1969; Parakkal, 1969). Recently (Jeffrey and Gross, 1970), collagenase has been identified both in culture medium in which slices of involuting post-partum rat uterus were incubated (Jeffrey and Gross, 1970) and in homogenates of involuting post-partum rat uterus (Ryan and Woessner, 1971). Jeffrey and coworkers have correlated this collagenase activity with the normal degradation of collagen in post-partum uterus (Jeffrey et al., 1971a) and have shown that progesterone inhibits this activity (Jeffrey et al., 1971b), in agreement with the known ability of progesterone to

reduce involution in the post-partum uterus (Goodall, 1966) if given in the first 24 hours after parturition.

There is some disagreement as to whether age and parity effect the collagen content of the uterus (Woessner, 1963; Woessner and Brewer, 1963; cf. Dilts and Greene, 1964). The collagen of uterus is unusual in that it has a low shrinkage temperature, does not fluoresce until after menopause (Brown et al., 1958) and has a high rate of turnover (Kao et al., 1961a, 1961b).

Elastin

In contrast to collagen, relatively little is known about elastin in uterus. Histological studies have shown that elastin is present in the walls of uterine arteries (Goodall, 1909) and in networks in the myometrium (Hunt and Evans, 1946). During pregnancy, elastin becomes more abundant (Goodall, 1909; Hunt and Evans, 1946; Maibenco, 1960), although some workers have had difficulty finding the stretched elastic fibers in the hypertrophied tissue (Hunt and Evans, 1946; Iversen and Christensen, 1963; Albert and Pease, 1968). Woessner and Brewer (1963), in the only biochemical study to date on the elastin of uterus, reported an elastin content (as non-collagen hydroxyproline) in nulliparous human uterus

of about 0.3g/uterus or 0.7% of wet weight of uterus (cf. 3.2g/uterus or 7.5% of wet weight, for collagen). During pregnancy the elastin content of the uterus increases 5- to 6-fold. Loss of elastin from uterus during post-partum involution was essentially complete at 11 days. During involution, uterine elastica exhibits anomalous histological properties (Maher, 1959). Although the mechanism of rapid resorption of elastin in involution is not known, there is some evidence from other tissues that suggests that elastin, like collagen, could be phagocytized (Gilfillan et al., 1962; Gilfillan, 1968; Papajiannis et al., 1970).

Multiple pregnancies leave the uterus with more elastin than in an age-matched, nulliparous uterus (Goodall, 1909; Dawson, 1959; Woessner and Brewer, 1963; Dilts and Greene, 1964; Albert and Bhussry, 1967). Up to age thirty, elastin content of the uterus probably also correlates with age in uteri matched for parity (Woessner, 1963; Albert and Bhussry, 1967; cf. Dilts and Greene, 1964).

Mischler and Welaj (1971) have examined the effect of the hormonal conditions prevailing during pregnancy on the stress/strain characteristics of the collagen-elastin framework of the rat uterus.

Ground Substance

Almost all our knowledge about ground substance in uterus is derived from histological studies. The mucopolysaccharides of ground substance have been identified by their metachromatic staining with toluidine blue (Bensley, 1934; Wislocki et al., 1947; Wislocki and Dempsey, 1948; McKay, 1950) and by various other stains (e.g. Hale, 1946), and the glycoproteins by staining with periodic acid-leucofuchsin (Schiff's reagent) (Hotchkiss, 1948; Leblond et al., 1957). The mucopolysaccharides of ground substance have been shown to contribute markedly to the increase in bulk of the uterine wall during pregnancy (Wislocki and Dempsey, 1948; Craig, 1956; Bassett, 1958; Mailbenco, 1960; Iversen and Christensen, 1963) or after estrogen treatment (Leppi and Kinnison, 1971). In post-partum involution, ground substance is lost rapidly and in great quantity (Craig, 1956; Maher, 1959; Maibenco, 1960). Maibenco (1960) suggested that modifications in ground substance are part of a 'softening' mechanism in the uterus for adapting to the increase in intrauterine pressure resulting from growth of the fetus.

It can be shown biochemically, that hexosamine is greatly increased in uterus during pregnancy and undergoes a

rapid and extensive reduction during post-partum involution (Montfort and Perez-Tamayo, 1961; Grant, 1965).

The glycoproteins of ground substance have not been studied in uterus. In cervix, Harkness and Harkness (1959b) noted that the patency of the collagenous framework depended more on the presence of some unknown, trypsin-susceptible, protein component than on mucopolysaccharide, and related this unknown protein to the 'cementing substance' of Day (1947). The nature of this substance has not been studied further in uterus. Recently (Groschel-Stewart and Hermann, 1971), two structural proteins have been isolated from uterus by the method of Timpl and associates (1969). Their amino acid compositions are stated to be similar to that of human amyloid protein (see Table I).

TERMINOLOGY

It is perhaps an indication of the uncertainty prevalent in the field of connective tissue biochemistry that there are several names in use in the literature which may refer to the same group of proteins. Timpl and coworkers use the term 'acidic structural proteins' to represent a class of proteins extracted from skin and placenta by the use of 8M urea and 0.2N NaOH (Timpl et al., 1969). 'Structural glycoproteins' was used for proteins extracted from aorta with 8M urea (Robert et al., 1965; Moczar and Robert, 1970). Keeley and coworkers (1969) used the term 'alkali-soluble protein' (ALSP) to refer to a protein fraction extracted from chick aorta and bovine ligament with hot, dilute alkali. All of the proteins mentioned have approximately the same amino acid composition and may represent simply different fractions of the same large pool of protein. In this thesis, the term 'alkali-soluble protein' (ALSP), will be used when an alkali extract is being referred to. It was felt, however, that a more general term should be used to denote the class of proteins to which ALSP belongs. This is necessary because (a) proteins of similar composition can be isolated by much milder procedures than extraction in hot, alkali, and (b) proteins of similar com-

position remain insoluble after hot alkali extraction in uterus. This general class of proteins will be referred to in this thesis as 'acidic protein(s)'.

II. STATEMENT OF THE PROBLEM

Uterus is the only tissue in the normal, mature animal where insoluble collagen has a high rate of turnover. Although the chemistry and metabolism of collagen from uterus has been studied in detail, there are few biochemical studies in uterus on elastin and the proteins of ground substance.

Relatively insoluble, acidic proteins, apparently derived, at least in part, from the ground substance, have been isolated from all tissues examined, but their importance to the structure and integrity of the connective tissue matrix is not well understood. There is some evidence, especially in fetal elastic tissues, that an intimate relationship exists between elastin and the acidic proteins of ground substance.

In preliminary experiments of a study designed to examine the turnover of elastin in pregnant and postpartum uterus, an apparent complex of elastin and an acidic protein was isolated from uterus of non-pregnant rat. This complex from uterus was similar to those reported to be present in fetal tissues, suggesting that non-pregnant uterus also, might be active in elastin synthesis and metabolism.

The purpose of this study was to isolate and charac-

terize the acidic protein in the ground substance of uterus, and to examine its possible relationship to elastin.

III. ISOLATION AND CHARACTERIZATION OF AN ACIDIC PROTEIN FROM UTERUS

INTRODUCTION

Elastin is defined as the protein residue remaining after exhaustive extraction of tissues according to well-defined procedures (Lansing et al., 1952; Partridge et al., 1955) and is characterized by an unusually high proportion of non-polar amino acids. To date, only one biochemical study on the elastin content of the uterus has been done. Woessner and Brewer (1963) estimated elastin content by determining hydroxyproline in a residue assumed to be collagen-free; no further purification or analysis was reported.

In the first part of this section, the preliminary observations that formed the basis for the rest of this study are reported. Initial experiments were carried out on rat uterus, and later studies were extended to other species.

The alkali-soluble protein isolated from rat uterus had an amino acid composition very similar to that of various proteins isolated by several workers from various tissues (Partridge and Elsden, 1961; Gotte et al., 1963b; Barnes and Partridge, 1968; Robert and Comte, 1968; Keeley et al., 1969; Timpl et al., 1969; Moczar and Robert,

1970). The alkali-insoluble residue from rat uterus had almost the same amino acid composition as that of the alkali-extracted material. Elastins isolated in certain circumstances have been observed to have a more polar character than conventional mature elastin (Gotte et al., 1963a; Farrar et al., 1965; FitzPatrick and Hospelhorn, 1965a, 1965b; Anwar, 1966; Cleary et al., 1966, 1967; Steven and Jackson, 1968; Keeley, 1970, Keeley and LaBella, 1972), though none exhibits as much polar character as elastin from uterus.

Evidence is presented below that the recovery of a very polar residue in uterus is not peculiar to our extraction procedure, and that a family of acidic proteins exists in various stages of aggregation, resulting in protein fractions of different solubilities with very similar amino acid compositions. The concentrations of certain fluorescent compounds in these various acidic protein fractions were examined.

METHODS

Preparation of Acidic Protein Fractions by Extraction with Hot Alkali

Rat, cat and dog uteri. Rats of the Long-Evans strain, 80-100 days old were used in the preliminary experiments. Subsequent experiments employed virgin rats of the Sprague-Dawley strain, 110 days old. Pregnant rats (Long-Evans) were estimated to be within one day of parturition, based on the date of mating. Cats and a dog of unknown age and parity were also used. The expected time to parturition in pregnant cats was unknown. Rats were killed by decapitation, and cats and the dog by thiopental overdosage. Uteri were removed, minced with scissors, and homogenized in a VirTis '45' blender (VirTis Research Co.). Fat was extracted with chloroform:methanol (3:1 by volume) for three hours at room temperature. Collagen was removed by multiple 12- or 24-hour extractions with distilled water in an autoclave at 110°C. In the final studies, from which amino acid analyses are reported, three 24-hour extractions were used. The insoluble material at this stage is referred to as 'collagen-free residue'.

Bovine and human uteri. Uteri from cows killed at a local abbatoir were transported in ice buckets to our labora-

tory. A uterus from a 47-year old woman of unknown parity was obtained at autopsy. The uteri were freed of fat and adventitia and sliced into thin strips about one inch long. The strips were placed in a flask of distilled water and heated to 110°C in an autoclave for 30 minutes to disrupt the tissue. The mixture was filtered, washed with distilled water, dehydrated in acetone and dried in air. The dried tissue pieces were mixed with dry ice powder and pulverized in a Weber Pulverizing Mill (Cole-Parmer Instrument and Equipment Co.) to 0.05 inch mesh. Lipid was removed with chloroform:methanol (3:1 by volume) for three hours at room temperature. Collagen was extracted by four 3-hour treatments with distilled water at 110°C in an autoclave.

Extraction with hot alkali. The collagen-free residue was suspended at a concentration of 1% in 0.1N NaOH at 95°C. Extraction was carried out in a boiling water bath for three (rat, cat), four (cow) or six (dog, human) 10-minute periods. After each 10-minute extraction, the residue was separated by centrifugation (rat, cat, dog) in a Model CL International Clinical Centrifuge or by filtration (bovine, human). The supernatants were dialyzed against distilled water and lyophilized. These successive alkali-soluble

protein (ALSP) fractions were designated ALSP-1 through ALSP-6. The alkali-insoluble protein (AIP) residue from the last extraction was washed in distilled water, dialyzed against distilled water, and lyophilized. In some preparations of bovine uterus, the collagen-free residue was pulverized to 60 mesh in a Wiley Micro Mill (A.H. Thomas Co.) prior to extraction with alkali.

Extraction with boiling alkali. AIP from human uterus, prepared as above, was suspended at 2 mg/ml in 0.1N NaOH in a round-bottomed flask fitted with an Allihn condenser. The suspension was refluxed for 45 minutes. The residue was washed extensively with water and both residue and supernatant were dialyzed against distilled water and lyophilized.

Preparation of Acidic Protein from Bovine Uterus by Milder Procedures

Extraction with alkali at room temperature. Collagen-free residues from bovine uterus were milled to 60 mesh in a Wiley Micro Mill and suspended at concentrations of 1% in 0.1N NaOH. The suspensions were stirred for 24 hours at room temperature and filtered. The filtrates and residues were dialyzed against distilled water and lyophilized.

Digestion with trypsin. Collagen-free residue from bovine uterus was milled to 60 mesh in a Wiley Micro Mill and suspended at a concentration of 1% in 0.05M phosphate buffer at pH 8.0. Trypsin (Sigma Chemical Co., Type III, 2x crystallized, from bovine pancreas) was added to give an enzyme/substrate ratio of 1/100 (w/w). The suspension was stirred gently for 24 hours at room temperature. The reaction was stopped by bringing the suspension to pH 3 and placing the flask in boiling water for 15 minutes. The residue was separated by filtration, washed, and resuspended in distilled water. The supernatant and the washes of the residue were pooled. Both the supernatant solution and the residue were dialyzed against distilled water and lyophilized.

Extraction with mercaptoethanol-urea. Flow diagrams of the methods used are given in Figure 1. A 1% suspension of fat-free residue or trypsin-resistant residue was prepared in 8M urea (Baker Chemical Co., analyzed reagent) containing 0.2% EDTA. The solution was bubbled with nitrogen and maintained at pH 8.5 with 0.2M tris(hydroxymethyl)methylamine (TRIS) (BDH, Laboratory Regent Grade) solution. To the suspension was added dropwise, and with gentle stirring, 2-mercaptoethanol (ME) (Sigma Chemical Co., Type I) to give a 0.05M solu-

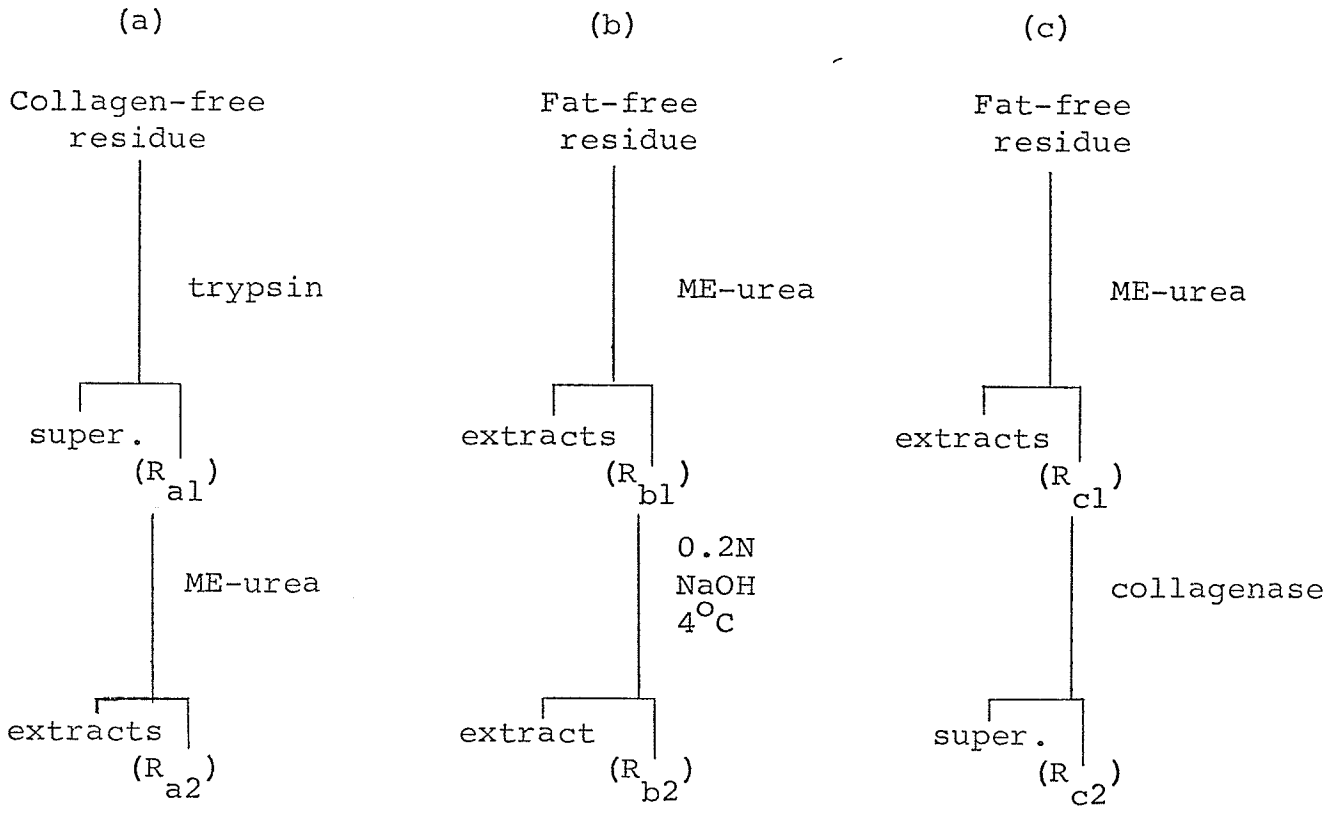


Figure 1: Preparation of acidic proteins from bovine uterus by mercaptoethanol-urea-based procedures. Abbreviations in parentheses are used in the text for the designation of the residues.

tion. The pH was maintained at 8.5 with 2N NaOH; the solution was bubbled again with nitrogen and the flask was sealed with Parafilm. The extraction was allowed to proceed with gentle stirring for 24 hours at room temperature. The suspension was centrifuged at 30,000 g-minutes and the pellet was re-suspended in distilled water and centrifuged. The first supernatant and the wash were pooled. The supernatant was dialyzed against distilled water and lyophilized. In scheme (a) (Figure 1) a single extraction of R_{a1} yielded R_{a2}. In schemes (b) and (c) (Figure 1) extraction was repeated three or four times and the resulting non-dialyzable, lyophilized material (R_{b1} or R_{c1}, Figure 1) was milled to 60 mesh in a Wiley Micro Mill. A portion of the ME-urea-resistant material was suspended at a concentration of 1% in 0.2N NaOH at $4^{o}C$ (Figure 1, scheme (b)). The suspension was stirred for 24 hours and the residue (R_{b2}) was separated by centrifugation at 30,000 g-minutes. Both supernatant and pellet were dialyzed against distilled water and lyophilized. Another portion of ME-urea-resistant material (R_{c1}) was suspended at a concentration of 1% in 0.1M $CaCl_2$. The pH was adjusted to 8.0 with 0.2M TRIS and collagenase (Sigma Chemical Co., Type III, chromatographically purified, from Clostridium Histo-

lyticum) was added to give an enzyme/substrate ratio of 1/50 (w/w). Digestion was carried out with gentle stirring for five hours at 37°C (Figure 1, scheme (c)). The residue (R_{C2}) was separated by centrifugation at 30,000 g-minutes. Both pellet and supernatant were dialyzed against distilled water and lyophilized.

Attempts to Isolate Elastin from Cat Uterus Using Special Techniques

Preparation of fat-free material. Cat uteri were minced with scissors and suspended in chloroform:methanol (3:1 by volume) for three hours at room temperature. The residue was separated by filtration, dehydrated in acetone, and dried in air. The dried material was milled to 20 mesh in a Wiley Micro Mill.

Method of Steven and Jackson. The 'Nishihara Technique' (Steven, 1964; Steven and Jackson, 1968) employs α-amylase and collagenase to extract collagen. It was used by Steven and Jackson (1968) to prepare a purified elastin residue from aorta and ligamentum nuchae.

Fat-free material from cat uterus was suspended at 1% concentration in 0.2M phosphate buffer at pH 5.3. Crude bacterial α-amylase (Sigma Chemical Co., Type III-A, from

Bacillus Subtilis) was added to give a 0.1% concentration. After stirring at room temperature for six days, the suspension was centrifuged at 270,000 g-minutes. The pellet was resuspended in 0.1M acetic acid and stirred at 4°C for 24 hours. The suspension was centrifuged as before and again resuspended in acetic acid. Extraction was carried out for 48 hours at 4°C, and the residue was again separated by centrifugation. A portion of the residue was suspended in 0.1M $CaCl_2$ at pH 8.0 to give a concentration of 0.3%. Collagenase (Sigma Chemical Co., Type III, chromatographically purified, from Clostridium Histolyticum) was added to give a concentration of 0.006%. The digestion was carried out twice for four hours at 37°C. The residue remaining was extracted successively with 0.1M acetic acid, 0.1N NaOH and 0.1M sodium acetate, each overnight at 4°C.

Method of Timpl and coworkers. Timpl and coworkers proposed a method for extracting 'acidic structural proteins' from skin, placenta and joint capsula (Timpl et al., 1968; Timpl et al., 1969).

Fat-free material from cat uterus was suspended twice at 1% concentration in 0.2M NaCl and stirred at 4°C for 24 hours. The residue was extracted three times by suspending

it in 0.2M citrate-phosphate buffer at pH 2.5 and stirring at 4°C for 24 hours. The residue was washed in distilled water and suspended at 1% concentration in borate-KCl-NaOH buffer (0.2M with respect to both KCl and boric acid) at pH 9.0. Four 24-hour extractions at 4°C were carried out in this medium. The residue was then treated with 5% trichloroacetic acid for 5-, 15- and 30-minute periods at 90°C, and the residue washed extensively with water. The washed residue was suspended in 8M urea and the pH adjusted to 7-8 using NaOH. Extraction was carried out at 4°C for 24 hours. The residue was washed with distilled water and suspended in 0.2N NaOH. The urea extract was dialyzed against distilled water and lyophilized. Extraction in NaOH was carried out at 4°C for 24 hours. The residue was washed extensively with distilled water and lyophilized. The extract was dialyzed against distilled water and lyophilized.

Digestion with trypsin. Digestion of 40 mg of collagen-free material from cat uterus was carried out in 40 ml of 0.05M phosphate buffer at pH 8.0, containing 1 mg trypsin (Sigma Chemical Co., Type III, 2x crystallized, from bovine pancreas) for 24 hours at room temperature.

Protein Hydrolysis and Amino Acid Analysis

Proteins were hydrolyzed in 6N HCl (Harleco Division, American Hospital Supply Corp., fluorometric grade) for 24 hours at 110°C in sealed ampoules under nitrogen. Amino acid analyses were carried out on a Technicon instrument using a 75 cm column and 5.5. hour gradient system. No corrections were made for destruction of amino acids during hydrolysis. In this system, methionine sulfone and hydroxyproline were eluted in the same position. Since the relative intensities of absorption at 570 nm and 440 nm of the reaction products of these two amino acids are known, their contributions to the combined peak could be calculated. Methionine is expressed as the sum of methionine and methionine sulfone. Crysteine and cystine were destroyed in these acid hydrolyzates. The effluent stream from the column was split so that one part of the eluent was reacted with ninhydrin and the other mixed with 2N NaOH and monitored for fluorescence at A/F:320/405 nm in an Aminco-Bowman spectrophotofluorometer using a 5 mm x 5 mm quartz flow cell. In some experiments, a G.K. Turner Assoc. Model 111 fluorometer with a 10 mm (i.d.) flow cell was equipped with a narrow pass #7-60 primary filter and a sharp cut #2A secondary filter to monitor fluorescence at A/F of

approximately 360/ > 415 nm. Dityrosine was eluted in the position of ornithine in the amino acid chromatogram and was detected by its fluorescence at A/F:320/405 nm. Another fluorescent fraction ('A'), detected on the Turner fluorometer, was eluted immediately after alanine. The areas of the fluorescent peaks were measured by weighing peaks traced from the recorder chart. Assuming a ninhydrin color equivalent of 2.0 (relative to norleucine) for dityrosine, each cm^2 of fluorescence was estimated to represent 1.4×10^{-4} μmoles of dityrosine (Keeley, 1970).

Since the amino acid composition of AIP (R_{AIP}) from uterus was found to be intermediate between those of elastin ($R_{elastin}$) and acidic protein (R_{acidic}), it was convenient to regard AIP as a composite of these two proteins. AIP could then be characterized in terms of the fraction of elastin ($F_{elastin}$) and acidic protein (F_{acidic}) in this composite, which would yield the composition of AIP sample. Thus, the following relationship would hold for each amino acid (r_{AIP}) in the composition of AIP (R_{AIP}):

$$r_{AIP} = (f_{acidic})\, r_{acidic} + (f_{elastin})\, r_{elastin}$$

where: f is the fraction of elastin ($f_{elastin}$) or acidic protein (f_{acidic}) in the AIP composite, determined for an individual amino acid. These are the unknown quantities being calculated.

r is the concentration of a given amino acid (e.g. ASP) in residues/1000 present in: elastin ($r_{elastin}$ = 4.5 for ASP in bovine elastin); acidic protein (r_{acidic} = 98 for ASP in bovine acidic protein); the given preparation of AIP (r_{AIP}) for which f_{acidic} or f_E is being calculated.

The standard compositions ($R_{elastin}$ and R_{acidic}) which were used in these calculations are given in Table II. The AIP compositions for which these calculations were made are given in Tables IX, XI, XIX, XX and XXII.

Since $f_{acidic} + f_{elastin} = 1$

the previous equation could be solved for either f_{acidic} or $f_{elastin}$ for each amino acid (except hydroxyproline and methionine) in the composition of AIP. The values of f_{acidic}

TABLE II

STANDARD AMINO ACID COMPOSITIONS USED IN THE CALCULATION OF CONTRIBUTION OF ACIDIC PROTEIN TO AMINO ACID COMPOSITION OF AIP
(residues/1000 total amino acid residues)

	R_{acidic}		$R_{elastin}$	
	BOVINE*	HUMAN+	BOVINE**	HUMAN++
ASP	98	66	4.5	4.3
THR	46	39	7.3	5.3
SER	51	39	7.6	3.1
GLU	148	106	14	19
PRO	70	58	116	114
GLY	88	122	347	314
ALA	82	90	237	251
VAL	66	78	136	135
ILE	39	44	23	23
LEU	91	116	57	58
TYR	27	33	7.2	19
PHE	38	47	28	22
LYS	73	60	3.5	4.8
HIS	24	18	0	0
ARG	46	52	3.8	3.8

* mean of six ALSP-2 preparations from uterus (see also Table VIII)
\+ single determination (ALSP-3) from 47-year old uterus
** from ligamentum nuchae (Partridge, 1962)
++ single determination from aorta of 51-year old male (LaBella and Vivian, 1967)

or $f_{elastin}$ calculated for each amino acid were then averaged to obtain the overall fraction of acidic protein (F_{acidic}) and elastin ($F_{elastin}$) which would yield the amino acid composition of the sample of AIP in question (R_{AIP}). These average fractions (F_{acidic} and $F_{elastin}$) were expressed as %acidic protein or %elastin in Tables IX, X, XI, XIX and XX.

Pulse-Labelling of ALSP and AIP in Rat Uterus

Twenty-four virgin female rats of the Sprague-Dawley strain, 110 days old, were injected intraperitoneally with 10 μC L-glycine-C^{14}(U) and 20 μC L-tyrosine H^3 (side chain-2,3) (Amersham/Searle) per 100 g body weight. Another six rats served as controls for spurious radioactivity, since very low levels of incorporation were anticipated. On each of days 1, 3, 5 and 10, six rats were killed by decapitation and the uteri excised and pooled. Uterine ALSP and AIP were prepared as described earlier. One mg samples of AIP and ALSP were weighed on a Cahn electrobalance and placed in scintillation vials in a hot water bath. Partial solubilization was accomplished with 0.5 ml 6N HCl. Hydrogen peroxide was used to decolorize the samples. Final solubilization was accomplished with 300 μl NCS Tissue Solubilizer (Amersham/Searle) in 20 μl of distilled water at 50^{o}C for 1 hour. The

digest was neutralized with acetic acid and 10 ml of a scintillation medium was added. The medium was composed of the following ingredients: 11.3 g PPO (Packard Instrument Co., scintillation grade), 0.2 g dimethyl POPOP (Packard Instrument Co., scintillation grade), 500 ml methyl cellosolve (Pierce Chemical Co., sequanal grade) and 2 l toluene. Samples were counted for 10 minutes each in a Philips Liquid Scintillation Analyzer. Counts were corrected for counting efficiency, background and quenching. Specific activities were expressed as dpm/mg protein.

Fluorescence Studies

Five mg samples of AIP and various extractable fractions from cat and bovine uterus and elastin from bovine ligamentum nuchae were solubilized either by 48-hour treatment in 2.5 ml 1.7N KOH at 37°C or 50°C or by 5- to 10-minute treatment with 2.5 ml 6N HCl at 98°C. The solutions were neutralized with HCl or NaOH and one portion adjusted to pH 2 - 3 and the other to pH 10 - 12. Final concentrations in all cases were approximately 1 mg/ml, and accurate determinations of protein concentration were made by an automated method based on the procedure of Lowry (Lowry et al., 1951) with bovine serum albumin as standard. Fluorescence spectra were determined using an

Aminco-Bowman spectrophotofluorometer. The intensities at A/F:340/405 nm were also measured.

Determination of Tryptophan

The concentration of tryptophan in various extractable fractions from cat and bovine uterus were determined using N-bromosuccinimide (Peters, 1959) as described by Blackburn (1968).

RESULTS AND DISCUSSION

Preliminary Studies Suggesting the Presence of a Relatively Insoluble Acidic Protein in Uterus

The material extracted from the collagen-free uterus tissue by hot, dilute alkali (ALSP) had a composition similar to that of acidic proteins isolated by various workers from tissues such as chick aorta (Keeley et al., 1969), rabbit placenta and skin (Timpl et al., 1969), rat skin (Timpl et al., 1969), human aorta (Barnes and Partridge, 1968), porcine and equine aorta (Moczar and Robert, 1970), calf skin (Bowes et al., 1958; Timpl et al., 1969), bovine nasal cartilage (Partridge and Elsden, 1961) and bovine ligamentum nuchae (Gotte et al., 1963b) (see Table III, cf. Tables I and VIII). In rat uterus (nulliparous or gravid, at term), this material represented 1 - 1.5% of the original wet weight of the tissue. The amount of ALSP solubilized in the third extract from rat uterus accounted for only about 2% of the total alkali-extractable material, indicating that extraction was essentially complete. The residue (AIP) remaining after hot alkali treatment represented about 0.5% of the wet weight of the rat uterus and was found to have an amino acid composition similar to that of ALSP (Table III). The finding of small amounts of hydroxyproline suggested the presence of elastin

TABLE III

AMINO ACID COMPOSITION OF ALKALI-SOLUBLE (ALSP) AND ALKALI-INSOLUBLE (AIP) PROTEINS FROM RAT UTERUS*

	ALSP+	AIP++
HPRO	3 ± 1	2 ± 1
ASP	82 ± 2	76 ± 3
THR	48 ± 2	45 ± 2
SER	54 ± 3	47 ± 3
GLU	136 ± 5	118 ± 8
PRO	61 ± 5	58 ± 4
GLY	92 ± 4	119 ± 11
ALA	87 ± 3	97 ± 6
VAL	71 ± 3	71 ± 2
CYS	not determined	not determined
MET	14 ± 1	13 ± 1
ILE	46 ± 2	47 ± 2
LEU	94 ± 4	98 ± 2
TYR	24 ± 2	27 ± 1
PHE	39 ± 2	41 ± 2
LYS	63 ± 3	61 ± 2
HIS	18 ± 1	18 ± 1
ARG	49 ± 2	53 ± 2

* residues/1000 total residues. Mean ± standard error.

\+ average of ALSP from 7 preparations of nulliparous and 7 of gravid uteri.

++ average composition of AIP from 7 nulliparous uteri.

in this residue.

The amounts of ALSP and AIP in rat uterus (as mg/uterus) increased during pregnancy (Table IV). This increase was especially prominent for ALSP.

The insoluble uterine protein (AIP) described here appears to be unique to the uterus, since apparently identical species of proteins from other tissues have been extracted by relatively gentle means (Bowes et al., 1958; Partridge and Elsden, 1961; Gotte et al., 1963b; Barnes and Partridge, 1968; Timpl et al., 1969; Moczar and Robert, 1970). The similarity in compositions suggests that AIP might be derived from ALSP.

These preliminary studies served to indicate that elastin in uterus was closely associated with a relatively insoluble polar protein. The rest of this thesis is concerned with attempts to characterize this polar protein and its relationship with elastin.

Effectiveness of Extraction with Water

It is apparent from Figure 2 that the first 24-hour autoclaving in water removed most of the water-extractable material from the fat-free residue of nulliparous or gravid rat uterus. Extractions in water carried out beyond three

TABLE IV

CONTENT OF ALKALI-SOLUBLE (ALSP) AND ALKALI-INSOLUBLE (AIP) PROTEINS IN RAT UTERUS*

	ALSP		AIP	
	mg/uterus+	% of collagen-free residue	mg/uterus+	% of collagen-free residue
NULLIPAROUS	13 ± 2 (9)	87	3 ± 1 (27)	13
GRAVID	97 ± 20 (8)	95	5 ± 2 (25)	5

* Both horns were used.
\+ mean ± standard error. Figures in parentheses refer to number of preparations.

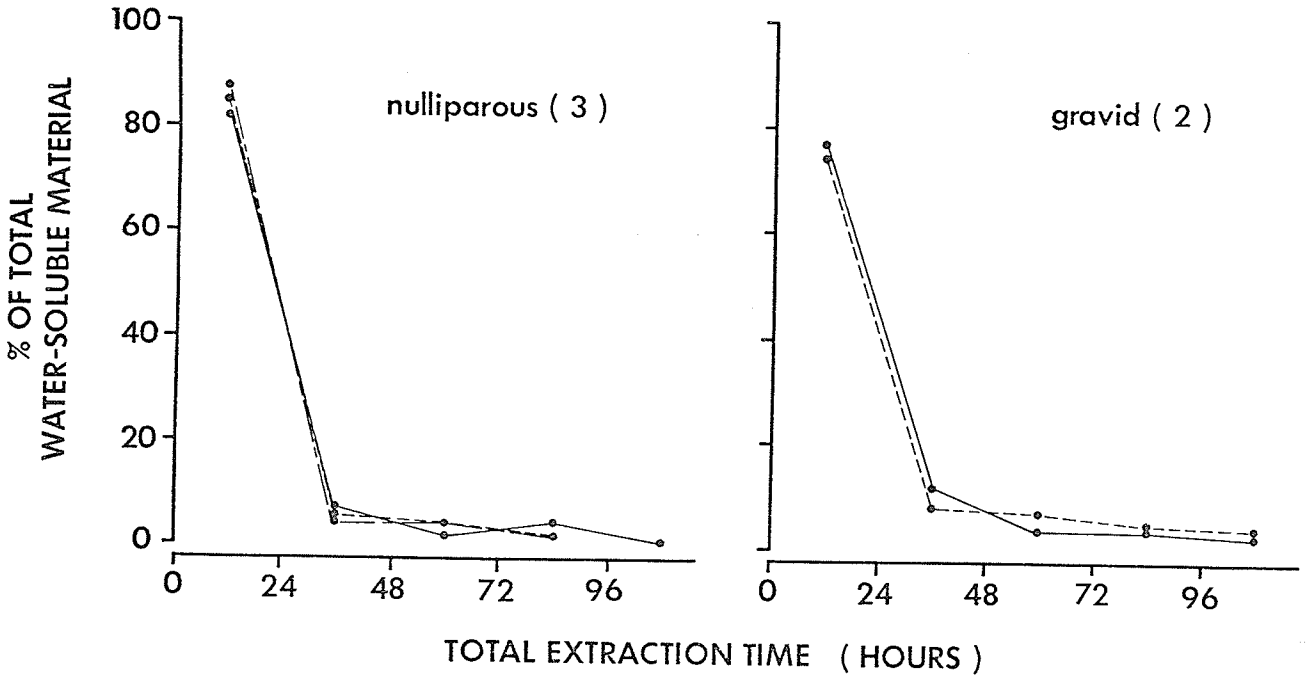


Figure 2: Effectiveness of extraction of rat uterus by autoclaving in distilled water.

24-hour periods resulted in a relatively low, constant yield which contributed negligibly to the overall quantity of protein extracted (Figures 3 and 4). Furthermore, as Table V indicates, these later extracts from dog uterus were not at all collagen-like. In fact, the amino acid compositions of the late water extracts were very similar to that of the alkali-soluble protein. These observations suggest the presence of a very large range of solubilities for this species of acidic protein.

Figure 5 shows that, as in rat and cat uterus, the first extraction in water removed most of the water-extractable material from bovine and human uterus. Three-hour extraction periods were used in bovine and human uteri.

Extraction in Hot Alkali

As seen in the preliminary experiments, hot, dilute alkali treatment effectively extracted ALSP from the collagen-free residue of rat uterus. In the Sprague-Dawley rats used in later experiments, the AIP recovered was less polar (equivalent to 53% ALSP-like and 47% elastin). We are unable to account for the difference in amino acid composition of AIP between these two strains of rats.

The amino acid compositions of the three alkali ex-

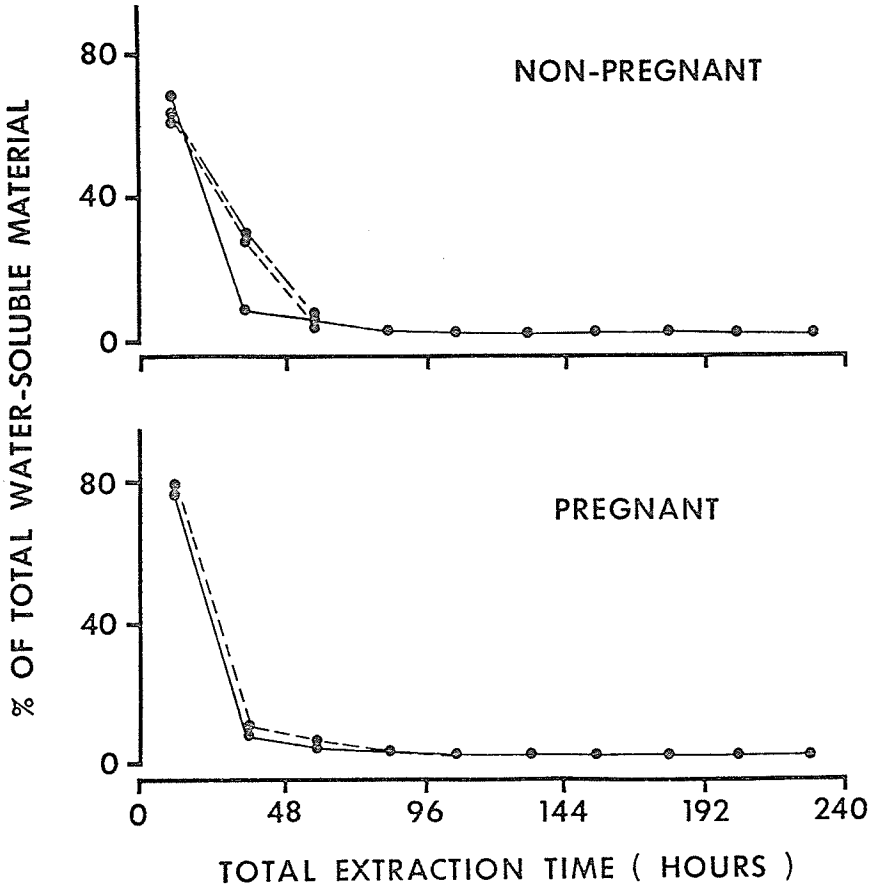


Figure 3: Effectiveness of extraction of cat uterus by autoclaving in distilled water.

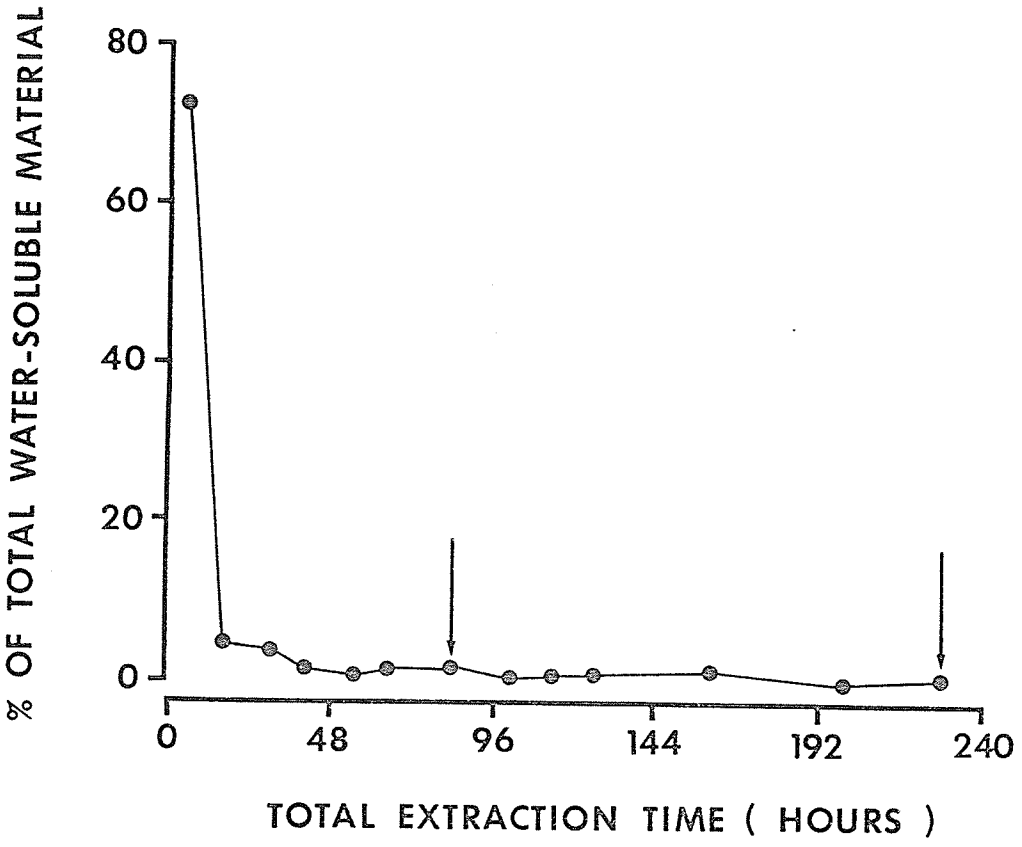


Figure 4: Effectiveness of extraction of non-pregnant dog uterus by autoclaving in distilled water. Amino acid compositions of extracts #7 and #13 (arrows) are given in Table V.

TABLE V

AMINO ACID COMPOSITIONS OF PROTEIN FRACTIONS FROM DOG UTERUS

(residues/1000 total amino acid residues)

	water extracts		insoluble collagen*	ALSP-6	AIP
	#7	#13			
HPRO	0	14	102	0	0
ASP	86	88	49	70	65
THR	64	49	17	40	31
SER	59	52	39	29	28
GLU	166	139	77	136	116
PRO	65	58	113	45	49
GLY	101	122	340	113	132
ALA	85	102	101	103	113
VAL	61	61	22	77	88
CYS	7.5	2.3	0	0	0
MET	14	3.0	5	23	20
ILE	35	36	11	49	49
LEU	73	78	24	98	99
TYR	23	22	4	32	31
PHE	30	35	12	40	38
LYS	50	59	18	66	68
HIS	20	19	5	16	15
ARG	53	54	52	42	39

* from rat uterus (Kao et al., 1968)

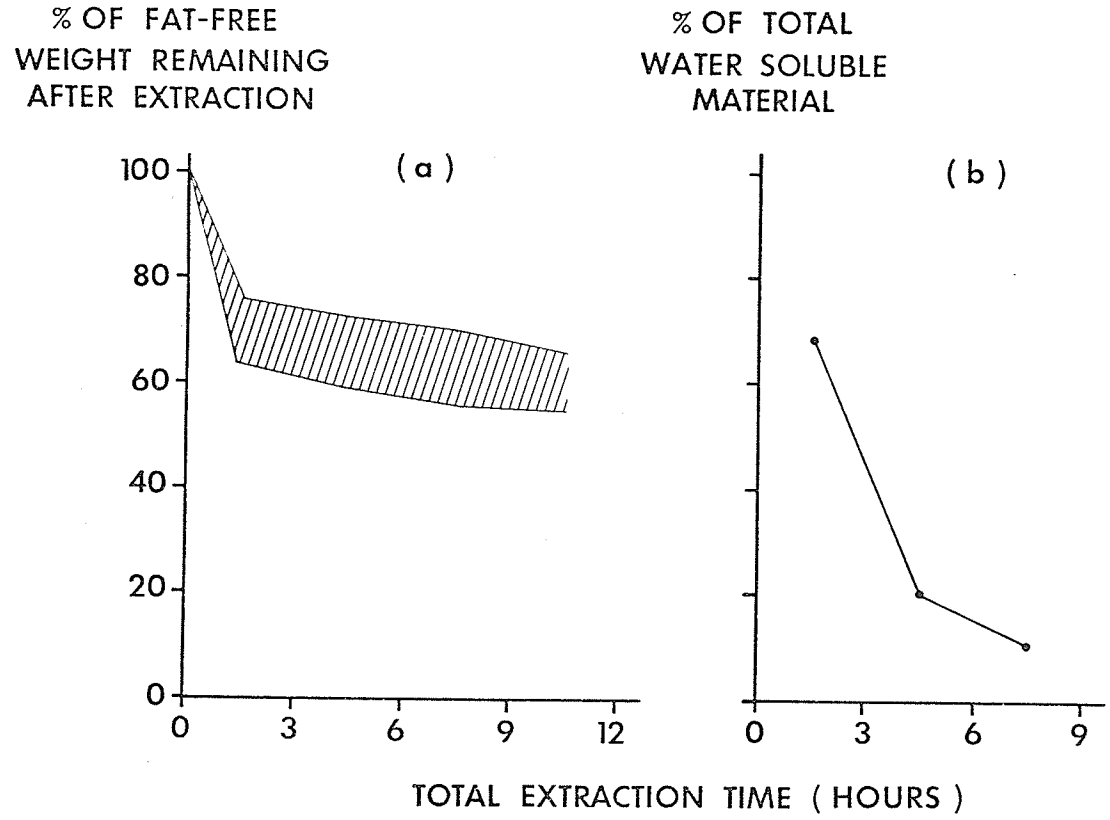


Figure 5: Effectiveness of extraction of bovine (a) and human (b) uterus by autoclaving in distilled water. Panel (a) shows the range of values obtained in 7 non-pregnant bovine uteri. Four 3-hour extraction periods were used. Panel (b) depicts the progress of water extractions in one human uterus sample.

tracts in Sprague-Dawley rats are given in Table VI. The coefficient of variation is a measure of the variability in concentration of a given amino acid among the three extracted proteins shown. Only proline differed substantially over the three extracts, but this might have been due to difficulties in resolving proline from glutamic acid at these particular concentrations on our analysis system. The low coefficients of variation suggest that there are no significant differences in amino acid composition among these extracts. Thus, ALSP from uterus appears to be a polydisperse protein, with varying degrees of aggregation accounting for differences in extractability. That a similar situation occurs in other tissues as well has been concluded by other workers (Timpl et al., 1969; Keeley, 1970; Wolff et al., 1971).

Table VII shows the amino acid compositions of the five alkali extracts from a bovine uterus. Again, coefficients of variation were low and there appeared to be no major difference in composition among the extracts. Hydroxyproline was found in the first extract and may represent slight contamination by collagen. The trace amount of hydroxyproline seen in the pooled fourth and fifth extracts might indicate solubilization of elastin by the prolonged treatment with

TABLE VI

COMPOSITION OF ALSP EXTRACTED BY SEQUENTIAL HOT ALKALI TREATMENTS OF COLLAGEN-FREE MATERIAL FROM RAT UTERUS

	ALSP			Coeff. var. (%)
	1	2	3	
	(residues/1000 total amino acid residues)			
ASP	106	102	98	3.9
THR	56	51	44	12.0
SER	45	48	44	4.6
GLU	141	115	116	11.9
PRO	47	50	73	25.1
GLY	87	79	90	6.7
ALA	87	87	90	2.0
VAL	59	64	62	4.1
ILE	40	45	46	7.4
LEU	89	104	101	8.1
TYR	27	30	26	7.5
PHE	34	44	42	13.2
LYS	76	80	79	2.7
HIS	20	22	20	5.6
ARG	44	53	49	9.3

TABLE VII

AMINO ACID COMPOSITIONS OF ALSP EXTRACTED BY SEQUENTIAL HOT ALKALI TREATMENTS OF COLLAGEN-FREE MATERIAL FROM BOVINE UTERUS
(residues/1000 total amino acid residues)

	ALSP				Coeff. var. (%)
	1	2	3	4&5	
HPRO	8.1	0	0	0.1	
ASP	91	96	98	98	3.5
THR	54	43	43	46	11.3
SER	52	45	47	38	13.1
GLU	173	164	149	157	6.2
PRO	51	63	66	55	12.5
GLY	86	99	98	118	13.2
ALA	81	79	90	89	6.5
VAL	71	73	67	72	3.5
MET	12	13	16	3.0	15.0
ILE	38	35	35	34	5.1
LEU	86	89	90	92	6.2
TYR	27	24	26	22	8.1
PHE	33	35	37	40	7.9
LYS	70	69	70	59	8.1
HIS	18	18	20	19	5.9
ARG	44	41	40	39	5.8
'A'*	2.6	2.3	3.7	3.5	
Dityrosine	0.10	0.13	0.24	0.19	

* expressed as peak area (cm^2)/μmole total amino acids

alkali. Because of the possibilities of contamination in the first and last extracts, only intermediate extracts (e.g. NaOH-2 or NaOH-3) were used in the determination of an average amino acid composition for ALSP in bovine uterus (Table VIII). Hydroxyproline was present in two of the six samples and in both cases in a concentration of less than 1 residue per 1000 total amino acid residues. The amino acid composition of ALSP from bovine uterus agreed well with compositions for ALSP from bovine ligamentum nuchae and day-old chick aorta (Keeley et al., 1969; Keeley, 1970) (Table VIII) and was used as the reference composition of acidic protein from bovine uterus (see Table II).

Treatment of AIP from human uterus with boiling 0.1N NaOH caused a considerable hydrolysis since total recoverable protein amounted to only 57%. The residue represented 17% of the starting material and was much more elastin-like (Table IX), although still more polar than elastin from ligamentum nuchae.

In summary, treatment of the collagen-free residue of uterus with hot alkali results in the extraction of a species of proteins of uniform amino acid composition but apparently with a spectrum of molecular weights. The amino acid composi-

TABLE VIII

AVERAGE AMINO ACID COMPOSITION OF ALSP FROM BOVINE UTERUS COMPARED TO ALSP FROM OTHER SOURCES (residues/1000 total amino acid residues)

	BOVINE UTERUS ALSP*	LIGAMENTUM NUCHAE ALSP**	CHICK AORTA ALSP**
HPRO	0.3 ± 0.2	0	0
ASP	98 ± 4	102	103
THR	46 ± 2	35	37
SER	51 ± 4	51	60
GLU	148 ± 9	139	125
PRO	70 ± 5	87	61
GLY	88 ± 4	103	115
ALA	82 ± 2	75	85
VAL	66 ± 3	73	69
MET	11 ± 3	13	12
ILE	39 ± 2	38	43
LEU	91 ± 1	81	93
TYR	27 ± 1	31	29
PHE	38 ± 1	42	39
LYS	73 ± 4	62	54
HIS	24 ± 2	17	16
ARG	46 ± 3	37	40

* average of 6 ALSP-2 preparations ± S.E.

** compositions of ALSP from bovine ligamentum nuchae and chick aorta reported by Keeley (1970).

TABLE IX

AMINO ACID COMPOSITIONS OF RESIDUES FROM HUMAN UTERUS AFTER TREATMENT WITH ALKALI AND AFTER SUBSEQUENT REFLUXING IN ALKALI
(residues/1000 total amino acid residues)

	AFTER 6x NaOH	AFTER REFLUXING
HPRO	7.5	11
ASP	38	11
THR	22	8.2
SER	22	11
GLU	65	27
PRO	83	92
GLY	198	262
ALA	177	259
VAL	116	133
MET	4.8	2.3
ILE	35	23
LEU	83	64
TYR	27	26
PHE	33	26
LYS	40	12
HIS	9.7	2.9
ARG	29	8.7
Dityrosine	0.06	0.02
% Elastin (Calculated)	49	83

tion of the alkali-insoluble residue (AIP) is more polar than that of elastin, but less polar than AIP recovered in the preliminary experiments. Even the drastic procedure of boiling in alkali failed to free the elastin completely of acidic protein.

Milder Methods of Extraction -- Bovine Uterus

In bovine uterus, when a finely divided preparation of collagen-free residue was used as starting material for extraction in hot alkali, yields of AIP varied from zero to 0.5% of the weight of the starting material. It was also evident that a considerable proportion of the elastin in the preparation had been solubilized by this procedure (Table X). For comparative purposes, extraction was carried out at room temperature (23°C) for three 24-hour periods. The yields in this case were much greater and the residue was more ALSP-like in composition (Table X). However, a further three 24-hour extractions at room temperature failed to alter substantially the composition of the residue. It is difficult to determine at what point extraction stops and hydrolysis of protein begins when extraction in alkali is used with bovine uterus; thus, alternative extraction procedures were examined. The amino acid compositions of the residues obtained from

TABLE X

INFLUENCE OF TEMPERATURE ON EXTRACTION OF PROTEIN WITH ALKALI FROM COW UTERUS

	95°C		23°C	
Exp.#*	Protein Residue**	%Elastin in residue	Protein Residue**	%Elastin in residue
1 a)	0.4	79	8	10
b)	0			
2 a)	0		11	10
b)	0			
3 a)	0.02		8	12
b)	0.5	69		

* a) and b) are replicates of the same sample.
** expressed as percent of collagen-free starting material.

these milder treatments (see Figure 1) are given in Table XI. ME-urea/collagenase failed to remove all the acidic protein. The amino acid composition of the residue (R_{c2}) was identical to that of the extractable acidic protein. The fact that no elastin could be detected probably reflects the presence of a large excess of acidic protein. This result is in contrast to calf ligamentum nuchae (Robert et al., 1971) and rabbit and rat skin (Furthmayr and Timpl, 1970) in which ME-urea removed essentially all of the acidic protein material from elastin. The other two milder methods (see Figure 1) yielded residues (R_{a2} and R_{b2}) which contained little or no acidic protein; the composition in each case was essentially that of elastin (Table XI). We attribute this result to hydrolysis of the acidic protein by trypsin or by 0.2N NaOH. It must be concluded that the differences between uterus and ligamentum nuchae in the nature of the residues left by various extraction procedures represent tissue-specific differences in the character of elastin and in its association with a polar, non-elastin protein (Gotte et al., 1963a; Anwar, 1966).

The compositions of fractions from bovine uterus which most closely resembled that of elastin are compared to elastin from ligamentum nuchae in Table XII. The estimates

TABLE XI

COMPOSITIONS OF RESIDUES FROM COW UTERUS TREATED WITH MILD MERCAPTOETHANOL-UREA-BASED PROCEDURES*

	R_{a2}	R_{b2}	R_{c2}
	(residues/1000 total amino acid residues)		
HPRO	20	12	0
ASP	9.8	10	95
THR	10	8.4	60
SER	11	9.8	67
GLU	18	24	109
PRO	100	80	63
GLY	348	404	94
ALA	202	177	86
VAL	145	128	45
MET	0.5	1.4	19
ILE	26	20	30
LEU	54	57	91
TYR	9.2	8.6	35
PHE	32	30	39
LYS	5.4	7.8	73
HIS	1.5	1.7	18
ARG	7.4	7.6	51
% Elastin (Calculated)	91	89	0

* See Figure 1 for a flow diagram of the extraction procedures.

TABLE XII

AMINO ACID COMPOSITION OF ELASTIN FROM BOVINE UTERUS
(residues/1000 total amino acid residues)

	AFTER HOT ALKALI	R_{a2}	R_{b2}	LIGAMENTUM NUCHAE*
HPRO	18	20	12	9.8
ASP	10	9.8	10	4.5
THR	7.2	10	8.4	7.3
SER	8.5	11	9.8	7.6
GLU	20	18	24	14
PRO	97	100	80	116
GLY	324	348	404	347
ALA	212	202	177	237
VAL	152	145	128	136
MET	0.5	0.5	1.4	0
ILE	24	26	20	23
LEU	64	54	57	57
TYR	6.7	9.2	8.6	7.2
PHE	28	32	30	28
LYS	6.3	5.4	7.8	3.5
HIS	1.4	1.5	1.7	0
ARG	6.0	7.4	7.6	3.8

* elastin from bovine ligamentum nuchae (Partridge, 1962).

for hydroxyproline might be expected to be less reliable than the others since they were calculated, but they were higher than that seen in elastin from ligamentum nuchae. There is an indication of a slightly more polar character in the uterine 'elastins'. Both methionine (as methionine sulfone) and histidine are found in uterine 'elastin' whereas they are absent from typical elastin from various tissues (Partridge, 1962).

Milder Methods of Extraction -- Cat Uterus

The Nishihara Technique (Steven, 1964; Steven and Jackson, 1968) was found to be unsuitable for isolating elastin from cat uterus. The residue contained a considerable amount of collagen, reflecting the very high collagen content of uterus.

As in Timpl's work on skin, placenta and joint capsula (Timpl et al., 1968, 1969), the 'acidic structural proteins' extracted in 8M urea and in cold 0.2N NaOH from cat uterus were very similar to ALSP in amino acid composition (Table XIII). Timpl indicated that the residue after these extractions 'contains elastin' (Timpl et al., 1969), and in uterus, a polar residue with an amino acid composition intermediate between those of ALSP and elastin was recovered (Table XIII).

TABLE XIII

AMINO ACID COMPOSITIONS OF FRACTIONS ISOLATED FROM CAT UTERUS BY THE PROCEDURE OF TIMPL AND COWORKERS*
(residues/1000 total amino acid residues)

	8M UREA EXTRACT	0.2N NaOH EXTRACT	RESIDUE
HPRO	0	0	11
ASP	105	94	34
THR	46	50	30
SER	54	54	29
GLU	146	173	50
PRO	79	65	77
GLY	88	76	234
ALA	100	79	202
VAL	67	69	112
MET	1.2	16	5.0
ILE	40	39	29
LEU	84	89	69
TYR	22	26	25
PHE	32	36	34
LYS	64	63	23
HIS	17	19	7.4
ARG	49	45	24

* reported in Timpl et al., 1969.

Assuming that no elastin was solubilized by any of the procedures, it could be calculated that elastin would represent about 0.1% of the wet weight of the uterus.

Prior treatment of collagen-free material with trypsin was studied as a possible means of increasing the yields of alkali-soluble material and obtaining true elastin. Digestion of the collagen-free material from gravid cat uterus with trypsin for 2 x 24 hours yielded a residue of typical ALSP-like composition (Table XIV, column 1). Subsequent extraction of this residue with hot, 0.1N NaOH yielded an insoluble residue with the amino acid composition of elastin (Table XIV, column 2). This elastin residue, however, was less than 0.2% of the starting weight of the collagen-free material and represents only 0.01% of the wet weight of the gravid cat uterus (cf. 0.6% for the gravid human uterus (Woessner and Brewer, 1963).

Thus, the unusual amino acid composition of AIP is probably not due to ALSP being embedded in an insoluble elastin matrix, because prior treatment with trypsin (which has little or no effect on elastin) enables NaOH to extract all of the acidic protein material. Acidic protein must, therefore, be accessible both to the enzyme and to NaOH. The

TABLE XIV

COMPOSITIONS OF RESIDUAL PROTEINS FROM CAT UTERUS AFTER DIGESTION WITH TRYPSIN AND AFTER TRYPSIN THEN NaOH (residues/1000 total residues)

	After Trypsin	After Trypsin then NaOH
HPRO	2	18
ASP	74	6
THR	56	11
SER	73	12
GLU	156	19
PRO	67	77
GLY	86	372
ALA	90	225
VAL	86	145
MET	16	0
ILE	41	21
LEU	90	38
TYR	29	16
PHE	38	23
LYS	37	4
HIS	19	2
ARG	34	6

action of trypsin is attributed either to the conversion of a highly polymerized acidic protein material to alkali-soluble fragments or to the cleavage of a peptide link between acidic protein and elastin. The low yield of elastin in this experiment is consistent with the known susceptibility of certain elastins to solubilization in hot alkali (Gotte et al., 1963a; Anwar, 1966; LaBella et al., 1966; Cleary et al., 1967).

Turnover of ALSP and AIP in Rat Uterus

Collagen turnover in the uterus is very rapid, even in the mature rat (Kao et al., 1961a, 1961b). Elastin turnover has not been measured in uterus, but is very slow in tissues where it has been measured in the mature animal (Slack, 1954; Kao et al., 1961a, 1961b; Walford et al., 1964).

The specific activities of some solubilized protein fractions from rat uterus after pulse labelling with glycine-C^{14} and tyrosine-H^3 are seen in Figure 6. For simplicity, only the specific activity of C^{14} is shown but H^3 followed the trends shown by C^{14} except in AIP (see Table XV). The alkali-soluble fractions were evidently turning over quite rapidly, since peak activity occurred before 24 hours, the time of first measurement. The early parts of the curves

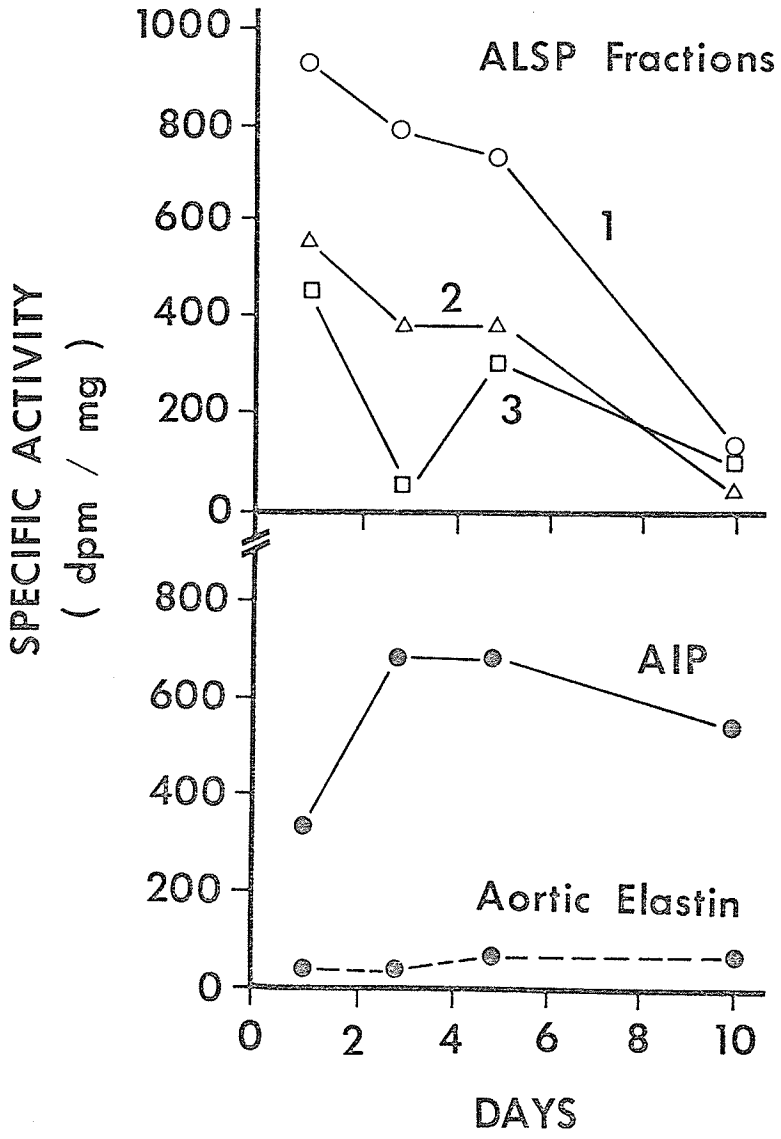


Figure 6: Specific activity of C^{14} in some protein fractions from rat uterus and aorta after a single injection of glycine-C^{14}. Time between administration of label and collection of tissues is shown on the abscissa. In hot alkali extracted fractions 1 (○——○), 2 (△——△), and 3 (□——□) from uterus, specific activity decreases with time. Uterine AIP (●——●) reaches its maximum specific activity by day 3 and declines only slowly after that. Elastin from rat aorta (●- - -●) shows only very low incorporation of label.

suggested that, initially, ALSP-1 attained the highest specific activity, followed by ALSP-2, then ALSP-3. This is consistent with the transfer of label from ALSP-1 through ALSP-2 to ALSP-3. From the time relationships of specific activity in fractions of ALSP and AIP, it would appear, also, that a progressive decrease in solubility of the acidic protein in ALSP yielded a highly insoluble component in AIP. The relatively rapid loss of specific activity ($t_{\frac{1}{2}}$ = 11 days) from AIP indicated a fairly rapid turnover of the acidic protein in AIP, in spite of its relative insolubility. Elastin in rat aorta showed negligible turnover.

The specific activity ratios of C^{14}/H^3 in the various ALSP fractions (Table XV) were approximately what would be expected assuming that both amino acids had equal access to the nascent acidic protein molecule. There was no apparent trend in specific activity ratio with time or with difficulty of extraction, again suggesting that the spectrum of proteins extracted in hot alkali were of a uniform amino acid composition. In AIP, the ratios were lower than those predicted if the amino acids had equal access to incorporation into the protein (i.e. glycine was relatively less readily incorporated into AIP than was tyrosine). This suggested the possibility

TABLE XV

SPECIFIC ACTIVITY RATIOS* IN ALSP AND AIP FROM RAT UTERUS

	DAY	S.A. RATIO *
ALSP-1	1	1.1
	3	1.1
	5	1.2
	10	1.1
ALSP-2	1	1.1
	3	1.0
	5	1.2
	10	2.2
ALSP-3	1	1.0
	3	2.1
	5	1.0
	10	1.4 (1.5)
AIP	1	1.3
	3	2.4 (7.6)
	5	2.5 (4.6)
	10	3.4 (8.3)

* ratio of C^{14} to H^3 corrected for the fact that tyrosine was administered at twice the amount per gram of rat body weight. Figures in parentheses indicate the expected ratios, based on the amino acid compositions and assuming non-selective incorporation of tyrosine and glycine.

that the residue was non-homogeneous and that a glycine-poor protein (e.g. acidic protein) was incorporated into AIP at a greater rate than was a glycine-rich protein (e.g. elastin). This is consistent with the aggregation and deposition of an insoluble form of acidic protein.

Fluorescent Compounds in Acidic Protein

Dityrosine, an o,o'-biphenol analogue of tyrosine, and a potential protein crosslink, was first identified in resilin, an elastin-like protein from the locust (Andersen, 1964, 1966), and later, in young elastin (LaBella et al., 1967) and ALSP (Keeley et al., 1969).

Dityrosine was identified in ALSP and AIP from rat and cat uterus, and its content in these proteins was determined in gravid and nulliparous rats of the Long-Evans strain (Table XVI). The dityrosine content of AIP was found to be greater than that of ALSP, and at term, was increased in both proteins.

Keeley (1970) correlated dityrosine content with molecular weight of various fractions of ALSP isolated from chick aorta and was able to promote aggregation and insolubility associated with increased dityrosine content of ALSP by treatment with peroxidase. Table XVII shows the concen-

TABLE XVI

DITYROSINE CONTENT IN ALKALI-SOLUBLE (ALSP) AND ALKALI-INSOLUBLE (AIP) PROTEINS FROM RAT UTERUS

	Residues/100,000 Total Residues	
	ALSP*	AIP*
NULLIPAROUS	2.1 ± 0.2 (4)	3.5 ± 0.8 (7)
GRAVID	5.5 ± 0.5 (4)	8.5 ± 2.0 (6)

* mean ± standard error.

(Figures in parentheses refer to number of preparations.)

TABLE XVII

CONCENTRATION OF FLUORESCENT COMPOUNDS
IN RAT UTERUS PROTEINS

	ALSP			AIP	
	1	2	3	total residue	acidic protein portion (calculated)+
DT*	8.1	9.9	15.8	5.7	13.5
A**	2.8	4.3	5.9	3.4	8.2

* dityrosine expressed as residues/100,000 total amino acid residues.

** expressed as peak area in cm^2/umole of protein.

\+ Last column of figures is obtained by assuming that each of the fluorescent compounds is present only in the acidic protein part of AIP.

trations of dityrosine and another fluorescent compound detected in the hydrolyzates of ALSP and AIP from the uterus of a rat of the Sprague-Dawley strain. Dityrosine concentration increased with apparent insolubility of ALSP but its concentration in AIP was quite low. Peak 'A' shows a similar trend. If the assumption is made that the fluorescent compounds occur only in acidic protein, then their concentrations there will be greater than indicated in AIP (Table XVII, column 4), since this sample of AIP was calculated to contain only about 42% acidic protein moiety. The corrected concentrations of the two fluorescent compounds are given in the last column of Table XVII.

Keeley (1970) found that, although dityrosine concentration correlated well with the aggregation of ALSP in chick aorta, the actual number of dityrosine residues could not account for crosslinking of all the protein chains. In uterus, a similar correlation is observed in ALSP fractions. It has been suggested (LaBella et al., 1967; Keeley, 1970) that dityrosine might be only one of a family of aromatic compounds serving as crosslinks. Although several fluorescent compounds have been detected in elastin (LaBella, 1961; Loomeijer, 1961; Walford et al., 1961; LaBella, 1962; Partridge, 1962;

LaBella and Thornhill, 1965; Thornhill and LaBella, 1965; Blomfield and Farrar, 1969), their identities and inter-relationships have not yet been established.

In spite of the correlation of dityrosine concentration with decreasing solubility in ALSP fractions, it could not be extended to AIP, since dityrosine concentration was not greater in AIP than in the alkali-soluble acidic protein. However, the unknown fluorescent compound 'A' showed the pattern that might be expected of a crosslink responsible for the aggregation of acidic protein from the most readily extracted (ALSP-1) to the non-extractable (acidic protein in AIP)(Table XVII) forms in rat uterus. 'A' may represent one of the aromatic crosslinks referred to above. However, compound 'A' would not seem to be the major crosslink in bovine acidic protein, since there was no trend in the ALSP fractions (Table VII) and its concentration in bovine AIP was extremely variable.

It is known that dityrosine alone cannot account for the fluorescence of ALSP from chick aorta (Keeley, 1970). ALSP and AIP from uterus are also fluorescent both in the dry state and in solution. A typical fluorescence spectrum of ALSP from bovine uterus is shown in Figure 7. Two major

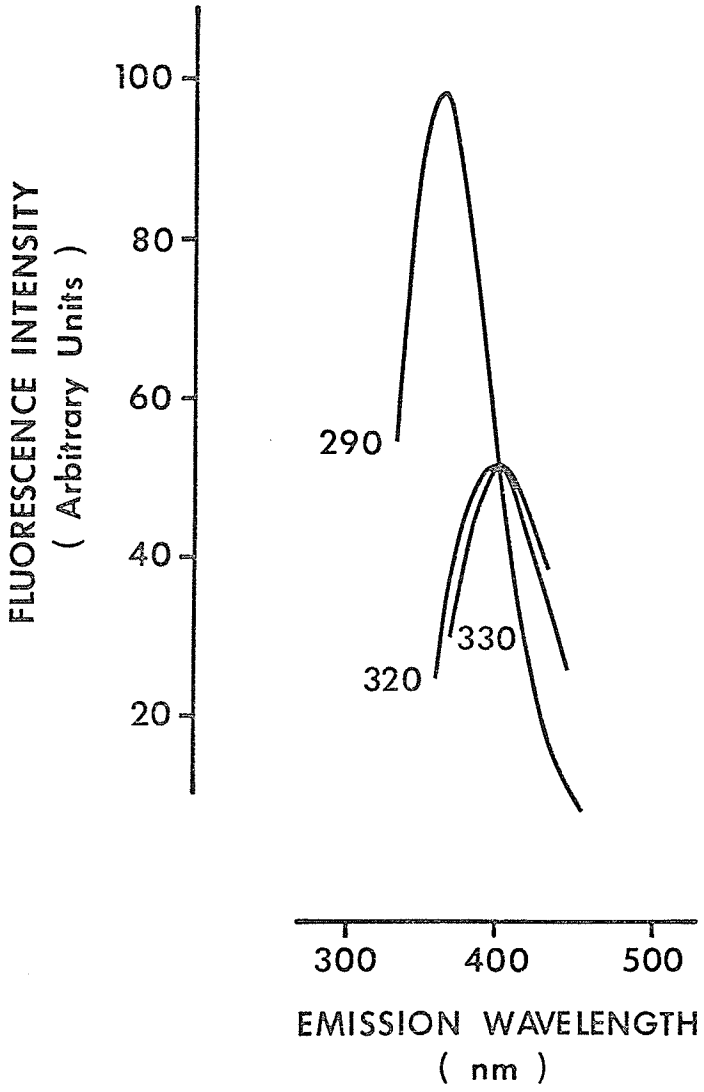


Figure 7: Fluorescence spectrum at pH 10 of second hot alkali extract from bovine uterus.

peaks are seen. The fluorescence maximum at 290/355 nm is due to tryptophan. Preliminary determinations of tryptophan in extractable fractions from uterus using N-bromosuccinimide gave values in the range of 2 - 6%. The compound responsible for the maximum at A/F:320/405 nm has not yet been identified, but comparison with the fluorescence spectrum of elastin from mature bovine ligamentum nuchae which contains no dityrosine suggested that this peak was not due to dityrosine. On the other hand, compounds containing a carbonyl group, which LaBella (1971) believes may be the major source of fluorescence in connective tissue proteins, exhibit fluorescence in this region. Previous work by LaBella and associates (LaBella and Lindsay, 1963; LaBella and Thornhill, 1965; Thornhill and LaBella, 1965; LaBella et al., 1966; Thornhill, 1972) indicated that elastin contains fluorescent compounds (A/F:340/405 nm), some of which may be derived from tyrosine. These fluorescent, putative crosslinks are concentrated in the enzyme-resistant regions of the protein and accumulate with age. The results of fluorescence measurements at A/F:340/405 nm in bovine and cat uterus are illustrated in Table XVIII. No correlation between fluorescence and difficulty in extraction was seen in the extractable fractions from either species.

TABLE XVIII

FLUORESCENCE* OF SOLUBILIZED AIP AND EXTRACTS FROM CAT AND COW UTERUS

Extracts	Cat	Cow
Urea	n.d.	46
ME-urea	70	87
Cold alkali	41	n.d.
Hot alkali-1	47	n.d.
Hot alkali-2	50	60
AIP	167	276

* fluorescence measured at A/F:340/405nm in solution at pH 10. Intensity is expressed as arbitrary units/100 mg% total protein. Intensity at pH 3 was proportionately lower.

n.d. = not determined

In both species, however, AIP showed a much greater fluorescence intensity than did the extracts.

GENERAL DISCUSSION

The presence of a large amount of acidic protein in bovine uterus and the difficulty in extracting this protein without concomitant solubilization of the elastin has been described. There are other reported examples of a polar protein intimately associated with elastin, particularly in fetal connective tissue (Cleary et al., 1966, 1967; Steven and Jackson, 1968; Keeley, 1970; Keeley and LaBella, 1972), where there is active elastin fibrogenesis; and elastin from young animals has been reported to be more susceptible to solubilization in hot alkali (LaBella et al., 1966; Cleary et al., 1967). Since it is known that collagen, the other major structural protein in uterus, turns over rapidly (Kao et al., 1961a, 1961b), it is possible that uterus might contain considerable amounts of newly synthesized elastin as well. Gotte and coworkers (1963a) and Anwar (1966) noted that, among mature tissues, ear cartilage of cattle and rabbits contained considerable amounts of polar protein which was extracted in alkali only with difficulty. Persistent attempts to purify elastin from these tissues by extraction with hot alkali resulted in considerable hydrolysis of the 'contaminating' protein, and much of the elastin as well.

Several interpretations of these observations are possible. The simplest suggestion is that elastin and the polar protein have a close physical association but are not covalently linked. Ross and Bornstein (1969, 1971) have suggested that a polar protein is first elaborated and forms a nidus for the orientation of the growing elastin fibril. This network of microfibrillar, polar protein may be insoluble and could correspond to the acidic protein in AIP. Partridge (1962) interpreted the work of Gotte and associates (1963a) as suggesting the possibility of covalent bonding between elastin and the proteins of ground substance during deposition of the elastin fibril. Keeley (Keeley, 1970; LaBella, 1971), on the basis of studies on chick embryo aorta, suggested that elastin may be synthesized in the form of 'proelastin', which contained a polar region with an amino acid composition like that of the acidic protein described above.

Observations in cat uterus that the acidic protein in AIP is accessible to trypsin and yet cannot be removed by alkali discount a physical mixture as the likely explanation for the existence of AIP. Evidence to support the hypothesis that acidic protein progressively becomes insoluble is provided by experiments on the incorporation of radioactive amino acids

into ALSP and AIP. Previous work by Keeley (1970) on chick aorta correlated diryrosine concentration with the degree of aggregation of ALSP. A similar correlation is apparent in rat uterus, but does not extend to the insoluble form of acidic protein except, perhaps, for a small fraction like that seen in AIP from human uterus (see p. 111). Other fluorescent compounds, as yet unidentified, may account for the gradual loss of solubility of acidic protein in uterus.

IV. EVIDENCE FOR A COVALENT ASSOCIATION BETWEEN ELASTIN AND AN ACIDIC PROTEIN IN UTERUS

INTRODUCTION

Previous speculations that elastin may be covalently linked to an acidic protein were based on the known difficulty in extracting the acidic protein without solubilizing elastin as well (Partridge, 1962; Petruska and Sandberg, 1968; Keeley, 1970; Keeley and LaBella, 1972). Much stronger evidence would be the isolation of a protein or large peptide fragment with an amino acid composition intermediate to those of elastin and acidic protein. To this end, AIP was partially hydrolyzed, and the fragments fractionated by ion-exchange chromatography.

Sandberg and coworkers developed procedures for the extraction of a soluble, elastin-like protein from aortas of copper-deficient swine (Smith et al., 1968; Sandberg et al., 1969a, 1969b, 1971a, 1971b). This 'tropoelastin' was rich in lysine and contained no desmosine crosslinks. The most recent modification of their procedure (Sandberg et al., 1971b), employing extraction in propanol/butanol/water, was used in this study. Pregnant bovine uterus was chosen because, in the absence of copper deficiency, the levels of soluble precursors of elastin might be expected to be very low. Uterus from pregnant cow furnished a large amount of

tissue; and it is known that elastin is synthesized rapidly in pregnancy (Woessner and Brewer, 1963).

METHODS

Non-pregnant Human Uterus

Preparation of AIP. The uterus of a 47-year old woman of unknown parity and medical history was obtained at autopsy and treated as indicated in Section III (p. 36).

Solubilization of AIP. To 300 mg of AIP was added 60 ml of 6N HCl at 95°C. The suspension was maintained at 95°C with agitation for 15 minutes, diluted 2:1 with cold distilled water and filtered. The residue was washed with distilled water, dehydrated in acetone, and air dried. The filtrate was neutralized, concentrated on a rotary evaporator under vacuum, dialyzed against distilled water and lyophilized.

Chromatography and DEAE- and CM- cellulose. A 1 cm x 100 cm DEAE-cellulose (Bio-Rad Laboratories, Cellex-D) column was equilibrated in 0.05M phosphate buffer at pH 8.2. Samples prepared in the same buffer were applied to the column and eluted with a linear gradient of 0 - 0.5M NaCl in the phosphate buffer (400 ml total volume). A CM-cellulose (Bio-Rad Laboratories, Cellex-CM) column, 2.5 cm i.d., with a bed volume of 70 ml, was equilibrated in 0.1M acetate buffer at pH 4.5. Samples were applied to the column in the same

buffer and eluted with a linear gradient of 0 - 0.3M NaCl in acetate buffer (200 ml total volume).

Fractions were monitored for protein in an automated procedure based on the method of Lowry (Lowry et al., 1951). Pooled fractions corresponding to the peaks were reduced in volume on a rotary evaporator under vacuum and either desalted on Sephadex G10 (Pharmacia Fine Chemicals) or dialyzed against distilled water and lyophilized.

Pregnant Bovine Uterus

Isolation of a putative soluble elastin precursor. The method used was based on that of Sandberg (Sandberg et al., 1971b) for the isolation of 'tropoelastin' from copper-deficient swine. The bovine uterus contained a single fetus estimated to be about 150 days old, based on the crown-to-rump length (Bogart, 1959). The fetus was removed and the uterus sliced into pieces about one inch square and frozen in liquid nitrogen. The frozen pieces were pulverized in a stainless steel mortar and ground to 0.05 inch mesh in a hammer mill in the presence of powdered dry ice. A flow chart of the subsequent manipulations, which were carried out at 4°C, is given in Figure 8. The uterus homogenate was suspended in 0.02M formic acid at 10 ml buffer/g wet tissue

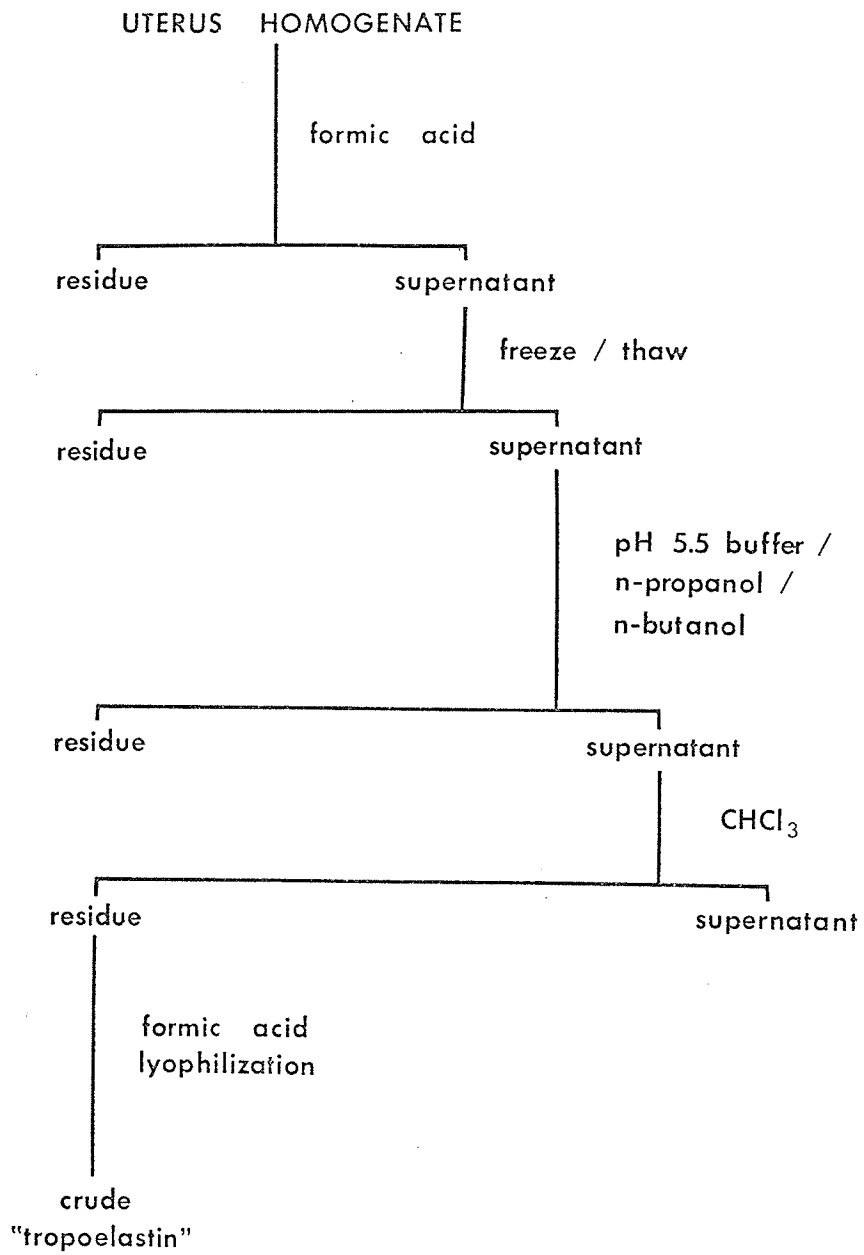


Figure 8: Flow chart of extraction procedures applied to pregnant bovine uterus. All manipulations were carried out at 4°C. This scheme is based on the method of Sandberg and coworkers (1971b) for the isolation of tropoelastin from aortas of copper-deficient swine.

for two 24-hour periods. At the end of each extraction period, the supernatants were separated from the residue by centrifugation at 810,000 g-minutes. Supernatants were frozen and the final residue was discarded. Thawed extracts were pooled and recentrifuged as before. The residue was discarded. Ammonium sulfate was added to the supernatant to give a 40% saturation (242.3 g/l) and the solution was stirred for 24 hours. The residue was collected by centrifugation at 810,000 g-minutes and the supernatant discarded. The residue was dissolved in 0.1M ammonium formate buffer at pH 5.5 (20 ml buffer/g of wet precipitate) and dialyzed against the same buffer for 24 hours. The pH of the retentate was adjusted to 5.5. To 2 volumes of the retentate were added slowly 3 volumes of n-propanol, followed by 5 volumes of n-butanol. The addition of each of the alcohols was carried out over 2 - 3 hours. When addition was complete, the flask was sealed with Parafilm and the mixture was stirred for 24 hours. The suspension was filtered and the precipitate discarded. The filtrate was taken to dryness on a rotary evaporator under vacuum, extracted twice with chloroform (BDH, 'ANALAR' grade) and filtered. The residue was dissolved in a small amount of 0.02M formic acid, dialyzed

against the same solution for 24 hours and lyophilized. This material was referred to as crude 'tropoelastin'.

Gel filtration chromatography. A Sephadex G100 (Pharmacia Fine Chemicals) column (bed volume = 153 ml; void volume = 54 ml) was prepared in 10% acetic acid. A 10 mg sample of crude 'tropoelastin' was suspended in 1 ml of the same solution. Since not all of the sample dissolved, the suspension was filtered and only the supernatant was applied to the column. A Sephadex G100 column (bed volume = 310 ml; void volume = 86 ml) was prepared in 0.13M pyridine-0.07M collidine at pH 9.9. A 10 mg sample of crude 'tropoelastin' was dissolved in 1 ml of the same solution and applied to the column.

The positions of the peaks were determined, either by absorbance at 280 nm monitored on an Isco model UA-2 Ultraviolet Analyzer or by measuring protein in an automated procedure based on the method of Lowry (Lowry et al., 1951). Pooled fractions corresponding to the peaks were evaporated to dryness on a rotary evaporator under vacuum.

Disc electrophoresis. Gels of 7.5% polyacrylamide were prepared in borate-NaOH buffer (I = 0.44). Samples of 100 to 150 μg were prepared in a solution of 0.1N NaOH con-

taining 15.6% sucrose. Final volume applied to the gels was 100 μl. Electrophoresis was carried out at 3 mA/tube for 9 hours. A marker dye mixture (Gelman Instrument Co., RBY Reference Dye) was used to monitor the progress of the electrophoresis. A preliminary 2-hour run had established that no component of the crude 'tropoelastin' moved faster than the red component of the dye mixture. After nine hours, the red component had travelled about 80% of the length of its gel column. Simultaneous fixing and staining was carried out with 0.25% Coomasie blue in 20% trichloroacetic acid for 90 minutes. Destaining was carried out in 2% acetic acid.

RESULTS

Non-pregnant Human Uterus

Brief treatment with 6N HCl under the conditions indicated solubilized 99% of the AIP from human uterus, but only 33% remained non-dialyzable. Table XIX shows the amino acid compositions of AIP, the solubilized, non-dialyzable material and the non-solubilized residue.

The HCl-solubilized fraction from human uterus AIP was applied to CM-cellulose (Figure 9) or to DEAE-cellulose (Figure 10). The early, broad peak on DEAE-cellulose (D1, Figure 10) was concentrated, desalted and applied to CM-cellulose. It gave a single, major discrete peak (E1, Figure 11). The amino acid compositions of some of these fractions from ion-exchange chromatography are given in Table XX. The fractions were found to have compositions estimated to contain 40 - 65% acidic protein.

Pregnant Bovine Uterus

The crude 'tropoelastin' fraction from bovine uterus had considerable polar character (Table XXI). Unlike the material isolated from aortas of copper-deficient swine by Sandberg and associates (1971b), it did not dissolve completely in 10% acetic acid. The acetic acid-soluble fraction

TABLE XIX

COMPOSITIONS OF AIP FROM HUMAN UTERUS AND DIALYZED FRACTIONS DERIVED FROM AIP BY SOLUBILIZATION IN 6N HCl

	AIP	HCl-insoluble	HCl-solubilized
	(residues/1000 total amino acid residues)		
HPRO	7.5	0	2.1
ASP	38	77	56
THR	22	42	29
SER	22	47	41
GLU	65	98	95
PRO	83	66	68
GLY	198	75	110
ALA	177	99	197
VAL	116	77	79
MET	4.8	15	6.1
ILE	35	56	40
LEU	83	99	79
TYR	27	35	29
PHE	33	53	34
LYS	40	42	57
HIS	9.7	18	12
ARG	29	50	31
Dityrosine	0.06	0.11	0.06
% Acidic Protein	51	100	73

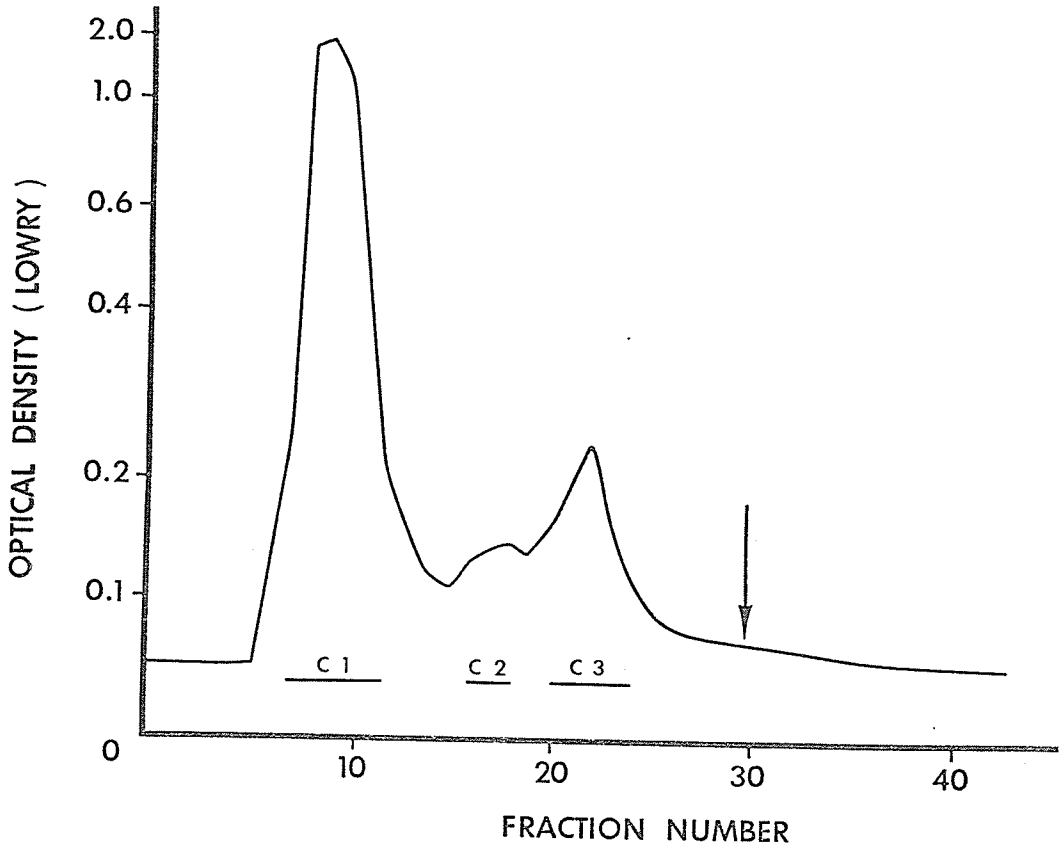


Figure 9: Ion-exchange chromatography of solubilized AIP from human uterus. Sample was applied in 5 ml 0.1M acetate buffer at pH 4.54 to a CM-cellulose column equilibrated in the same buffer. Elution was carried out with a linear gradient of 0 - 0.3M NaCl, followed by a wash with 0.5M NaCl (arrow). Fractions of 7 ml volume were collected.

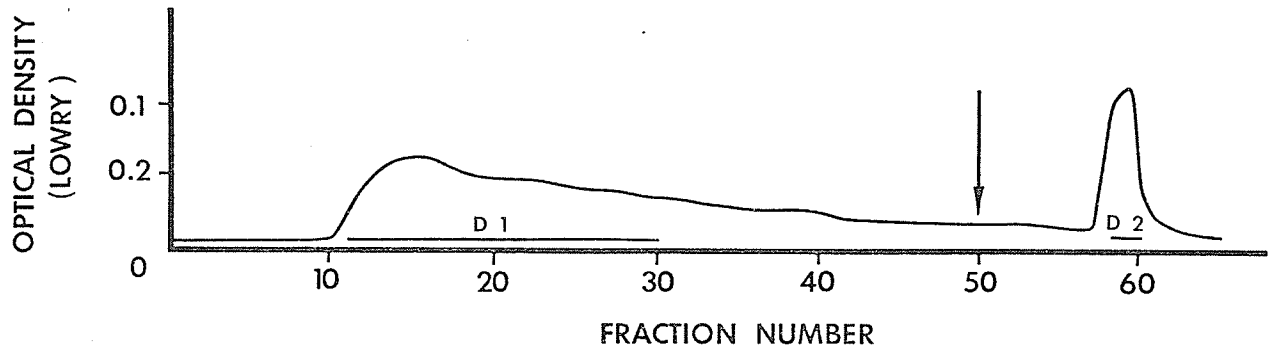


Figure 10: Ion-exchange chromatography of solubilized AIP from human uterus. Sample was applied in 5 ml 0.05M phosphate buffer at pH 8.2 to a DEAE-cellulose column equilibrated in the same buffer. Elution was carried out with a linear gradient of 0 - 0.5M NaCl followed by a wash with 1.0M NaCl (arrow). Fractions of 8 ml volume were collected.

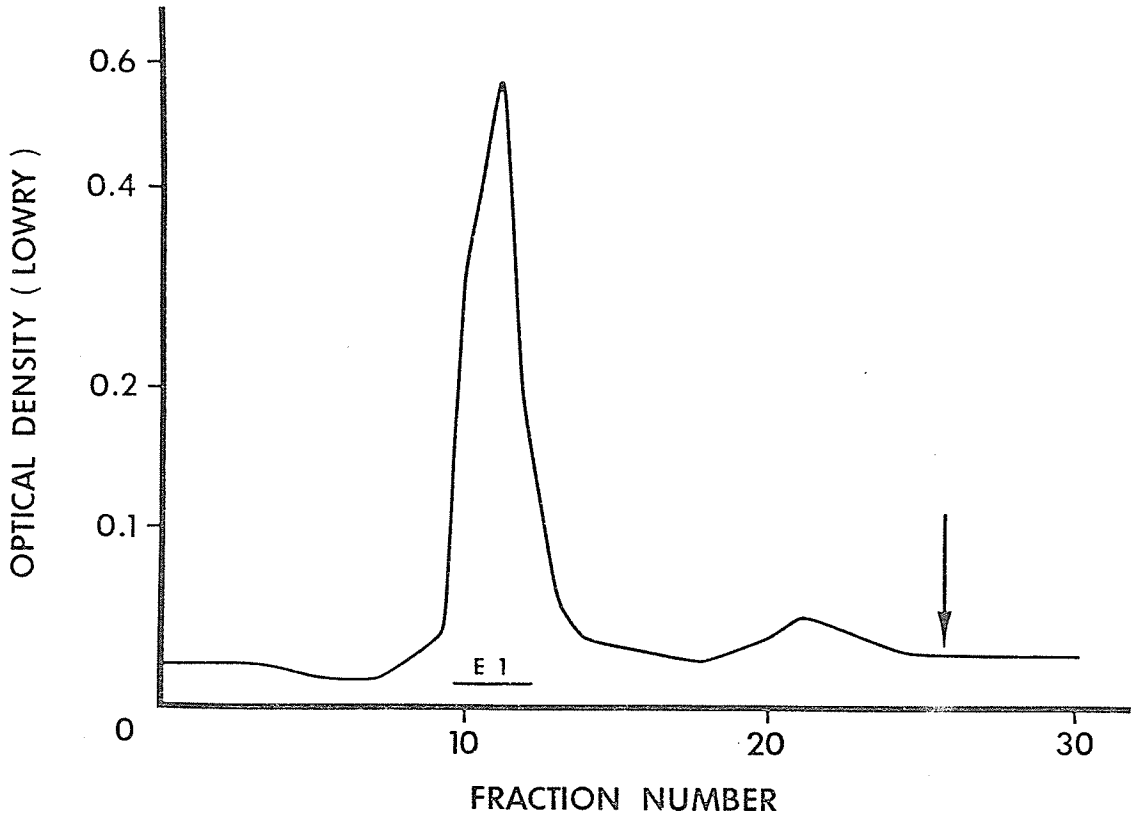


Figure 11: Rechromatography of peak D1 from DEAE-cellulose on CM-cellulose. Sample was applied in 5 ml 0.1M acetate buffer at pH 4.54 to a CM-cellulose column equilibrated in the same buffer. Elution was carried out with a linear gradient of 0 - 0.3M NaCl, followed by a wash with 0.5M sodium acetate at pH 7.0 (arrow). Fractions of 8 ml volume were collected.

TABLE XX

COMPOSITIONS OF FRACTIONS OBTAINED BY ION-EXCHANGE CHROMATOGRAPHY OF SOLUBILIZED HUMAN UTERUS AIP

	C1	C3	E1
	(residues/1000 total amino acid residues)		
HPRO	0	7.9	0
ASP	46	59	44
THR	15	23	11
SER	17	30	18
GLU	90	108	82
PRO	79	80	79
GLY	158	122	155
ALA	281	190	311
VAL	73	62	75
MET	8.6	8.2	6.7
ILE	28	32	26
LEU	62	78	63
TYR	33	34	31
PHE	22	26	21
LYS	41	68	39
HIS	2.3	5.4	1.1
ARG	5.1	10	2.7
Dityrosine	0.05	0.08	0
% Acidic Protein	46	66	39

TABLE XXI

AMINO ACID COMPOSITIONS OF CRUDE 'TROPOELASTIN' FROM BOVINE UTERUS AND ITS ACETIC ACID-SOLUBLE FRACTION (residues/1000 total amino acid residues)

	'tropoelastin'	
	crude	acetic acid-soluble
HPRO	1.7	0
ASP	77	84
THR	63	43
SER	69	59
GLU	92	79
PRO	92	295
GLY	101	152
ALA	67	40
VAL	70	38
MET	8.8	4.4
ILE	47	20
LEU	83	63
TYR	26	4.5
PHE	32	18
LYS	51	36
HIS	24	32
ARG	86	26
'A'*	n.d.	23
Dityrosine	<0.005	<0.005

* expressed as peak area in cm^2/μmole total amino acids.

was eluted as a single peak on Sephadex G100 and had an amino acid composition which was less polar than the original material and had an extraordinarily high proline content (Table XXI). The crude 'tropoelastin' dissolved completely at pH 9.9 and was eluted from Sephadex G100 in three peaks (Figure 12). Peak B had an amino acid composition calculated to be equivalent to 80% $\pm$ 10% acidic protein (Table XXII). Peak C was very similar to acidic protein (calculated to be equivalent to 120% $\pm$ 17% acidic protein) (Table XXII). Polyacrylamide gel electrophoresis showed that peak B contained a single component and was different from the authentic tropoelastin of Sandberg and coworkers (Figure 13).

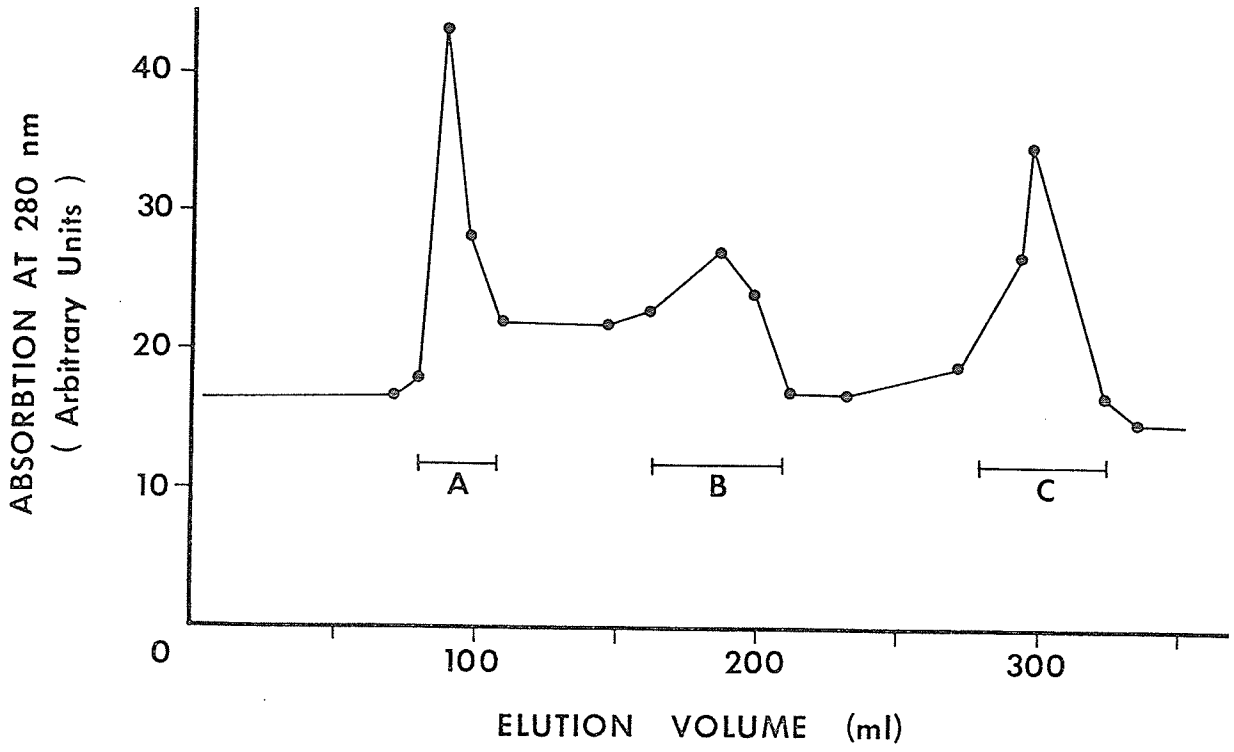


Figure 12: Gel filtration chromatography of crude 'tropoelastin' fraction from pregnant bovine uterus. Ten mg sample was applied in 1 ml 0.125M pyridine-0.074M collidine solution to a Sephadex G100 column equilibrated in the same buffer. Fractions of 7.3 ml volume were collected.

TABLE XXII

AMINO ACID COMPOSITION OF FRACTIONS FROM GEL FILTRATION CHROMATOGRAPHY OF CRUDE 'TROPO-ELASTIN' FROM BOVINE UTERUS
(residues/1000 total amino acid residues)

	G100B	G100C
HPRO	0	0
ASP	114	81
THR	51	65
SER	62	74
GLU	152	76
PRO	99	78
GLY	112	88
ALA	68	62
VAL	66	75
MET	4.4	5.3
ILE	40	45
LEU	78	95
TYR	19	33
PHE	25	62
LYS	39	59
HIS	25	26
ARG	36	65
'A'*	>14	>14
Dityrosine	0.01	0.05

* expressed in cm^2 peak area/μmole total amino acids.

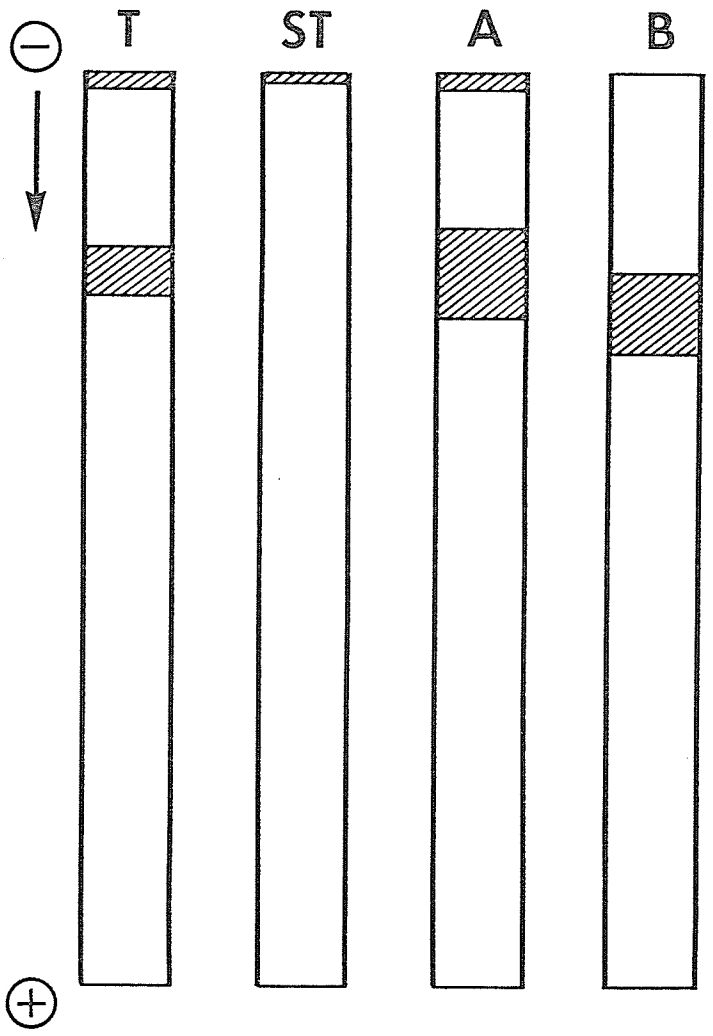


Figure 13: Sketch of stained columns after polyacrylamide gel electrophoresis. Crude 'tropoelastin' fraction (T) from bovine uterus contained two components, one of which did not migrate. Authentic tropoelastin from aorta of copper-deficient swine (ST) showed a single, non-migrating component. Peak A from G100 gel filtration chromatography of the crude 'tropoelastin' (A) showed both components of the crude preparation. Peak B (B) showed only the migrating component absent from authentic tropoelastin.

DISCUSSION

The 1% of the AIP from human uterus that was not solubilized by 6N HCl under the conditions used was rich in dityrosine, suggesting that at least part of the AIP represented a more highly polymerized form of acidic protein, with dityrosine as a possible crosslink. Comparison of the amino acid composition of the starting material with that of the insoluble residue and the solubilized material indicated that elastin was hydrolyzed to dialyzable fragments to a greater extent than was acidic protein. Interpretation of this result is difficult. The most significant observation, however, is that each of the fragments recovered from ion-exchange chromatography had a composition intermediate to those of elastin and ALSP. This is strong evidence for a covalent connection between elastin and acidic protein in uterus. However, it cannot differentiate between acidic protein and elastin moieties synthesized as a single chain (Keeley, 1970; LaBella, 1971) and a covalent, elastin-acidic protein complex occurring during the formation of the elastic tissue network (Partridge, 1962; Hall, 1971).

The pregnant uterus is a good source of newly synthesized elastin (Woessner and Brewer, 1963), but attempts

to isolate a soluble elastin resulted in the extraction of a soluble protein with an amino acid composition intermediate to those of elastin and acidic protein. It is possible that this material is the 'proelastin' suggested by Keeley (Keeley, 1970; LaBella, 1971) to be the soluble, precursor form of elastin.

Many workers have reported the intimate association of elastin with a more polar protein in very young (Cleary et al., 1966, 1967; Steven and Jackson, 1968; Keeley, 1970; Keeley and LaBella, 1972) and very old (Lansing et al., 1951; Hall, 1955; Lansing, 1955; FitzPatrick and Hospelhorn, 1965b; Gotte et al., 1965; LaBella et al., 1966; Hall, 1971) elastic tissues. There may not be any relationship between these apparently similar situations in young and old elastic tissues. It is at least possible that a more polar elastin precursor, analogous to procollagen (Bellamy and Bornstein, 1971; Lapiere et al., 1971; Layman et al., 1971; Muller et al., 1971; Speakman, 1971) is synthesized and accounts for the polar composition of elastin seen in fetal elastic tissues (Keeley, 1970; LaBella, 1971; Keeley and LaBella, 1972). It is possible also, that with age, proteins which are turned over only slowly (e.g. elastin or insoluble

collagen) may aggregate covalently and link to other proteins by their constituent reactive groups or through the mediation of circulating reactive substances (Verzar, 1956; Bjorksten, 1968; Butzow and Eichorn, 1968; Carpenter, 1969; LaBella, 1971, 1972) to form accretion complexes (LaBella, 1972). Thus, we may be examining an elastin precursor in the case of pregnant bovine uterus and an accretion complex in the case of human uterus. In both situations, however, the presence of a covalent connection between elastin and acidic protein is indicated.

V. ELECTRON MICROSCOPY OF UTERINE ELASTIN

INTRODUCTION

As reported above (Section III), the amino acid composition of 'elastin' isolated from rat uterus is atypical. Experiments on the turnover of ALSP and AIP in rat uterus (Section III) indicated that these protein fractions were very active metabolically. The presence of a metabolically active, atypical elastin is characteristic of rapidly growing, fetal elastic tissue.

Microfibrils (MF) have been described as components of connective tissue which are distinct from the other recognized fibrous elements (Low, 1962; Haust, 1965). The work of Greenlee and associates (1966) indicated that MF are stained with uranyl acetate/lead citrate, whereas elastin is unstained. Electron microscopic studies of fibrogenesis in fetal connective tissues have shown the existance of this microfibrillar component on the periphery of the growing elastin fibril (Fahrenbach et al., 1966; Greenlee et al., 1966; Takagi and Kawase, 1967; Fyfe et al., 1968; Ross and Bornstein, 1969; Takagi, 1969a). It has been suggested that the MF are involved in elastin fibrogenesis (Haust, 1965; Fahrenbach et al., 1966; Greenlee et al., 1966; Ross and Bornstein, 1969, 1971) and perhaps are actually continuous

with the elastin core (Keeley, 1970). Biochemical studies (Ross and Bornstein, 1969; Takagi, 1969b; Robert et al., 1971) have indicated that MF have an amino acid composition similar to that of ALSP.

METHODS

Rats of the Long-Evans strain, 110 days old, were used in this study. Pregnant rats were estimated to be within one day of parturition, based on the date of mating. The rats were killed by decapitation and the uteri removed. A small portion of the uterus was excised and immediately immersed in cold 2% glutaraldehyde in Millonig's phosphate buffer at pH 7.2 (Millonig, 1962). Blocks about 1 mm^3 were cut from the tissue and fixed initially for one hour, washed in the phosphate buffer and post-fixed for one hour in osmic acid (Millonig, 1962).

The tissue was stained *en bloc* in 2% aqueous uranyl acetate, dehydrated in alcohol and embedded in Epon (Luft, 1961). Sections were cut on a Reichert Om U_2 ultramicrotome, mounted on uncoated copper grids, stained with Reynold's lead citrate (Sjostrand, 1967), and examined with an Hitachi HS-8 electron microscope, operating at 50 kV.

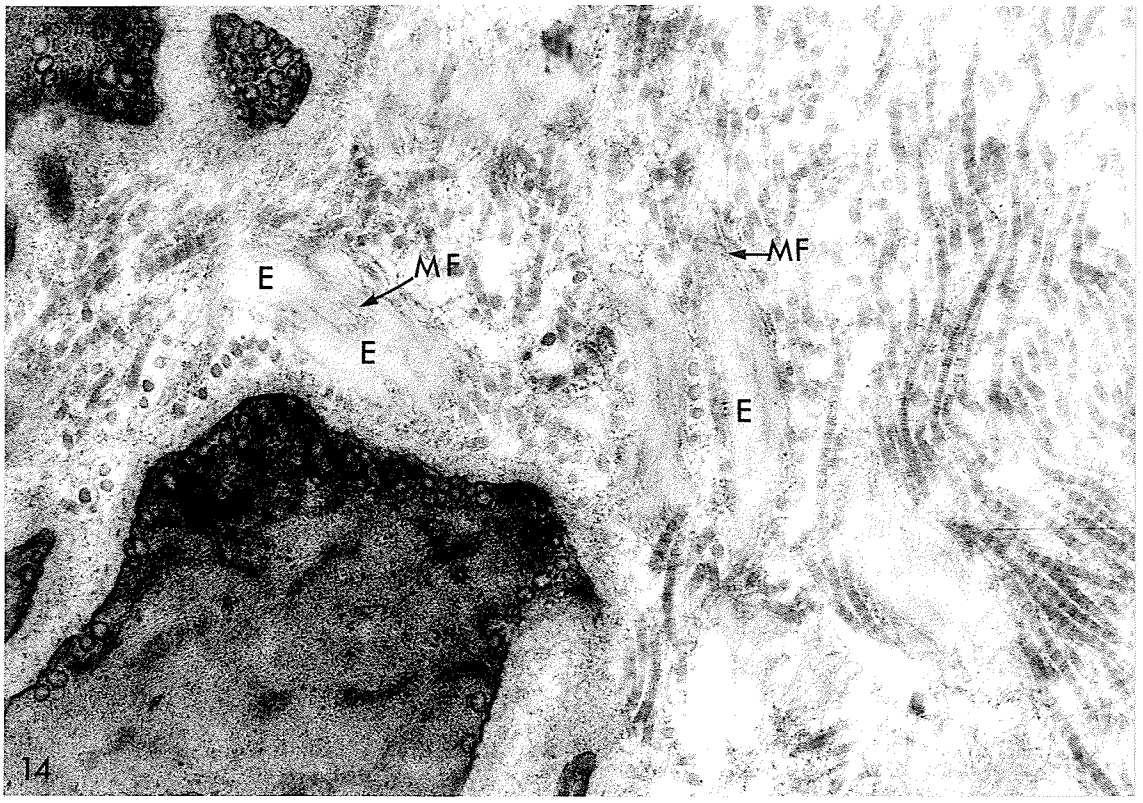
RESULTS AND DISCUSSION

Elastin in the uterus of pregnant rat showed the same characteristics as elastin from fetal bovine ligamentum nuchae (Greenlee et al., 1966). Sections from both non-pregnant (Figure 14) and pregnant (Figure 15) uteri contained MF surrounding, and sometimes embedded in, the electron-lucent elastin core. There was considerable orientation of MF about the elastin fibers (Figure 16). There appeared to be a greater amount of MF associated with elastin in the uterus of pregnant (Figures 15, 16, 17) than in non-pregnant (Figure 14) rats. In fact, the masses of MF seen (Figure 17) were unusually dense, even compared to such tissues as healing wounds (Williams, 1970a, 1970b), fetal aorta (Haust et al., 1965; Takagi and Kawase, 1967; Takagi, 1969a) or fetal ligamentum nuchae (Fahrenbach et al., 1966; Greenlee et al., 1966; Ross and Bornstein, 1969), where elastin synthesis is rapid. Tangled masses of MF without an apparent central core, the first step in elastin fibrogenesis (Greenlee et al., 1966) were observed also in pregnant uterus (Figure 18).

Thus, most of the elastin in uterus of mature rat is apparently newly synthesized and rapidly growing. This picture is intensified in the pregnant rat, where elastin

Figure 14: Section from uterus of non-pregnant rat. Electron lucent elastin core (E) is surrounded by a few electron-dense microfibrils (arrows). x 35,700

Figure 15: Section from uterus of pregnant rat. Elastin (E), collagen (C) and microfibrils (MF) are seen in longitudinal section. Note that, in contrast to non-pregnant uterus, there is considerable inclusion of electron-dense material in the core of the elastin fiber (arrow). x 35,900


E
MF
E
MF
E
14

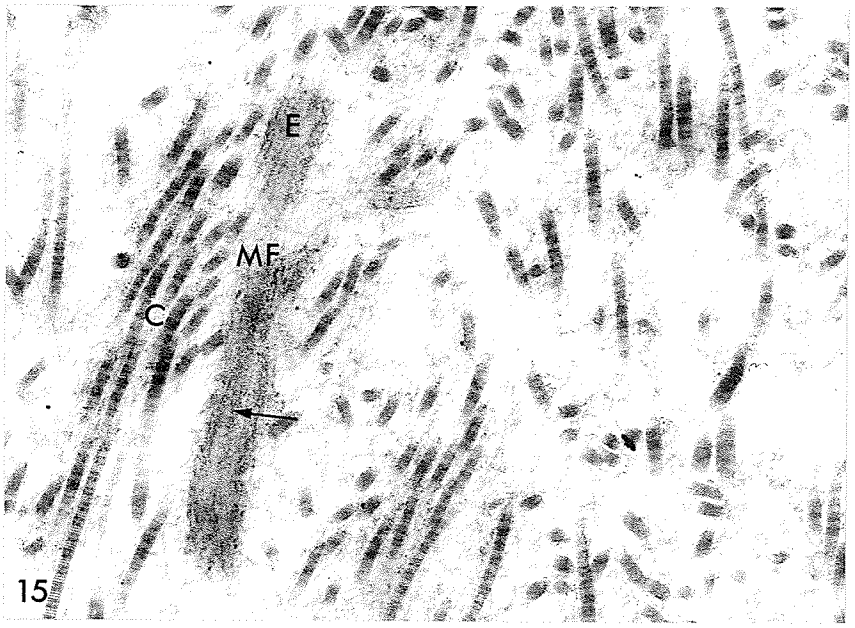
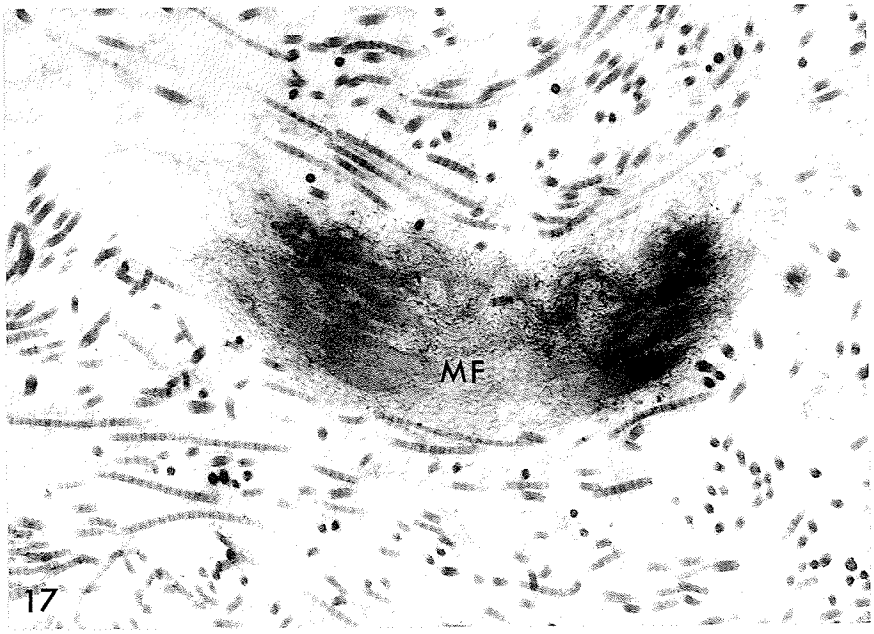

E
MF
C
15

Figure 16: Section from uterus of pregnant rat. Collagen (C), elastin (E) and microfibrillar material (MF) are seen in cross-section in the region of a fibroblast. x 48,100

MF
C

Figure 17: Section from uterus of pregnant rat, showing a dense mass of microfibrils (MF). The core area is not electron-lucent. x 24,200

Figure 18: Section from uterus of pregnant rat, showing a mass of microfibrils without an associated amorphous core (arrow). x 23,200



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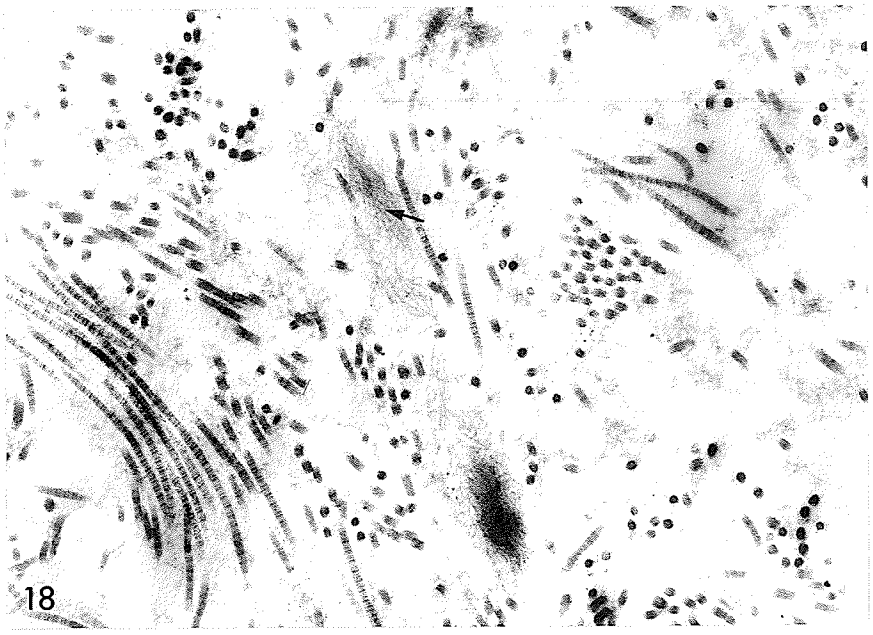

18

biosynthesis evidently is increased markedly, compatible with the finding of a 5- to 6-fold increase in non-collagen hydroxyproline during pregnancy in human uterus (Woessner and Brewer, 1963). No direct demonstration of newly synthesized uterine elastin or of its fetal-like morphology in pregnancy has been reported previously.

VI. CONCLUSIONS

(1) An acidic protein, similar to that postulated to serve a structural function in various other connective tissues, has been extracted from uterus in hot alkali or in mercaptoethanol-urea.

(2) Treatment with hot alkali, an accepted procedure for purification of elastin in other tissues, yields, in uterus, a protein residue of considerable acidic character. This insoluble residue may be considered as a composite of elastin and an acidic protein identical in amino acid composition to that soluble in alkali. Although fetal elastin also shows some degree of acidic character, the composite from uterus is considerably richer in polar amino acids.

(3) Evidence has been obtained for the aggregation of acidic protein in situ. Newly synthesized acidic protein is extracted easily from uterus, but becomes less soluble with time. The form of acidic protein which evidently is aggregated most highly cannot be separated from elastin by treatment with hot alkali.

(4) An apparent covalent attachment of acidic protein to elastin has been found in mature uterus. (a) Special extraction procedures applied to the uterus of pregnant cow yielded a soluble apparent composite of elastin and acidic protein

which may represent a precursor of elastin. If the apparent covalent connection between elastin and acidic protein persists after the crosslinking of elastin, a more polar elastin residue may result. (b) In human uterus, evidence for a covalent connection between elastin and acidic protein has been found in the alkali-resistant residue. Although this insoluble composite could be due to crosslinking of a soluble complex, e.g. the putative precursor of elastin, the possibility of age-related accretion of elastin and acidic protein cannot be dismissed.

(5) The results of this study suggest that uterus might be a suitable organ in which to investigate the interplay of processes which results in deposition, remodelling and removal of the fibers and matrix of connective tissue, as well as their maturation and aging.

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