CELL-MATRIX INTERACTIONS

by

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Ph.D.

UNIVERSITY OF EDINBURGH

1992

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DECLARATION

I declare that this thesis was composed by me, that the work of which it is a record was done by me, except where stated in the thesis. This work has not been submitted elsewhere in any previous application for a degree. All sources of information have been acknowledged.

This thesis is

dedicated

to the memory of my

mother

ACKNOWLEDGEMENTS

I would like to thank Dr D.J.S. Hulmes for his excellent supervision and for providing plenty of time for useful discussions. I am grateful to Mr I.F. Purdom for providing the procollagen C-propeptides, to Dr D.K. Apps for radio-iodination of the C-propeptides, to Dr G.L. Atkins for analysis of the binding data, and to Dr J.H.P. Sizer, Dr J. Perez-Castineira and Dr J. Torbet for helpful discussions. The work was funded by a Wellcome Trust Prize Studentship.

I wish to thank Dr D.J.S. Hulmes, Dr D.R.Shackleton, Dr T.J.Wess, Mr G.W. Fiaux, Mr I.F. Purdom, Mr M.E. Marsden, Mr E.W. Forbes and Mr J.R.E. MacBeath for providing a pleasant environment to work in. Special thanks are due to Dad, Jamil and Shaheen for their support and encouragement during my student years.

ABSTRACT

Cellular involvement in collagen fibril organisation is a poorly understood phenomenon. The localisation of procollagen to the cell membrane coincident with secretion, the identification of cell surface receptors for collagen, and the assembly of collagen fibrils within extracellular compartments suggest a role for plasma membranes in the assembly and organisation of collagen fibrils. Cell-collagen interactions may be mediated by collagen receptors, cell surface proteoglycans and also membrane lipids. The interactions of type I collagen with phosphatidylcholine have been described and there have been several reports of lipid association with collagen.

The interactions of [^{3}H]procollagen I with various phospholipids were studied by density gradient centrifugation. At physiological conditions of pH, ionic strength and temperature, there was no evidence of procollagen binding to phosphatidylcholine phosphatidylethanolamine, phosphatidylinositol or phosphatidylserine liposomes. In contrast type I procollagen bound strongly to sphingomyelin liposomes, in a reversible and saturable manner, with an apparent dissociation constant (K_d) of 2.6 nM. Binding occurred over a range of temperatures (4 °C to 35 °C) and was relatively unaffected by salt concentrations up to 1.2 M NaCl. Binding was observed in phosphate buffers but not in the presence of high concentrations of Tris or Hepes. Bovine serum albumin had no effect on procollagen binding to sphingomyelin, neither did unlabeled type I collagen, with or without the non-helical telopeptides. Type II procollagen and denatured type I procollagen also bound to sphingomyelin. Procollagen binding to sphingomyelin at 35 °C was considerably reduced when small amounts of phosphatidylcholine were present, though binding was partially restored when the temperature was reduced below the corresponding phase transition temperature. Purified, unlabeled procollagen C-propeptides successfully competed for binding, and [^{125}I]C-propeptides bound to sphingomyelin in the absence of procollagen. Weaker binding to sphingomyelin, mediated by the collagen triple helical region, was also observed, but this was dominated by the sphingomyelin C-propeptide interaction. The data suggest a novel mechanism for matrix vesicle mediated biomineralisation.

Dextran sulphate enhanced the activity of both purified procollagen C- and N-proteinases. Dextran sulphate-induced procollagen aggregates observed with electron microscopy after rotary shadowing may provide better substrates for the procollagen proteinases. Heparin inhibited the activity of the C-proteinase, but had no effect on the N-proteinase. Cell surface associated proteoglycans may play a role in controlling the activity of the procollagen processing enzymes.

CONTENTS

Page

CHAPTER 2: GENERAL MATERIALS AND METHODS

ABBREVIATIONS

Amino acids

Ala	alanine
Asn	asparagine
Cys	cysteine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
Lys	lysine
Phe	phenylalanine
Pro	proline
Hyp	hydroxyproline

Other abbreviations

α-chain	Individual polypeptide chain of collagen
BAPN	β-aminopropionitrile
BSA	bovine serum albumin
C-propeptide	carboxy-terminal domain of procollagen
Ca^{2+}	calcium ions
Ci	Curies
COL	triple helical domain
cpm	counts per minute
DEAE	diethylaminoethyl
ΔH	enthalpy change
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid, disodium salt
EM	electron microscope
ER	endoplasmic reticulum
FCS	foetal calf serum
GAG	glycosaminoglycan
^{3}H	tritium
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HS	heparan sulphate

MEM	Minimal Essential Medium
ml	millilitre (s)
mmol	millimole (s)
mM	millimolar
Mr	molecular weight
μmol	micromole (s)
μg	microgram (s)
nm	nanometre (s)
nM	nanomolar
N-propeptide	amino-terminal propeptide of type I procollagen
NEM	N-ethyl maleimide
OI	Osteogenesis imperfecta
PBS	phosphate buffered saline
pC	pC-collagen: procollagen with the N-propeptide absent
PC	dimyristoylphosphatidylcholine
PEG	polyethylene glycol
PG	proteoglycan
pN	pN-collagen: procollagen with the C-propeptide absent
PPO	2, 5-diphenyloxazole
proα1	α1 chain of procollagen
proα2	α2 chain of procollagen
PS	penicillin-streptomycin
PTA	phosphotungstic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
SLS	segment-long-spacing
SP-A	surfactant protein-A
SPM	sphingomyelin
TEMED	N, N, N', N'-tetramethylethylenediamine
TGF-β	transforming growth factor-β
Tris	Tris (hydroxymethyl) aminomethane

CHAPTER 1

GENERAL INTRODUCTION

1.1 Extracellular matrix

The extracellular matrix (ECM) is the diverse structural material that surrounds connective tissue cells, and lies under epithelial and muscle cells (Brown *et al.*, 1991; Farquhar, 1991). It is composed of four major classes of macromolecules, collagen, proteoglycans (see chapter 4), elastin (Mecham and Heuser, 1991), and glycoproteins which are distributed in a tissue-specific manner, and provide the different ECMs with their distinctive structural and functional properties (for reviews, see Labar-Robert *et al.*, 1990; Brown *et al.*, 1991; Engel, 1991; Hay, 1991; Sage and Bornstein, 1991; Yamada, 1991). In addition to providing structural support in tissues, the ECM plays a major role in cell differentiation and cell migration during embryogenic morphogenesis, wound healing and metastasis. Cell-matrix interactions involve multiple mechanisms. For example, the fibrillar collagens, proteoglycans and glycoproteins such as fibronectin (Ruoslahti, 1988), laminin (Beck *et al.*, 1990) and vitronectin, have been shown to interact with integrins (Humphries, 1990; Ruoslahti, 1991), heparan sulphate proteoglycans (see chapter 4) or a variety of other cell surface receptors (for reviews, see Hay, 1991; Toole, 1991).

The formation of collagen fibrils and the arrangement of these fibrils into specific three dimensional patterns is an important element in establishing the ordered architecture of extracellular matrices. The precise molecular mechanisms that regulate the structure of collagen fibrils and their spatial arrangement are not understood. The following sections review current knowledge in the field of collagen research, with particular emphasis on the role of cells on collagen fibril assembly.

1.2 Collagens

The collagens are the major structural components of the extracellular matrix which include in their structure one or several domains with a characterisitic triple helical conformation (Figure 1.1). The amino acid sequence of the three constituent chains are made up of a repetition of Gly-X-Y triplets. Collagens constitute a family of related

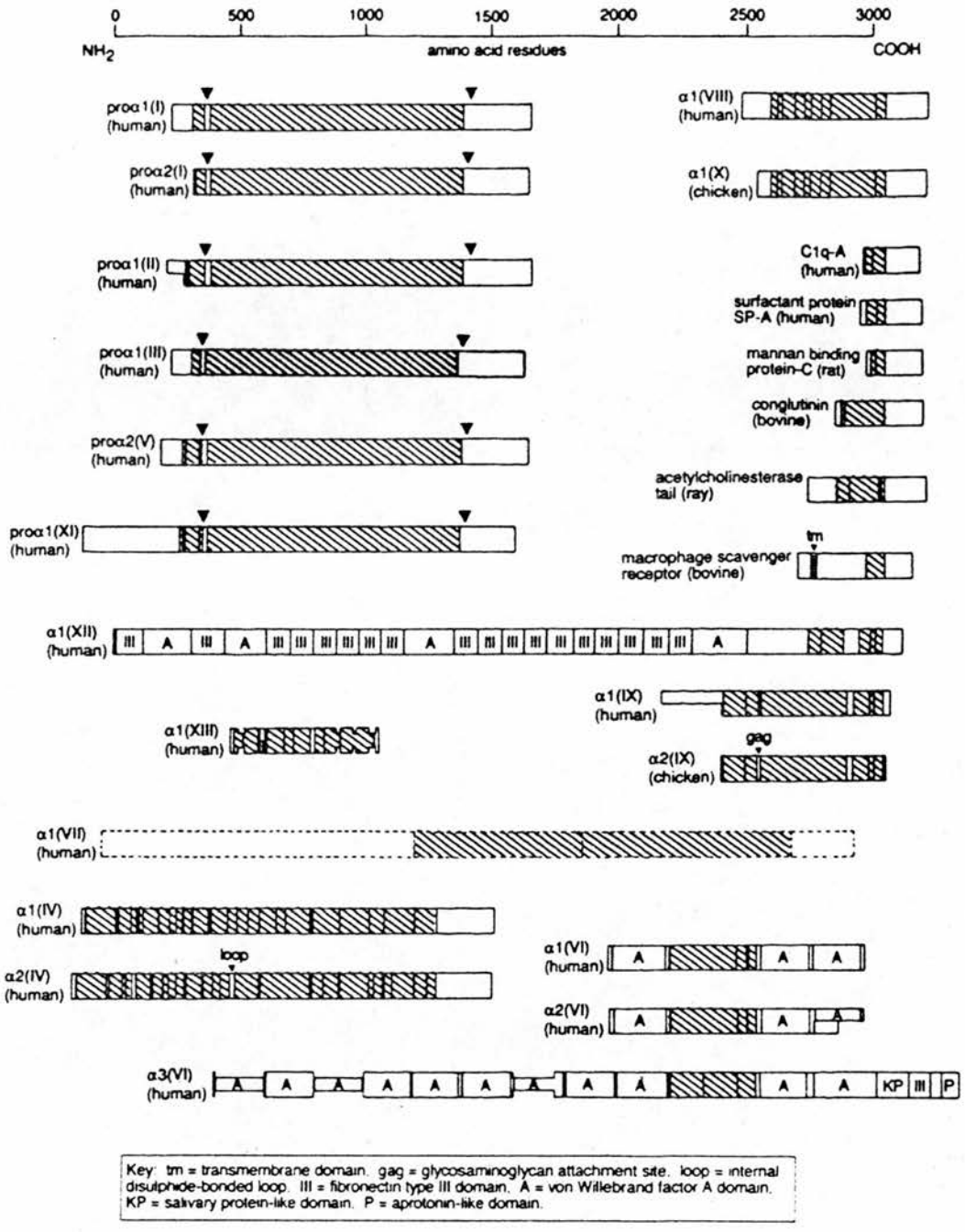


Figure 1.1 A diagrammatic representation of the amino acid sequence of collagen α (or proα) chains and collagen-like protein chains.

The shaded regions show the (Gly-X-Y) sequence. Short interruptions in the (Gly-X-Y) sequence are indicated by vertical black lines. The non-triple helical regions are shown as white boxes which are subdivided where appropriate into discreet structural domains. Boxes of half-height indicate the absence of certain sequences caused by alternative splicing. Propeptide cleavage sites are indicated by the arrows (from Hulmes, 1992, with permission).

Table 1.1 Vertebrate collagens

Type	α-chains	Molecular forms	Tissue distribution
I	α1(I), α2(I)	$[\alpha1(I)]_2\ \alpha2(I)$[1]	Skin, bone, tendon, cornea, dentin, vascular tissue
II	α1(II)	$[\alpha1(II)]_3$	Cartilage, vitreous homour
III	α1(III)	$[\alpha1(III)]_3$	Skin, vascular tissue
IV	α1(IV), α2(IV), α3(IV) α4(IV), α5(IV)	$[\alpha1(IV)]_2\ \alpha2(IV)$	Basement membranes
V	α1(V)[2], α2(V), α3(V)	$[\alpha1(V)]_2\ \alpha2(V)$	Skin, bone
VI	α1(VI), α2(VI), α3(VI)	α1(VI) α2(VI) α3(VI)	Most connective tissues
VII	α1(VII)	$[\alpha1(VII)]_3$	Anchoring fibrils
VIII	α1(VIII), α2(VIII)	$[\alpha1(VIII)]_3$?	Descemet's membrane endothelial cells
IX	α1(IX), α2(IX), α3(IX)	α1(IX) α2(IX) α3(IX)	Cartilage, vitreous humour
X	α1(X)	$[\alpha1(X)]_3$	Hypertrophic zone of cartilage
XI	α1(XI), α2(XI), α3(XI)[3]	α1(XI) α2(XI) α3(XI)	Cartilage
XII	α1(XII)	$[\alpha1(XII)]_3$	Skin, tendon
XIII	α1(XIII)	$[\alpha1(XIII)]_3$	Endothelial cells
XIV	α1(XIV)	$[\alpha1(XIV)]_3$	Skin, tendon

[1] $[\alpha1\ (I)]_3$ is a minor form present in skin.

[2] There is a high degree of primary structure similarity between the α1(V), α3(V), α1(XI) and α2(XI) collagen chains.

[3] α3(XI) is closely related to α1(II).

proteins that are assembled in a variety of supramolecular structures (Mayne and Burgeson, 1987; Vuorio and de Crombrugghe, 1990; van der Rest and Garrone, 1991; Hulmes, 1992). The variation in length, primary structure, and the presence, extent and location of both non-collagenous domains and interchain disulphide bonding, form the basis for categorising the different collagen types.

To date 14 collagen types have been identified and characterised (Robert *et al.*, 1990; van der Rest and Garrone, 1991; Hulmes, 1992; see Table 1.1). The collagens are divided into two main classes based on their supramolecular structure: fibril forming collagens and non-fibril forming collagens (Miller 1985; Vuorio and de Crombrugghe, 1990). The former group contains molecules with a single uninterrupted helical domain approximately 300 nm in length, which are constituents of banded collagen fibrils. The non-fibril forming collagens are more heterogeneous and have been further classified according to their molecular characteristics, supramolecular structures and types of extracellular networks that they form.

1.2.1 Fibrillar collagens (I, II, III, V, XI)

Based on the protein and gene structures, types I, II, III, V and XI collagens have been assigned to the fibril forming group (Miller 1985; Vuorio and de Crombrugghe, 1990; van der Rest and Garrone, 1991). They have the same overall molecular structure[4] , and form highly organised fibrils composed of quarter staggered molecules (see section 1.8.1), which provide the structural support for organs and connective tissues.

Type I collagen is the most abundant fibrillar collagen isolated from many adult connective tissues, such as skin, bone, tendon and cornea. It is a heterotrimer of two identical α1 (I) chains[5] and one α2 (I) chain, although small amounts of homotrimeric $[\alpha 1\ (I)]_3$ molecules have been observed. Homotrimeric type II collagen, $[\alpha 1\ (II)]_3$, is the major collagenous component of cartilage. Another homotrimer, type III collagen

[4] For the molecular structure of type I procollagen, see Figure 1.6.

[5] Nomenclature: Different chains within a single molecule are distinguished by arabic numerals, while different collagen types are distinguished by roman numerals in parentheses. For example, type I collagen has the chain compostion $[\alpha 1\ (I)]_2\,\alpha 2\ (I)$.

$[\alpha 1\ (III)]_3$ is found in skin and blood vessels (Kuhn, 1987). It has recently been demonstrated that in skin, fibrils are composed of both types I and III collagen (for more details see section 1.8.3.3). Other supramolecular interactions between fibrillar collagen types have been demonstrated for types I and V (Linsenmayer *et al.*, 1988) and types II and XI (Mendler *et al.*, 1989). The most common structure for type V is $[\alpha 1\ (V)]_2\ \alpha 2\ (V)$, but the homotrimer $[\alpha 1\ (V)]_3$ and heterotrimer $\alpha 1\ (V)\ \alpha 2\ (V)\ \alpha 3\ (V)$ have also been detected (Fessler and Fessler, 1987). The predominant form of type XI collagen is $\alpha 1\ (XI)\ \alpha 2\ (XI)\ \alpha 3\ (XI)$ (Eyre and Wu, 1987). The $\alpha 1\ (XI)$ chain has been identified as a constituent of bone type V collagen, exhibiting a chain composition $\alpha 1\ (V)\ \alpha 2\ (V)\ \alpha 1\ (XI)$ (Nigibizi and Eyre, 1989). Sequence data have demonstrated a remarkable degree of primary structure similarity between the $\alpha 1\ (V)$, $\alpha 3\ (V)$, $\alpha 1\ (XI)$ and $\alpha 2\ (XI)$ collagen chains (Kimura *et al.*, 1989; Bernard *et al.*, 1988). $\alpha 3\ (XI)$ and $\alpha 1\ (II)$ have been suggested to be products of the same gene (see van der Rest and Garrone, 1991).

Using type I procollagen as the prototype, the fibrillar collagens are discussed in further detail in the following sections.

1.2.2 Non-fibrillar collagens

The non-fibril forming class of collagens is very heterogeneous both structurally and functionally and can be separated into two groups: collagens that constitute the components of different extracellular matrix networks (types IV, VI, VII, VIII, and X) and collagens that interact directly with the fibril forming collagens (types IX, XII and XIV).

1.2.2.1 Fibril-associated collagens (IX, XII, XIV)

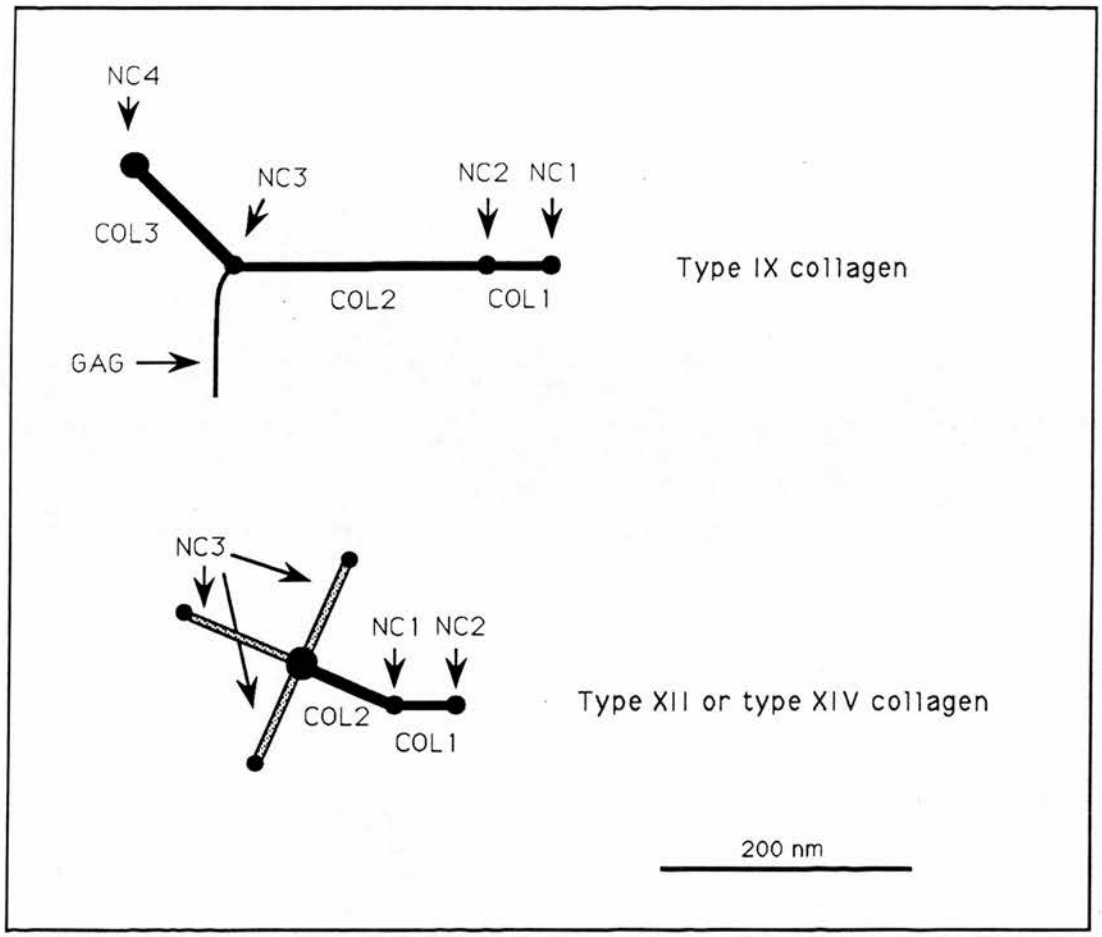
The fibril-associated collagens with interrupted triple helices (FACIT), contain the collagens IX, XII and XIV (Gordon *et al.*, 1989; Gordon and Olsen, 1990; van der Rest *et al.*, 1991; Shaw and Olsen, 1991). These proteins do not form fibrils, but

appear to be associated with the surface of fibrils. In each molecule, a non-collagenous domain acts as a flexible hinge, permitting a portion of the molecule to associate laterally with fibril surfaces, while other domains project out from the surface of the fibril (Vaughan *et al.*, 1988). This allows the projecting domain to interact with cells or other extracellular matrix components (Gordon *et al.*, 1989).

Type IX collagen is the best characterised molecule of this group (Vaughan *et al.*, 1988). The heterotrimeric, disulphide bonded type IX collagen is expressed in cartilage and in the primary corneal stroma of chick embryos (Svoboda *et al.*, 1988; Brewton *et al.*, 1991). The type IX molecule consists of three triple helical domains[6] COL1, COL2 and COL3 (with some single residue imperfections) interspersed between four non-collagenous domains (NC). Type IX molecules have been localised by immunoelectron microscopy on the surface of the type II collagen fibrils of cartilage, where they are found in register with the D-period of the fibril (Vaughan *et al.*, 1988). This arrangement allows covalent cross-linking to occur between a lysyl residue of the COL2 domain and a lysine in the amino telopeptide of type II collagen. The NC3 domain has a covalently bound chondroitin sulphate or dermatan sulphate side chain (van der Rest and Mayne, 1987; Yada *et al.*, 1990), and also provides the flexible hinge that allows the COL3 and NC4 domains to project from the fibril surface (Vaughan *et al.*, 1988; see Figure 1.2). The NC4 has an estimated pI of about 11, and is therefore likely to interact with the acidic proteoglycans found in the cartilage matrix. The localisation of type IX collagen on fibril surfaces has also prompted suggestions that it plays a role in regulation of the fibril diameter (van der Rest and Mayne, 1987). The type IX amino-terminal domains differ in cartilage and embryonic cornea (Shaw and Olsen, 1991). Such tissue specific domains may provide alternative structures suitable for each environment.

Type XII collagen is a homotrimer containing two triple helical domains and three non-collagenous domains (Gordon *et al.*, 1987; Dublet *et al.*,1989; Yamagata *et al.*, 1991). The collagenous domain represents only 8 % of the entire molecule. The COL1 domain of types IX and XII have been shown to be homologous (Gordon *et al.*, 1989) The association between type XII and type I collagen has not been demonstrated,

[6] COL is used as an abbreviation for the triple helical domain.



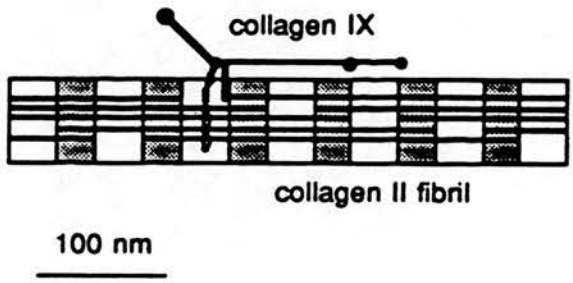


Figure 1.2 Schematic representation of the molecular structures and supramolecular assemblies of type IX, XII and XIV collagens

Triple helical domains are indicated by thick black lines and non-triple helical regions by closed circles or shaded domains. The GAG chain refers to the glycosaminoglycan chain attached to the α2 chain of type IX collagen. Type IX binds to the surface of type II collagen fibrils . The globular NC4 and triple helical COL3 domain project out of the fibril. The location of a cross-link between type II and IX collagen is shown by a short vertical line.

although immunolocalisation studies have shown coexpression of type I and XII collagen in several tissues (Sugrue *et al.*, 1989). Van der Rest and Garrone (1991) proposed that the presence of such a COL1 domain was an indication of a FACIT molecule. The existence of a homotrimeric molecule (type XIV collagen) with the characteristic FACIT COL1 domain has been demonstrated in skin and tendon (Dublet *et al.*, 1991).

1.2.2.2 Basement membrane collagens (IV and VII)

Type IV collagen is a major component of basement membranes (for reviews, see Glanville, 1987; Yurchenco and Schittny, 1990). The most common form of type IV is a heterotrimer [α1 (IV)]$_2$ α2 (IV)] (Glanville, 1987). Three additional chains α3 (IV), α4 (IV) and α5 (IV) also exist, although there is no evidence that they can form heterotrimers with α1 (IV) or α2 (IV) (Saus *et al.*, 1988; Hostikka *et al.*, 1990). The type IV molecule consists of a central triple helical domain, a large C-terminal globular domain (NC1) and an N-terminal globular domain (NC2). The α-chains of type IV collagen are not proteolytically processed in contrast to those of the fibrillar collagens. Type IV collagen molecules are assembled into a flexible three dimensional network (Timpl *et al.*, 1981; Yurchenco and Schittny, 1990). The basic repeating unit consists of four individual molecules, overlapping and joined together by disulphide and lysine derived cross-links at their amino-terminal ends (called the 7S domain). The molecules also associate at their carboxyl terminal NC1 domain (see Figure 1.3). Lateral associations are poorly understood, although, helical structures have been identified, which appear to result from molecules being entwined together (Yurchenco and Schittny, 1990). The lateral associations would effect the thickness of a basement membrane, and the pore size of its meshwork. A considerable number of interruptions of the gly-X-Y repeat are present in both the α1 (IV) chain and α2 (IV) chain (see Vuorio and de Crombrugghe, 1990). The non-triple helical interruptions in the relatively rigid structure of the triple helix are thought to modulate the flexibility of the molecule and the macromolecular network.

Type VII collagen is a large macromolecule of about 1000 kDa found close to the

basement membrane zone, beneath stratified squamous epithelia (for reviews, see Burgeson, 1987; Burgeson *et al.*, 1990). Rotary shadowing analyses suggest a molecule with a very long discontinuous 425 nm triple helical domain, and globular domains at either ends. The amino-terminal domain (NC1) appears in a trident-like arrangement (see Figure 1.3). Type VII collagen is capable of forming antiparallel dimers stabilised by intermolecular disulphide bonds through a 60 nm short overlap. The C-terminal NC 2 domain appears to be cleaved during this process, and the dimers then laterally aggregate in a non-staggered fashion. Immunolocalisation studies show type VII to be a major component of anchoring fibrils (Sakai *et al.*, 1986). The anchoring fibrils are believed to function by securing the lamina densa, which underlies stratified squamous epithelia, to its underlying stroma (Burgeson *et al.*, 1990). The model proposed by Burgeson *et al.* (1990) has the NC1 domain embedded within the lamina densa and the triple helix extending perpendicular into the stromal matrix. The NC1 domains at the other end of the dimer are condensed into electron dense structurestermed anchoring plaques. The plaques contain type IV collagen which has been shown to interact with the NC1 domain of type VII collagen using solid phase interaction studies (Burgeson *et al.*, 1990). There are a number of inherited conditions which are characterised by separation of skin basal cells from the dermal stroma. These conditions comprise the general classification of epidermolysis bullosa. The disease is characterised by blistering of the skin and external mucous membranes. Absence or abnormalities in type VII collagen and anchoring fibrils have been noted in several cases of epidermolysis bullosa (Bruckner-Tuderman *et al.*, 1989).

1.2.2.3 Type VI collagen

The ubiquitous, type VI collagen is a heterotrimer, α1 (VI) α2 (VI) α3(VI). It consists of a short 100 nm triple helix containing two small interruptions, and is unusual among collagens in that its globular domains comprise more than two-thirds of its total mass (Timpl and Engel, 1987; Bonaldo *et al.*, 1990; Chu *et al.*, 1990). It is characterised by the presence of 11 arginine-glycine-aspartate (RGD) sequences for the three chains (Chu *et al.*, 1990). The RGD sequences have been shown to play a central

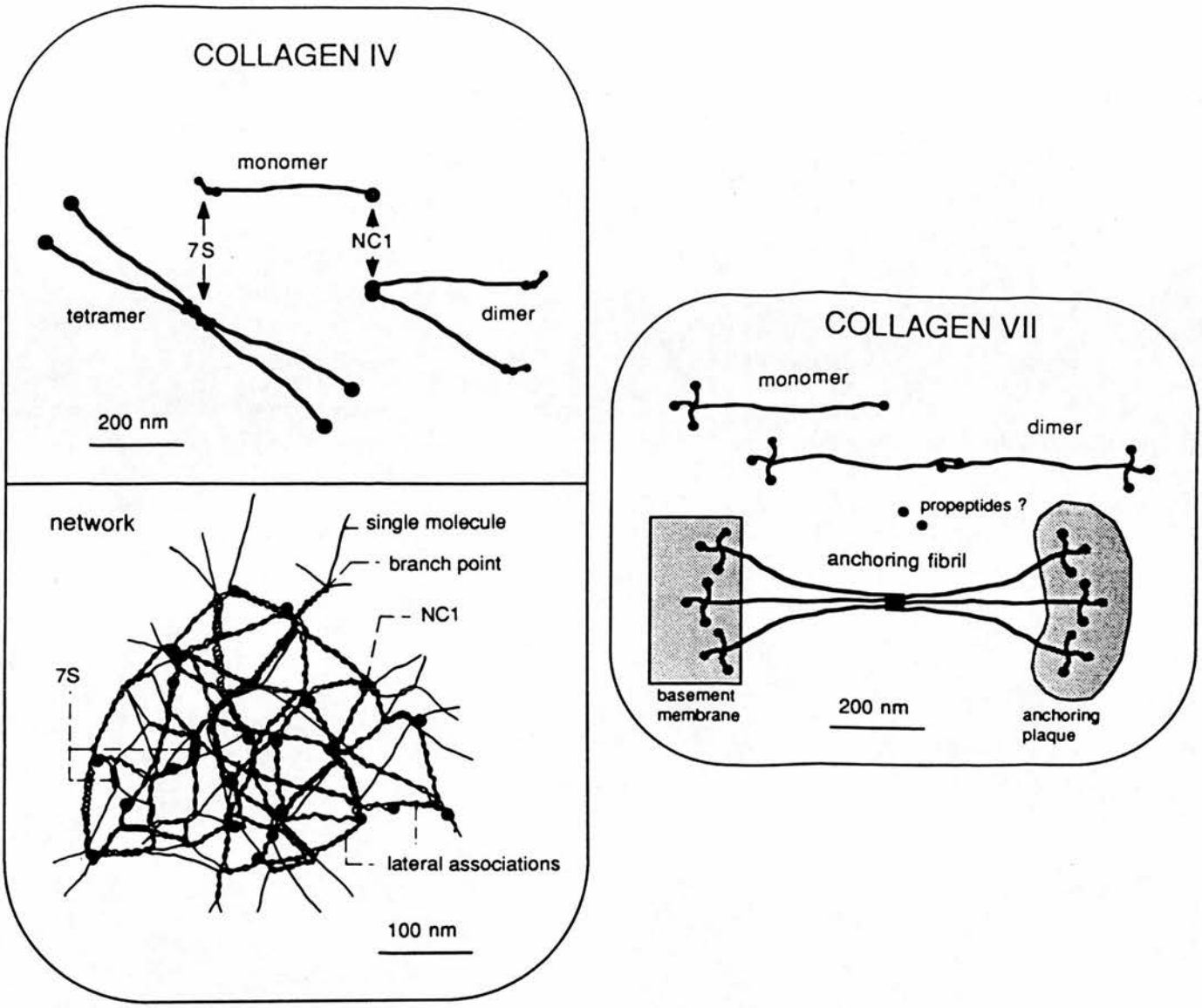


Figure 1.3 Schematic representation of the molecular structures and supramolecular assemblies of type IV and VII collagen

Triple helical domains are represented by thick black lines and non-triple helical domains by closed circles. 7S is the domain of antiparallel interaction of the type IV collagen triple helices to form a tetramer. In the lower part of the figure is the complex branching network of type IV collagen observed in basement membranes. Type VII collagen dimers form by antiparallel, partial overlapping of the amino-terminal domain. Several dimers aggregate laterally to form anchoring fibrils. The carboxyl-terminal domains interact with basement membrane and anchoring fibrils (from Hulmes, 1992, with permission).

role in the interaction of matrix constituents, such as collagens, fibrinogen and fibronectin with cell receptors of the integrin type (Humphries, 1990). The presence of 200 residue repeats showing significant similarities to the A domains of von Willebrand factor has been demonstrated in the non-helical N- and C-terminal region (Colombatti and Bonaldo, 1990). In addition, the C-terminal region has been shown to contain domains showing similarities to salivary proteins and fibronectin-type III repeats. The functions of these domains remain to be elucidated. Type VI collagen is a major constituent of a class of tissue microfibrils (Timpl and Engel, 1987). These microfibrils have a banding periodicity of 110 nm and are composed of tetramers of type VI collagen associated in an end to end fashion, which results in a beaded filamentous appearance (see Figure 1.4).The tetramers are assembled from defined dimers and monomers in a process involving disulphide bridges and not lysine derived cross-links like most other collagens (Timpl and Engel, 1987). The Type VI collagen containing microfibrils have been localised in the close vicinity of cells, large fibrillar collagen fibrils and some basement membranes. This collagen may have a role as an interface between cells and the extracellular matrix.

1.2.2.4 Short chain collagens (VIII,X)

The short chain type VIII collagen is the major collagenous constituent of Descemet's membrane (Sage and Bornstein, 1987). The structure derived from the cDNA sequence of the α1 (VIII) chain is that of a molecule with a single triple helical domain, containing 8 imperfections and two relatively short non-triple helical terminal domains (Yamaguchi *et al.*, 1989). The α2 (VIII) chain has a similar structure (Muragaki *et al.*, 1991). Descemet's membrane which seperates the corneal endothelial cells from the stroma, consists of stacks of hexagonal collagenous lattices made of type VIII collagen (Sawada *et al.*, 1990; see Figure 1.4). The lattice is built by nodes interconnected by rod-like structures. The highly organised lattice may function in stabilising the characteristic endothelial phenotype.

Type X collagen, a homotrimeric disulphide bonded collagen is limited to hypertrophic chondrocytes which suggests a specific function related to mineralisation

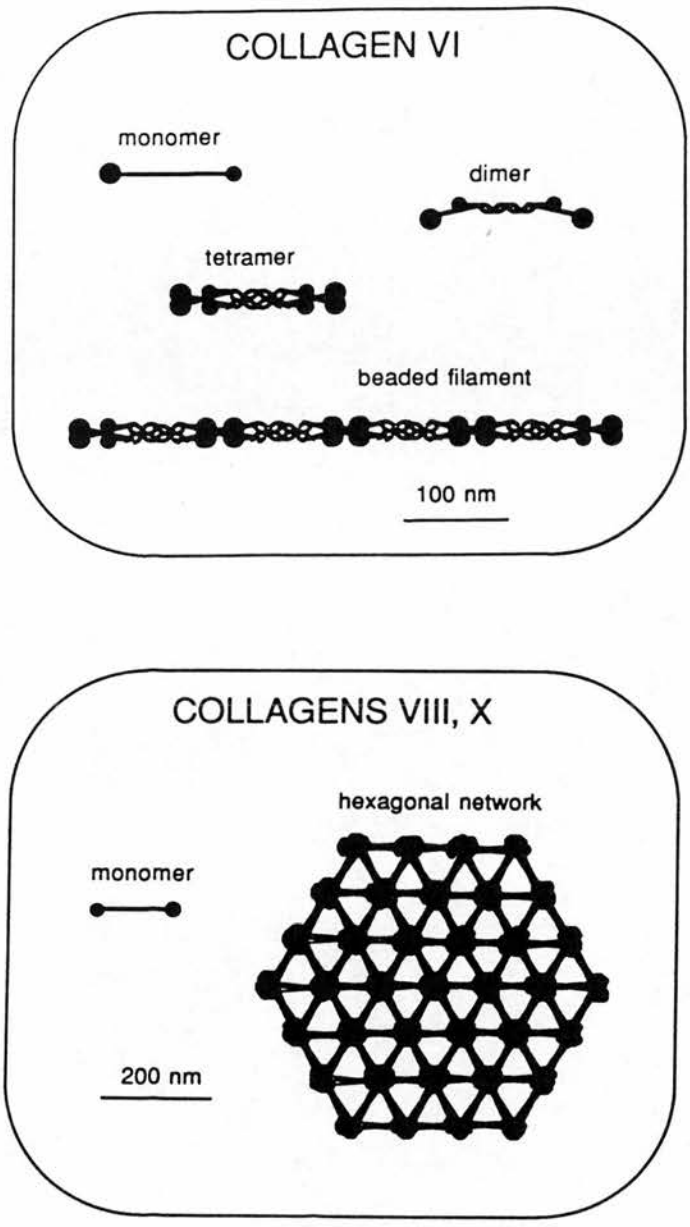


Figure 1.4 Schematic representation of the molecular structures and supramolecular assemblies of type VIII, X and VI collagen

Type VI dimers are formed by antiparallel association and tetramers by lateral association of the dimers. Beaded filaments can associate side by side to form 110 nm periodic fibrils.Type XIII and X collagen molecules form hexagonal lattices. The nodes are likely to be connected by bundles of molecules though the number of bends in each bundle is unknown (from Hulmes, 1992, with permission).

of cartilage (Schmid and Linsenmayer, 1990). The correlation between type X collagen deposition and matrix mineralisation *in vivo* has been demonstrated (Kwan *et al.*, 1991). Matrix vesicles (see section 3.4.9.2) are believed to have a role in the initiation of matrix mineralisation, and potential interactions between these vesicles and type X collagen have been proposed (Wu *et al.*, 1989). Both types VIII and X short chain collagens show considerable structural homology (Yamaguchi *et al.*, 1989; Ninomiya *et al.*, 1990). The length of the triple helical domain, the number and the relative imperfections in the Gly-X-Y repeat and the amino acid sequence of the NC1 domain of α1 (VIII) have a high similarity to that of α1 (X). Type X collagen has been shown to be associated with fibrillar structures that resemble the hexagonal lattice of Descemet's membrane (Kwan *et al.*, 1991; see Figure 1.4). This has led to the suggestion that the type X collagen lattice may influence the migration of invading endothelial cells during angiogenesis within the cartilage matrix.

1.2.2.5 Type XIII collagen

Type XIII collagen has only been characterised at the cDNA and genomic level (Pihlajaniemi and Tamminen, 1990; Tikka *et al.*, 1991). These studies describe a novel, short chain collagen with three triple helical domains and four non-collagenous domains. The mRNA has been shown to be distributed in skin, intestinal mucosa, bone and cartilage. There are five alternatively spliced forms of RNA, where uniquely amongst the collagens, splicing occurs in both collagenous and non-collagenous domains.

1.2.2.6 Collagen-like proteins

Collagen-like proteins such as the complement component C1q (Sellar *et al.*, 1991), the asymmetric form of acetylcholinesterase (Krejci *et al.*, 1991), mammalian lectins (Theil and Reid, 1989), and the macrophage scavenger receptor (Kodama *et al.*, 1990) are not classed as collagens because they do not participate in supramolecular structures of the ECM (see Figure 1.1).

The asymmetric form of acetylcholinesterase, a complex oligomer characterised by the presence of a collagen-like tail subunit attached to up to three catalytic tetramers through disulphide bridges is localised in differentiated muscule and neural cells (see Figure 1.5). The collagen-like tail consists of a proline-rich domain, a collagenous domain and a C-terminal domain composed of proline and cysteine-rich regions (Krejci *et al.*, 1991). The collagenous region attaches the enzyme to the neuromuscular endplates, and this may involve ionic interactions with membrane bound proteoglycans (Massoulie, 1980). This region is also important in the interaction of the enzyme with sphingomyelin (see section 3.1.3).

The complement component C1q is composed of six identical subunits. In each subunit, a collagenous region is attached to a C-terminal globular domain. The collagenous region interacts with the proenzyme complex[7] (C1r C1s), and a C1q receptor found in lymphoid cells (see below). The C-terminal globular regions are involved in the binding to the Fc regions of IgG and IgM. A strong similarity in amino acid sequence has been demonstrated between the globular C-terminal region and the C-terminal regions of type VIII and X collagens (Sellars *et al.*, 1991). The conserved structure may define the framework for chain alignment prior to triple helix formation.

The mammalian lectins, pulmonary surfactant protein A (SP-A; Voss *et al.*, 1988; Haas *et al.*, 1991), mannan-binding protein (MBP; Lu *et al.*, 1990), and conglutinin (Lee *et al.*, 1991), contain a short N-terminal region, followed by a collagen-like domain and a globular C-terminal domain (Theil and Reid, 1989). A C-type lectin domain in the C-terminal region is responsible for the Ca^{2+} dependent binding of these proteins to carbohydrates. Electron microscopic observations of SP-A and MBP show a marked similarity to C1q (see Figure 1.5).

An interruption in the gly-X-Y repeating pattern causes a characteristic 'bend' half way up the collagen-like domain of C1q, SP-A and MBP. The collagenous region of the plasma proteins, C1q, conglutinin and MBP has been demonstrated to bind to C1q-like receptors found in a wide range of lymphoid cells (Erdei and Reid, 1988; Malhotra *et al.*, 1990). A glutamic acid residue within the gly-X-Y repeating pattern preceding

[7] The proenzyme complex C1r C1s, are components of the C1 complex (see Seigel and Schumaker, 1983).

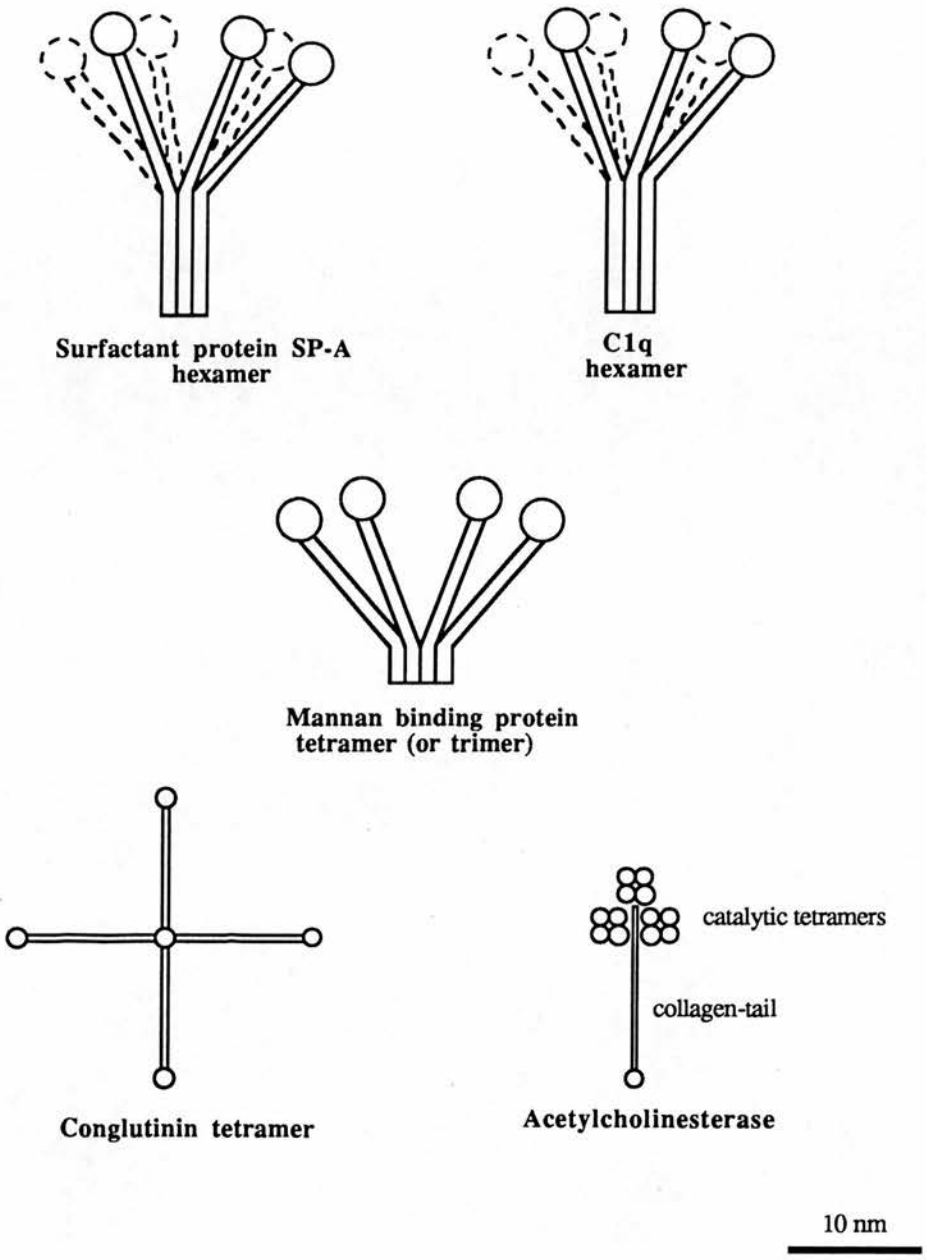


Figure 1.5 Schematic representations of the molecular structures of the collagen-like proteins

The triple helical domains are shown as columns and non-triple helical domains as empty circles.The small N-terminal non-triple helical regions of SP-A, C1q and mannan-binding protein are not indicated (see Figure 1.1). The mannan-binding protein can exist as a tetramer or a trimer. One quarter the scale has been used for conglutinin and acetylcholinesterase (adapted from Thiel and Reid, 1989).

the 'bend' in the triple helical region has been shown to be conserved within C1q and MBP (Sellar *et al.*, 1991). The conserved glutamic acid residue, and the 'bend' in the triple helix may be important structural features for the interaction of C1q with its receptor. The binding of the collagenous domain to a common receptor in lymphoid cells could be of physiological significance. Thiel and Reid (1989) suggested such an interaction could be a recognition and clearance mechanism for immune complexes and mannose coated proteins or cells.

SP-A is a major component of the lung lipid-protein complex (pulmonary surfactant). The collagenous domain has been shown to have a role in the interaction with surfactant lipids (See section 3.1.3).

1.3 Collagen genes

The 14 members of the collagen family of proteins are coded for by at least 25 different genes (for reviews, see Sandell and Boyd, 1990; Vuorio and de Crombrugghe, 1990). The various collagen genes in humans are distributed on different chromosomes (Myers and Emanuel, 1987; for other references, see Vuorio and de Crombrugghe, 1990). However, in three cases two genes have been mapped to the same chromosome: α1 (III) and α1 (V) to chromosome 2; α1 (IV) and α2 (IV) to chromosome 13; α1 (VI) and α2 (VI) to chromosome 21 (see Vuorio and de Crombrugghe, 1990).

1.3.1 Fibrillar collagen genes

The entire exon -intron structure for the α1 (I), α2 (I) and α1 (II) procollagen genes and partial structures for α1 (III), α2 (V) and α1 (XI) have been established (Sandell and Boyd, 1990). There are more than 50 exons in each of the fibrillar collagen genes. DNA sequence comparisons among the fibrillar collagen genes led to the observation that the arrangement of exons within the genes is identical in the region coding for the triple helical domains (Yamada *et al.*, 1984; Chu *et al.*, 1984; Upholt and

Sandell, 1986). There are three minor exceptions, for details, see Vuorio and de Crombrugghe (1990). The conservation of structure is independent of species or fibrillar collagen type. Each exon is a multiple of 9 base pairs (bp), encoding a single collagen triplet. The majority of the exons are 54 bp or multiples thereof (108 or 162). This suggests that the triple helical coding domain evolved by processes of duplication, fusion and deletion of an ancestral 54 bp unit. Each exon begins with a codon for glycine and ends with a codon for a Y-position amino acid.

There are features of structural conservation within the fibrillar collagen genes coding for the C-propeptide (Yamada *et al.*, 1983). The locations of the cysteine residues, their neighboring sequences, as well as the sequence around the carbohydrate attachment site are conserved (Yamada *et al.*, 1983). The exon organisation of the genes coding for the N-propeptide show much more divergence than that for the rest of the polypeptide (for more information, see Chu *et al.*, 1984; de Wet *et al.*, 1987; Benson-Chanda *et al.*, 1989; Su *et al.*, 1989).

1.4 Type I procollagen

Procollagen is the precursor of collagen, and is the form secreted into the extracellular space (see Figure 1.6). At either end of the central helical domain are globular domains termed the amino and carboxyl propeptides (Fessler and Fessler, 1978).

1.4.1 Triple-helical domain

The sequence of the main and central part of the α-chain is essential for the formation of the macromolecular structure of collagen. The region consists of three separate chains, two α1 and one α2 chain, each of which contains approximately 1000 amino acids twisted in the form of a left handed helix termed a polyproline type II helix (for a review, see Ramachandran and Ramakrishnan, 1976). These three triple helical structures are wrapped around one another to produce a right-handed superhelix. The

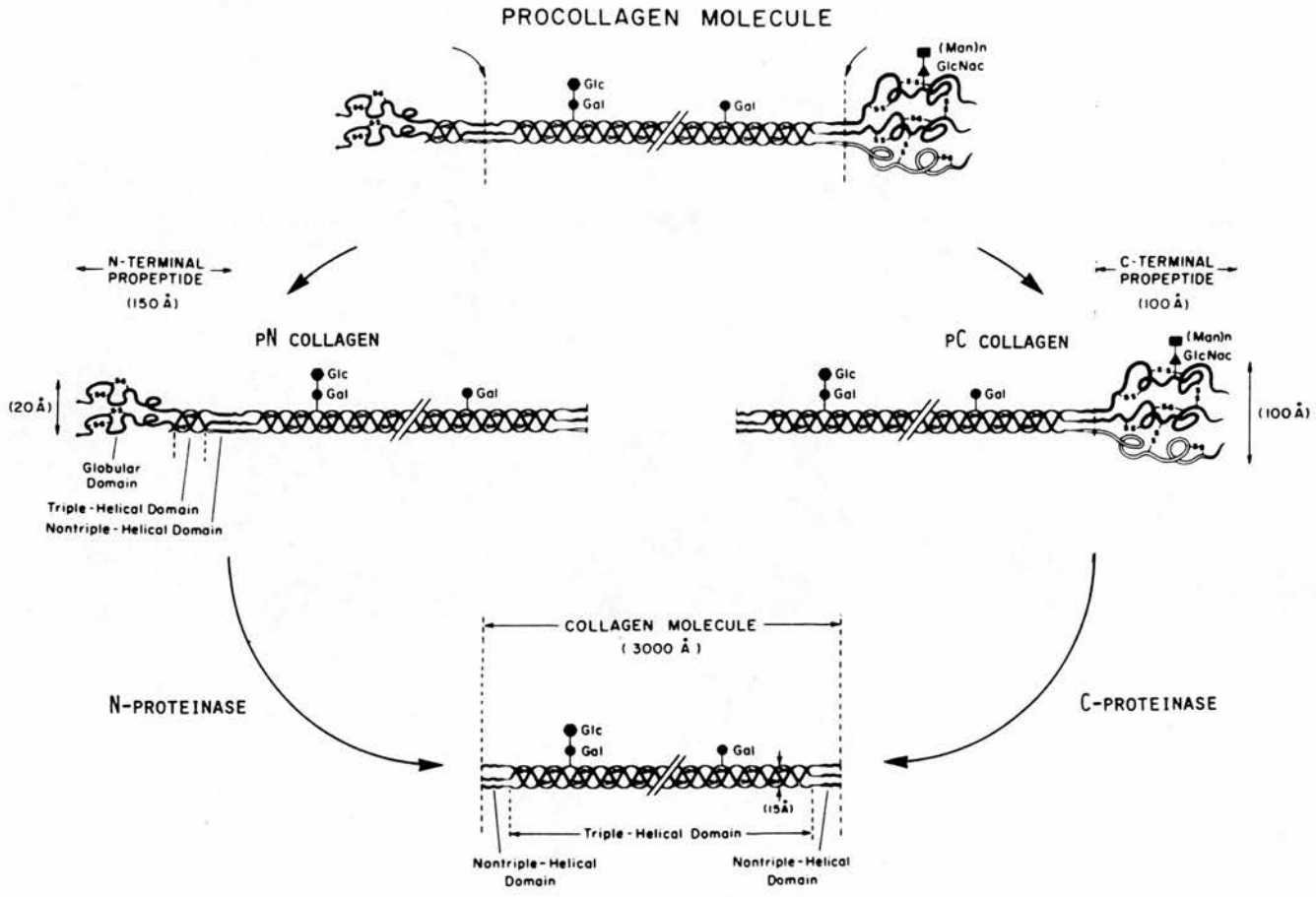


Figure 1.6 Pathways of procollagen processing by procollagen C-proteinase and N-proteinase (from Hulmes *et al.*, 1989, with permission).

folding of the component chains into the proper helical conformation requires that only glycine be present at the centre of the superhelix. Therefore, each of the three α-chains in collagen consists of repeating tripeptide sequences of gly-X-Y. The sidechains of X andY protrude from the helix and thus can be accommodated by a variety of amino acids. This offers a large potential for lateral interactions, particularly with other triple helices (van der Rest and Garrone, 1991). The triple helical conformation is wound such that the peptide bonds linking adjacent amino acids of the chains are buried within the interior of the molecule, and thus, the triple helical region is highly resistant to attack by proteases, such as pepsin. The triple helical conformation is stabilised by interchain hydrogen bonds. Disruption of these bonds during chemical or thermal denaturation results in unfolding of the molecule. The presence of the imino acids proline and hydroxyproline are important for the stabilisation of the triple helix. Proline, as a 5-membered ring, is rigid and does not permit rotation about the N-Cα bond. The 4-hydroxyproline residue stabilises the triple helix by forming extra hydrogen bonds and contributes to its thermal stability (Berg and Prockop, 1973). The rigidity and flexibility of distinct areas along the molecule can be adjusted by varying amounts of hydroxyproline and proline. The carboxyl terminal end of the triple helical domain of the α1 (I) and α2 (I) chain is sealed by a 5-fold repeat of the triplet gly-pro-X (Kuhn, 1987). The collagenase cleavage site and the carboxyl terminal helical cross-link area require a more relaxed and flexible structure and therefore do not contain tripetide units with imino acids (Kuhn, 1987).

1.4.2 Amino and carboxyl propeptides

Evidence for the presence of the amino (N-) and carboxyl (C-) propeptides in collagen came from both chemical and morphological data (reviewed in Fessler and Fessler, 1978). Immunological studies have shown that procollagen molecules contain two independent sets of antigenic determinants not present in collagen (Nist *et al.*, 1975).

Studies using vertebrate collagenase (Harris *et al.*, 1984) to cleave procollagen molecules into two fragments contained extra peptides that were not present when

collagen molecules were cleaved with the same enzyme (Tanzer *et al.*, 1974; Byers *et al.*, 1975). Also, procollagen segment-long-spacing crystallites (SLS; see section 1.6) contain extra pieces at both ends of the aggregates not found in collagen SLS (Tanzer *et al.*, 1974).

1.4.2.1 N-propeptide

The N-propeptides of fibrillar collagens exhibit a much higher degree of divergence both in length and domain structure than the rest of the polypeptide (see Figure 1.1). The N-propeptides of the fibrillar collagens adhere to one of two basic architectures (see Sandell and Boyd, 1990). The first, characteristic of proα1(I), proα1 (II)[8] , proα1 (III) and proα2 (V) consists of a cysteine-rich globular domain known also to be present in thrombospondin (Lawler and Hynes, 1986) and von Willebrand factor (Titani *et al.*, 1986), a triple helical domain and a short non-helical segment (Chu *et al.*, 1984). The second, characteristic of proα2 (I), lacks the entire cysteine-rich globular domain (De Wet *et al.*, 1987). The globular domain of proα1 (I) (66-68 amino acids) is rich in acidic and hydrophobic residues and stabilised by 5 intrachain disulphide bridges. In type I procollagen, the short non-helical region (21 amino acids) which connects the N-propeptide to the large triple helical domain contains the cleavage site for the specific procollagen amino (N-) proteinase. The conformation of the cleavage site is stabilised by lateral interactions of three hairpins formed by the proα chains (Dombrowski *et al.*, 1989). The cleaved N-propeptide may have a role in the regulation of collagen synthesis by feedback inhibition (see section 1.10). The telopeptide extension formed after the cleavage contains the sites where hydroxylysine derived intramolecular and intermolecular cross-links are formed (see sections 1.5.2 and 1.9).

1.4.2.2 C-propeptide

The carboxyl propeptides of the proα1 (I) and proα2 (I) are about 244 residues in

[8] The large Cys-rich coding domain is not present in all type II procollagen mRNA (Ryan and Sandell, 1990). This sequence probably undergoes alternative splicing in the α1 (II) collagen pre-mRNA.

length. The amino acid composition is very different from that of the central triple helical domain, and shows a relatively high content of acidic amino acids, tyrosine, phenylalanine and cysteine. The C-propeptide contains 8 cysteine residues of which six are involved in intrachain disulphide bridges, which stabilise the globular structure. Two cysteine residues form interchain disulphide bridges, and are a prerequisite for the formation of the triple helical molecule (Uitto and Prockop, 1974). One of the roles proposed for the C-propeptide is the association and correct registration of the α-chain prior to triple helix formation (for further details, see section 1.5.3). There is also evidence to suggest that the C-propeptide may function as a negative feedback inhibitor of collagen synthesis (see section 1.10). The C-propeptides may also play a role in the intracellular transport and packaging of procollagen in secretory vacuoles (Olsen, 1991). The C-propeptide of type II collagen (chondrocalcin) has been found to accumulate in the ECM of mineralising cartilage and may play a role in the mineralisation process (Poole *et al.*, 1984; van der Rest *et al.*, 1986; see chapter 3). The C-propeptide also contains a mannose and N-acetylglucosamine containing oligosaccharide not found within the main triple helix, the function of which is not known.

1.5 Biosynthesis of procollagen

Collagen biosynthesis is distinguished from other secretory proteins by the presence of many unusual reactions (for reviews, see Fessler and Fessler, 1978; Kivirikko and Myllyla, 1982; Kivirikko and Myllyla, 1984; Prockop and Kivirikko, 1984; Byers, 1990; Kuivaniemi *et al.*, 1991). The biosynthesis takes place in several stages.

1.5.1 Transcription and translation of proα (I) chains

The proα1 (I) and proα2 (I) chains are products of the COL1A1[9] and COL1A2 genes located on chromosome 17 and 7, respectively. The expression of the COL1A1 and COL1A2 genes are highly coordinated (for further information on collagen gene expression, see section 1.10). The mRNAs for the proα chains of collagen are synthesised and processed in the same way as those for other proteins in eukaryotes (Brown, 1981). The structure of the collagen gene implies that processing of the pre-mRNAs must involve at least 50 excision and splicing events (Kivirikko and Myllyla, 1984). The polypeptide chains are synthesised on membrane-bound ribosomes and pass through the membranes into the cisternae of the rough endoplasmic reticulum (RER) while being assembled. The initially synthesised polypeptide chains have N-terminal hydrophobic signal sequences which serve as vectors for ensuring that the nascent protein chains cross the membrane of the RER. As the amino terminal ends of newly synthesised proα chains enter the cisternae of the RER, they encounter protease(s) that rapidly remove the signal peptide (Olsen, 1991). The signal sequence of the proα (I) chain is different from the proα (II) chain (Prockop *et al.*, 1979).

1.5.2.1 Hydroxylation of proline and lysine residues

The amino acids 4-hydroxyproline, 3-hydroxyproline, and hydroxylysine are found in vertebrate proteins almost exclusively in collagens, and a few other proteins with collagen-like sequences (Kivirikko and Myllyla, 1984, 1985; Kivirikko *et al.*, 1990). All three amino acids are formed as post-translational modifications by the hydroxylation of peptide bound prolyl or lysyl residues, catalysed by three separate enzymes, proline 4-hydroxylase, proline 3-hydroxylase and lysine hydroxylase. The enzymes are located on the inner side of the membrane of the rough endoplasmic reticulum (RER; Kuhn, 1987). The active form of proline 4-hydroxylase is a tetramer

[9] Nomenclature: The gene for members of the collagen family have been given the names beginning with COL, followed by an arabic number denoting the collagen type, letter A (for α chain), and another arabic number for the α-chain. For example, COL1A2 is the gene for the α2 (I) chain.

($\alpha_2\beta_2$). The β-subunit (60 kDa) of prolyl 4-hydroxylase is a multifunctional protein residing in the RER. It has been shown to have protein disulphide isomerase (PDI) activity (Kiovu *et al.*, 1987; Freedman *et al.*, 1989), and also serve as the major thyroid hormone binding protein in the RER (Gong *et al.*, 1988). The β-subunit also retains the prolyl 4-hydroxylase in the RER, since it has the sequence lys-asp-glu-leu at its carboxyl end which is necessary for the retention of polypeptides in the RER (Kivirikko *et al.*, 1990). Lysyl hydroxylase is a dimer (both subunits 90 kDa) and its solubility properties suggest that it is tightly associated with the membrane of the RER (Kivirikko and Myllyla, 1985).

All three of the enzymes require Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate. They all decarboxylate 2-oxoglutarate into succinate, one atom of the O_2 being incorporated into the succinate, while the other is transferred as an hydroxyl group to the amino acid residues. The mechanism of the proline 4-hydroxylase and lysine hydroxylase reactions have been elucidated (Kivirikko and Myllyla, 1985). There is a binding of Fe^{2+}, 2-oxoglutarate, O_2, and the peptide substrate to the enzyme in this order, and ordered release of the hydroxylated peptide, CO_2, succinate and Fe^{3+}. Ascorbate is not used stoichiometrically which suggests it is required to prevent occasional oxidation of either the enzyme bound iron or some group of the enzyme molecule. In ascorbate deficiency (scurvy), proα chains are underhydroxylated and do not form stable triple helices at 37 °C. The hydroxylation of about 100 prolyl residues is essential for the three proα chains to form a triple helix that is stable at body temperature (Kuivaniemi *et al.*, 1991).

Most of the hydroxylations occur while the nascent polypeptide chains are growing in the ribosome, although, the reactions are continued after the release of the polypeptide chains from the ribosomes (Kivirikko and Myllyla, 1980). The minimum sequence requirement for the interaction with proline 4-hydroxylase is fulfilled by a gly-pro-X sequence, and that for interaction with lysine hydroxylase by a X-lys-gly triplet (Kivirikko and Myllyla, 1980). The N- and C-terminal non-triple helical regions that participate in cross-link formation do not obey this rule. In these regions, a lysine not followed by glycine is frequently hydroxylated. These hydroxylysines may be formed by a different hydroxylase (Linsenmayer, 1991). In contrast, proline 3-hydroxylase

probably requires a pro-4hyp-gly triplet, whereas a triplet of the form pro-pro-gly probably cannot be a substrate (Kivirikko and Myllyla, 1982). The interaction with all three enzymes is affected by amino acids in other parts of the peptide, by the peptide chain length, and by the peptide conformation. The hydroxylases have a requirement for a non-helical conformation which has a critical role in the processing of the protein (Prockop *et al.*, 1979).

1.5.3 Triple-helix formation

Upon completion of synthesis, the proα chains associate by hydrophobic and electrostatic interactions at the C-propeptides. This association is stabilised by the formation of interchain disulphide bonds (Schofield *et al.*, 1974; Uitto and Prockop, 1974). The disulphide bonded C-propeptide domain serves as the single nucleation site of triple helix formation (Bruckner and Eikenberry, 1984; Engel and Prockop, 1991). Protein disulphide isomerase catalyses the interchain disulphide bond formation in procollagen *in vitro* (Kiovu and Myllyla, 1987; Freedman *et al.*, 1989). The mechanism of disulphide bonding is poorly understood. Randomly cross-linked disulphide bonds are formed and then isomerised to native ones via a series of thiol disulphide interchange reactions. Native disulphide bond formation can proceed in the absence of enzyme, however, the bonding is rather slow and results in a delay in the formation of the triple helix.

The association directs chain selection to ensure that each type I molecule consists of two proα1 chains and one proα2 chain (Kuivaniemi *et al.*, 1991). The N-terminal amino acid sequence of types I, II and III procollagen C-propeptides were shown to be variable (Ninomiya *et al.*, 1984). This region could contain information important for the specific associations between proα chains during the assembly of procollagen molecules. The association and disulphide bonding of the C-propeptides provides the correct registration of the gly-X-Y tripeptide units in the three chains and allows a nucleus for triple helix formation. The nucleus is then propagated from the C-terminal to the N-terminal domain with a zipper-like mechanism (for further details see Engel and Prockop, 1991). Folding of the

proα chains is delayed until the chains aquire about 100 residues of hydroxyproline. Without any hydroxyproline, type I collagen will denature at 25 °C, whereas with 100 residues per α-chain the melting temperature is about 40 °C (Berg and Prockop, 1973). The delay in folding probably ensures that the nucleation and propagation of the triple helix occur in an orderly manner (Kuivaniemi *et al.*, 1991). Propagation of the triple helix stops whenever a cis-peptide bond is encountered. Therefore, the cis-trans isomerisation is a rate limiting step in collagen folding (Bruckner and Eikenberry, 1984). Triple helix formation is a requirement for secretion (Prockop *et al.*, 1979).

1.5.4 Glycosylation of hydroxylysine and asparagine

The hydroxyl group of hydroxylysine is the site of attachment for O-linked carbohydrate units (Kivirikko and Myllyla 1979). As lysyl residues in the newly synthesised proα chains are hydroxylated, sugar residues are added to some of the resulting hydroxyl residues. These glycosylations are catalysed by two specific enzymes, a galactosyltransferase which adds galactose to certain hydroxylysines, and a glucosyltransferase which adds glucose to certain galactosylhydroxylysines (Kivirikko and Myllyla, 1979). Both these transferases use sugar in a form of uridine diphosphate glycoside, and both reactions require the presence of a bivalent cation, preferably manganese (Kivirikko and Myllyla, 1979). The triple helical conformation of the substrate prevents interaction with both transferases, therefore glycosylation, like hydroxylation, must occur intracellularly prior to triple helical formation. The role of the disaccharide units remain unclear. The C-propeptide of type I procollagen is glycosylated by transfer of a mannose-rich oligosacchride side chain from a dolichol phosphate intermediate to an asparagine residue in the polypeptide chain. The acceptor site for the oligosacchride side chain contains the sequence asn-X-thr (Pesciotta *et al.*, 1981). The acceptor site recognition unit is identical to the β-subunit of prolyl 4-hydroxylase (Geetha-Habid *et al.*, 1988). Each domain of the C-propeptide has one oligosacchride side chain consisting of two residues of N-acteyl glucosamine and 6-9 residues of mannose (Marti *et al.*, 1984). The functional role of the N-linked

oligosacchride in procollagen is unknown.

Several mutations have been identified in human procollagen genes that lead to the abnormal biosynthesis of procollagen (Byers, 1990; Prockop *et al.*, 1990; Kuivaniemi *et al.*, 1991; see section 1.12).

1.6 Transport and secretion of procollagen

The passage of procollagen from the rough ER, through the Golgi complex and secretory vesicles was demonstrated by procollagen immunostaining (Leblond and Wright, 1981) and autoradiography (Weinstock and Leblond, 1974; Marchi and Leblond, 1983; Marchi and Leblond, 1984). Procollagen bundles which appear to be SLS-like aggregates[10] were first observed within the secretory vacuoles of epithelial cells (Trelstad, 1971), and have been seen subsequently in a number of other cell types (Weinstock and Leblond, 1974; Bruns *et al.*, 1979; Cho and Garant, 1981; Wright and Leblond, 1981). Dimeric SLS-like aggregates have been observed in tendon fibroblasts (Trelstad and Hayashi, 1979). These intracellular aggregates have an electron dense interface between their ends and the membrane of the vacuole. The possible affinity of the ends of the aggregates for the vacuole membrane could dictate the alignment of the molecules and represent an early stage in fibril development (Trelstad and Hayashi, 1979; see section 1.8.3.1). Many fibrils are heteropolymeric containing different collagen types (section 1.8.3.3) and proteoglycans (section 1.8.3.2). Birk et al (1991) suggests that mixing of the components would probably begin within the cell in one of the intracellular compartments.

Procollagen appears to be secreted in a biphasic process (Kao *et al.*, 1983). For chick embryo corneal cells in culture, approximately 55% of pulse-labelled type I procollagen is secreted with a first order rate constant of $0.071 min^{-1}$ (half-life 10 min), whereas the remaining procollagen has a rate constant for secretion of $0.0053\ min^{-1}$ (half-life 130 min; Kao *et al.*, 1983).

[10] Segment long spacing (SLS): procollagen molecules arranged side by side with the same amino to carboxyl terminal polarity.

An immunocytochemistry study of cultured human fibroblasts with type I procollagen C-propeptide antibodies suggests procollagen is bound at the cell surface coincident with secretion (Phelps *et al.*, 1985; see section 1.8.3.1).

The extent to which procollagen remains aggregated after secretion into the extracellular milieu is unclear. SLS-like aggregates have been observed in fibroblast culture medium after concentration to a pellet by prolonged ultracentrifugation (Hulmes *et al.*, 1983; Gross and Bruns, 1984). Bruns *et al.* (1979) also identified SLS aggregates of procollagen in the culture medium of fibroblasts from chick embryo tendon and human skin after negative staining. However, these results were probably due to surface induced aggregation during preparation of specimens for electron microscopy (Mould and Hulmes, 1987; for further details of surface induced aggregation, see section 1.8.3.1). The solubility limit of purified chick type I procollagen incubated at 37 °C in phosphate buffered saline was found to be in the range 1-1.5 mg/ ml (Mould *et al.*, 1990). Bundles of molecules packed as 300 nm long segments (SLS-like aggregates), and D-periodic assemblies of procollagen have been observed at concentrations greater than the 1.5 mg/ ml (Mould *et al.*, 1990). Therefore, SLS-like aggregates may exist in the extracellular milieu at the higher concentration of procollagen that may be present in the extracellular matrix.

In contrast with the ultrastructural studies of fibroblasts *in vivo* (Trelstad and Hayashi, 1979; Bruns *et al.*, 1979; Marchi and Leblond, 1983), SLS-like aggregates were not observed within golgi saccules or cell vacuoles *in vitro* (Phelps *et al.*, 1985). The abundance of collagen fibrils in the fibroblast culture system led Phelps *et al* (1985) to suggest that cytoplasmic SLS-like aggregates are not required for procollagen secretion and fibrillogenesis.

1.7 Processing of type I procollagen

In the ECM, enzymatic processing of procollagen to collagen occurs with the removal of the N and C-terminal propeptides (for a review, see Peltonen *et al.*, 1985; see Figure 1.6). The cleavages occur at the junction of the short non-helical telopeptide

sequences with the propeptide domains (see Figure 1.1), thus leaving the intact telopeptides at the N- and C-termini of the α-chain helix to fulfil their role of inter- and intramolecular crosslinking (Seyedin and Rosen, 1990).

Sequence data of released propeptides and tissue collagens show that the cleavage always takes place at the same sites which suggests that the proteinases are highly specific (Kivirikko and Myllyla, 1984). The conversion of type I procollagen to collagen involves two different enzymes, a procollagen amino (N) proteinase to remove the N-propeptide (Tuderman *et al.*, 1978; Tuderman and Prockop 1982; Berger *et al.*, 1985; Hojima *et al.*, 1989) and a procollagen carboxyl (C-) proteinase to remove the C-propeptide (Njieha *et al.*, 1982; Hojima *et al.*, 1985; Kessler *et al.*, 1989). Both enzymes share certain characteristics in that they are neutral metalloproteinases and require Ca^{2+} for maximal activity.

The exact site of procollagen processing in relation to the cell surface is unknown. The conversion of procollagen to collagen has been shown to take place at or near the surface of embryonic chick calvaria bone cells (Morris *et al.*, 1975). Layman (1981) has shown that fetal and neonatal skin cells retain the N-proteinase on the cell surface for a limited time before releasing it into the culture medium. However, adult skin cells were shown to secrete the enzyme directly into the culture medium. In the rapidly growing embryos, the rate of collagen synthesis and remodelling is high, and an efficient mechanism for collagen fibril orientation and deposition may be required. Layman (1981) proposed that the retention of the N-proteinase at the cell surface (and possibly procollagen) in the embryo may provide a mechanism for cellular control of fibril formation (see chapter 4). In mature animals, where collagen turnover is relatively low, such an efficient cellular control of fibril formation may not be required.

The charged polymer dextran sulphate was shown to promote the accumulation of collagen in the cell layer of human fibroblast cultures (Bateman and Golub, 1990). Procollagen was completely processed to collagen by the addition of 0.01 % dextran sulphate to the culture medium (Bateman and Golub, 1990; see chapter 4). The dextran sulphate induced processing was shown to occur by the rapid cleavage of the C-propeptide followed by the slower cleavage of the N-propeptide. The molecular mechanism for processing may involve the retention of procollagen in the pericellular

environment, and consequent exposure to the N- and C-proteinase. In contrast with the previous observations, Uitto *et al* (1979) observed a continuous conversion of secreted procollagen to collagen even after the cells had been removed by centrifugation.

Other proteinases with propeptide cleavage properties have been identified. In some human skin fibroblast cultures a cell layer associated telopeptide cleavage activity has been identified which could also remove the propeptides (Bateman *et al.*, 1987). Acidic proteases in the culture medium of chick tendon fibroblasts and in extracts of whole chick embryos, as well as chick liver cathepsin D were shown to cleave the C-propeptides of type I procollagen (Davidson *et al.*, 1979; Helseth and Veis, 1984).

1.7.1 N-proteinase

Type I procollagen N-proteinase has been purified and characterised from chick embryo tendons (Tuderman *et al.*, 1978; Tuderman and Prockop, 1982; Morikawa *et al.*, 1980; Hojima *et al.*, 1989), whole chick embryos (Tanzawa *et al.*, 1985; Berger *et al.*, 1985; Dombrowski and Prockop, 1989), human skin (Steinmann *et al.*, 1980) and bovine aorta (Gerstenfeld *et al.*, 1984). The purified proteinase was also shown to act upon type II procollagen and the homotrimer of proα1 (I) chains (Tuderman *et al.*, 1978; Tuderman and Prockop, 1982; Hojima *et al.*, 1989). A different proteinase is required for the removal of the N-propeptide of type III procollagen (Nusgens et al, 1980; Halila and Peltonen, 1984, 1986; see below for further details).

The molecular weight of the type I N-proteinase from chick tendons (Tuderman *et al.*, 1978) and whole chick embryos (Tanzawa *et al.*, 1985) was shown by gel filtration to be 260 kDa and 320 kDa, respectively. A different form of the chick tendon enzyme was shown to have a molecular weight of 500 kDa determined by gel filtration (Hojima *et al.*, 1989). This was suggested to be the intact form of the enzyme. The 500 kDa form of the enzyme was shown to degrade slowly to a 300 kDa form which had similar properties (Hojima *et al.*, 1989). The presence of $CaCl_2$ in the buffers used to purify the enzyme increased the conversion. The 500 kDa form of the enzyme was shown to contain polypeptides of 61, 120, 135 and 161 kDa when separated by SDS-PAGE in non-reducing conditions (Hojima *et al.*, 1989). The 135 and 161 kDa polypeptides

were catalytically active after elution from the polyacrylamide gel.

As is typical for many metalloproteinases, the activity of the purified N-proteinase was inhibited by the metal chelators, EDTA, α,α'-dipyridyl and o-phenanthroline (Tuderman and Prockop, 1982; Hojima *et al.*, 1989), and the thiol reagent dithiothreotol (Tanzawa *et al.*, 1985). Addition of physiological concentrations of Ca^{2+} (5-10 mM) was required for maximal activity under standard assay conditions. The addition of a 10-fold molar excess of Ca^{2+} to N-proteinase preincubated with EDTA does not fully restore activity (Tuderman and Prockop, 1982). This observation suggested that a second metal in addition to Ca^{2+} was required. The activity of N-proteinase pretreated with EDTA was partially restored by Mn^{2+} or Mg^{2+}. However, when Mn^{2+} or Mg^{2+} were added together with Ca^{2+} after EDTA treatment, they partially prevented Ca^{2+} from restoring the enzyme activity. The nature of the second cation has not been resolved.

The N-proteinase differs from several other proteinases of connective tissues in that it is not inhibited by phosphoramidon, ovastatin or α2-macroglobulin (Hojima *et al.*, 1989). Fetal calf serum (2 %) was shown to inhibit 60-100 % of the N-proteinase activity (Hojima *et al.*, 1989) which is in contrast to the observations of Tanzawa *et al* (1985).

The N-proteinase has a highly specific requirement for a substrate with the correct native conformation (Tuderman *et al.*, 1978; Tanzawa *et al.*,1985). Procollagen becomes resistant to cleavage by N-proteinase at temperatures higher than melting temperature T_m[11]; Tanzawa et al, 1985), and short synthetic peptides with amino acid sequences identical with those in the cleavage site of the proα2 (I) chain are not cleaved (Morikawa *et al.*,1980). The K_m was 0.3-0.5 μM (Tuderman and Prockop, 1982), and 54 nM (500 kDa enzyme; Hojima *et al.*, 1989) for the native substrate.

Type I N-proteinase cleaves a pro-gln bond in the proα1 (I) chain and an ala-gln bond in the proα2 (I) chain (Kivirikko and Myllyla, 1984). Small changes in the primary structure of type I procollagen can either make the protein totally resistant to cleavage or substantially alter the rate at which it is cleaved by the N-proteinase

[11] Human type I procollagen has a denaturation temperature (T_m) of 41°C (Bruckner and Prockop, 1981).

(Dombrowski *et al.*, 1989). The changes in primary structure can occur at sites over 900 amino acid residues away from the cleavage site. The conformation of the cleavage site may be stabilised by lateral interactions of three hairpins formed by the proα chains (Dombrowski *et al.*, 1989). The long range effects of changes in primary structure are probably explained by the disruption of the hairpin structure (see section 1.12).

The N-proteinase cleaves the three polypeptide chains of type I procollagen at different rates. An intermediate containing only one uncleaved proα chain has been detected (Berger *et al.*, 1985) demonstrating that most of the reaction proceeds by a pathway in which the third chain is cleaved more slowly than the first two. With most endoproteinases, the susceptible bonds in the substrate become more accessible, and the rate of cleavage increases as the protein is partially cleaved and begins to unfold (Fruton, 1974). If the N-proteinase conformed to the general pattern, then the most readily detectable intermediate would be the first cleavage product containing one cleaved and two uncleaved propeptides. The procollagen C-proteinase (Morris *et al.*, 1979; Njieha *et al.*, 1982) and vertebrate collagenase (Jeffrey, 1986) are two other exceptions to this general pattern of endoproteinases. A possible explanation for the generation of a slowly reacting intermediate is that the conformation of the peptide bonds around the cleavage site may change markedly after the first two proα chains are cleaved. It is possible that after the N-proteinase cleaves the first two proα chains, the region of the protein containing the cleavage site partially unfolds and thereby makes the remaining proα chains more resistant to cleavage.

A separate N-proteinase partially purified from calf tendons (Nusgens *et al.*, 1980), smooth muscle cells of fetal calf aorta (Halila and Peltonen, 1984), and human placental tissue (Halila and Peltonen, 1986) has been shown to cleave the N-propeptide from type III procollagen. The enzyme does not accept type I or type II procollagen as its substrate. The type III N-proteinase has a molecular weight of about 70 kDa as determined by SDS-PAGE. The enzyme requires an intact triple helical conformation in the substrate and Ca^{2+} for maximal activity (Halila and Peltonen, 1984). Removal of Ca^{2+} from the incubation mixture does not inhibit the human enzyme (Halila and Peltonen, 1986) in contrast with the bovine enzyme or type I procollagen N-proteinase (Tuderman *et al.*, 1978; Halila and Peltonen, 1984; Tanzawa *et al.*, 1985). This could

possibly be explained by Ca^{2+} being more tightly bound to the human placental enzyme than from the enzyme purified from calf aorta smooth muscle cells. The K_m for pN-collagen was 0.76 μM for the calf aorta enzyme and 2 μM for the human placental enzyme (Halila and Peltonen, 1984, 1986).

1.7.2 C-proteinase

Enzyme activities that remove the C-propeptides from type I procollagen at neutral pH have been demonstrated in the medium of cultured chick embryo tendons (Leung *et al.*, 1979; Hojima *et al.*, 1985), extracts of embryonic chick calvaria (Njieha *et al.*, 1982), and the medium of cultured mouse fibroblasts (Kessler *et al.*, 1986).

The chick tendon proteinase has been partially purified and characterised (Hojima *et al.*, 1985). The molecular weight of the enzyme was shown to be 97 kDa-100 kDa by gel filtration, although crude preparations of the enzyme suggested it had a molecular weight ranging from 200 kDa to 600 kDa (Hojima *et al.*, 1985). The enzyme may be present in tissues as a larger aggregate. The C-proteinase has been shown to require 5-10 mM Ca^{2+} for optimal activity (Njieha *et al.*, 1982; Hojima *et al.*, 1985). The enzyme is similar to the N-proteinase in that Ca^{2+} also increases its stability near body temperature (Tanzawa *et al.*, 1985; Hojima *et al.*, 1985). Several metal chelators, EDTA, EGTA, α,α'-dipyridyl or o-phenanthroline have been shown to inhibit C-proteinase activity (Njieha *et al.*, 1982; Hojima *et al.*, 1985).

The peptide bonds in the chick proα1 (I) and proα2 (I) chains that are cleaved by the C-proteinase have been identified by amino acid sequencing of the C-propeptide (Pesciotta, 1981) and by nucleotide sequencing of cloned cDNAs (Fuller and Boedtker, 1981; Dickson et al, 1981). The peptide bond cleaved in the proα1 (I) and proα2 (I) chain is an ala-asp bond. Similar data established that an ala-asp bond was also cleaved during the processing of type II procollagen (Ninomiya *et al.*, 1984). Analysis of cDNA clones suggested that an arg-asp bond is cleaved in type III procollagen (Yamada *et al.*, 1983).

Partially purified C-proteinase has been shown to be inhibited by synthetic peptides

with amino acid sequences similar to that around the cleavage site in the proα1 (I) chain (Njieha *et al.*, 1982). The C-proteinase has also been shown to be completely inhibited by 5 % fetal calf serum. The C-proteinase purified from chick embryo tendons (Hojima *et al.*, 1985) has many properties that are similar to the enzyme from chick calvaria (Njieha *et al.*, 1982). However, the latter has a smaller molecular weight (80 kDa) and a slightly different pattern of response to inhibition. The enzyme from chick calvaria has been shown to be insensitive to arginine and lysine (Njieha *et al.*, 1982), whereas inhibition of most of the chick C-proteinases by the basic amino acids has been demonstrated (Leung *et al.*, 1979; Hojima *et al.*, 1985; Kessler *et al.*, 1986).

The chick C-proteinase has similar properties to the mouse enzyme (Kessler and Goldberg, 1978; Kessler *et al.*, 1986; Kessler and Adar, 1989). Both enzymes were found extracellularly, they required Ca^{2+} for activity, acted at physiological pH, cleaved the carboxyl propeptides of types I, II and III procollagen, and exhibited similar inhibition properties. However, full expression of the mouse C-proteinase activity was shown to depend on an enhancer protein (Adar *et al.*, 1986; Kessler and Adar, 1989).

Three glycoproteins have been purified with molecular weights of 55 kDa, 36 kDa and 34 kDa (determined by SDS- PAGE) which enhance C-proteinase activity and bind to the C-propeptide of type I procollagen (Adar *et al.*, 1986; Kessler and Adar, 1989). The 55 kDa protein was shown to cross-react immunologically with both the 36 kDa and 34 kDa proteins. In the course of pp-sepharose chromatography (chromatography on a column of Sepharose coupled to the C-propeptide of type I procollagen) which was used during the purification of the mouse enzyme, a large proportion of the 55 kDa protein was shown to disappear with the concomitant appearance of the 36 kDa and 34 kDa proteins (Kessler and Adar, 1989). These results suggest that the 55 kDa protein is a precursor of the low molecular weight enhancer proteins. The enhancer was shown to change the kinetics of the C-proteinase reaction (Adar *et al.*, 1986). In the presence of the enhancer, the rate of procollagen processing was shown to increase by 20-fold. Such an enhancer was not found in the tendon conditioned medium which served as the source for the chick enzyme (Hojima *et al.*, 1985). A possible explanation for this difference could be that the enhancer is a cell-surface protein. Therefore, little of it

would be expected to be released into the medium under organ culture conditions as in the Hojima *et al* (1985) preparation. In contrast, a significant release of cell-surface proteins is likely to occur in the Kessler and Adar (1986) system, where cells are grown in roller bottles, and therefore subjected to a constant washing by the medium.

The turnover number of the chick enzyme was shown to be relatively low (k_{cat} = 41 h^{-1}) which led Hojima *et al* (1985) to propose that the C-proteinase is present in the tissue as an inactive precursor or is inhibited by an endogenous component of connective tissue. The demonstration of C-proteinase enhancement by Kessler and Adar (1989) provides an additional mechanism, i.e. stimulation of the C-proteinase by an enhancer protein. Kessler and Adar (1989) proposed that the enhancer may act by presenting the procollagen molecule to the C-proteinase in a favourable conformation, increasing thereby the catalytic rate, and possibly the specificity of cleavage.

1.7.3 Pathways of procollagen processing

The conversion of procollagen to collagen can occur via two different intermediates: pN-collagen (procollagen with the C-propeptide removed) or pC-collagen (procollagen with the N-propeptide removed; see Figure 1.6). The sequence of cleavage of type I procollagen has not been established, with reports of processing via pC-collagen (Fessler *et al.*, 1975; Davidson *et al.*, 1977; Limeback and Sodek, 1979; Leung *et al.*, 1979; Gerstenfeld *et al.*, 1984) or pN-collagen (Veis *et al.*, 1973; Fleischmajer *et al.*, 1981, 1983; Helseth and Veis, 1984). Other studies have suggested that N-propeptide and C-propeptide cleavage may occur independently (Sonohara *et al.*, 1981). Fibroblast culture experiments have generally supported a pC-collagen processing intermediate. However, the results from the cell culture studies are difficult to relate to the process occurring *in vivo* because of the relatively small amount of complete processing of type I procollagen that occurs (Taubman and Goldberg, 1976; Limeback and Sodek, 1979; Bateman and Golub, 1990). Also, the absence of a substantial extracellular matrix in cell culture experiments, and the consequent rapid dilution of the procollagen into the culture medium may effect the order of propeptide cleavage.

Pulse chase experiments and electrophoretic analysis of salt-soluble extracts have shown developmental changes in the processing pathway in chick embryo cornea (Mellor *et al.*, 1991). The relative flux through the pC-collagen pathway was shown to increase approximately 4-fold between days 12 and 17 of chick embryo corneal development (Mellor *et al.*, 1991). The developmental changes in procollagen processing may have an effect on the organisation of the extracellular matrix. Since pC-collagen is more soluble than pN-collagen (Hulmes *et al.*, 1989b) it will diffuse more freely from the cells before being fully processed and finally deposited in fibrils. Therefore, altering the flux through the pC-collagen and pN-collagen pathways provides a possible mechanism whereby cells could control the site of deposition of collagen fibrils following the secretion of procollagen and its processing enzymes. Mellor *et al* (1991) suggested such a mechanism may be important ECM changes during corneal development.

The study state levels of pN-collagen were shown to exceed those of pC-collagen in both proα1 (I) and proα2 (I) chain processing in the developing chick embryo cornea (Mellor *et al.*, 1991). However, pC-collagen was found to be the major kinetic intermediate. Such a situation appears to exist in the chick embryo tendon, where immunoblotting has indicated a pN-collagen/ pC-collagen ratio greater than 1 (Fleischmajer *et al.*, 1988), whereas pulse-chase experiments show pC-collagen as the major kinetic intermediate (Leung *et al.*, 1979). Fibril diameter may be affected by the pathway of procollagen processing (for details, see section 1.8.3.4).

1.8 Fibril formation

The cleavage of the propeptides leads to the spontaneous assembly of fibrils. The collagen molecule contains all the necessary structural information for the assembly of fibrils.

1.8.1 Structural features of the fibril

The details of the molecular architecture of collagen fibrils has been derived from electron microscopy, X-ray diffraction and modelling from amino acid sequence data (for reviews, see Chapman and Hulmes, 1984; Piez, 1984).

Collagen fibrils in the electron microscope show a regular transverse banding pattern with an axial periodicity (D) of 64-67 nm. A positively stained collagen fibril has a repeating asymmetric band pattern with up to 12 distinct bands per period (Chapman and Hulmes, 1984). With this type of staining, heavy metal ions bind to regions rich in polar amino acids, and therefore, each band locates clustered charged groups along the collagen molecule. The alternating dark and light bands of the negative staining pattern show that each D-repeat can be divided into a 'gap' zone penetrated by the stain and an overlap zone which is relatively impervious to the stain. The pattern arises from staggered molecules spanning about four periods (see Figure 1.7). The D-staggered arrangement of molecules in a fibril was first elucidated by comparing positive staining patterns from fibrils with those from segment long spacing (SLS) collagen. Superposition of four SLS patterns mutually staggered by D was shown to give a pattern resembling that from a fibril (Hodge and Schmitt, 1960). The individual molecules are approximately 4.4 times the length of the repeat period, D (Hodge and Petruska, 1963), and are staggered with a 0.4 D overlap and a 0.6 D gap region.

The observed band pattern of positively stained type I collagen fibril was shown to compare well with the pattern predicted from the sequence (Meek *et al.*, 1979; Chapman and Hulmes, 1984). Assuming uniform spanning of the residues in the triple helix, the best agreement was obtained when the assumed stagger between molecules was 234 residues. An independent derivation of the number of residues in a D-period was obtained from sequence data alone (Hulmes *et al.*, 1973). Assuming a regular spacing of residues, charged and hydrophobic interactions between the $\alpha 1$ chains were calculated (Hulmes *et al.*, 1973). The interactions were shown to be maximal at staggers of multiples of 234 residues. Repeated clusters of hydrophobic and charged residues divide the monomer into 4.4 D units and cause the monomers to associate so that each is staggered by one D-unit or a multiple of D-units relative to its neighbour

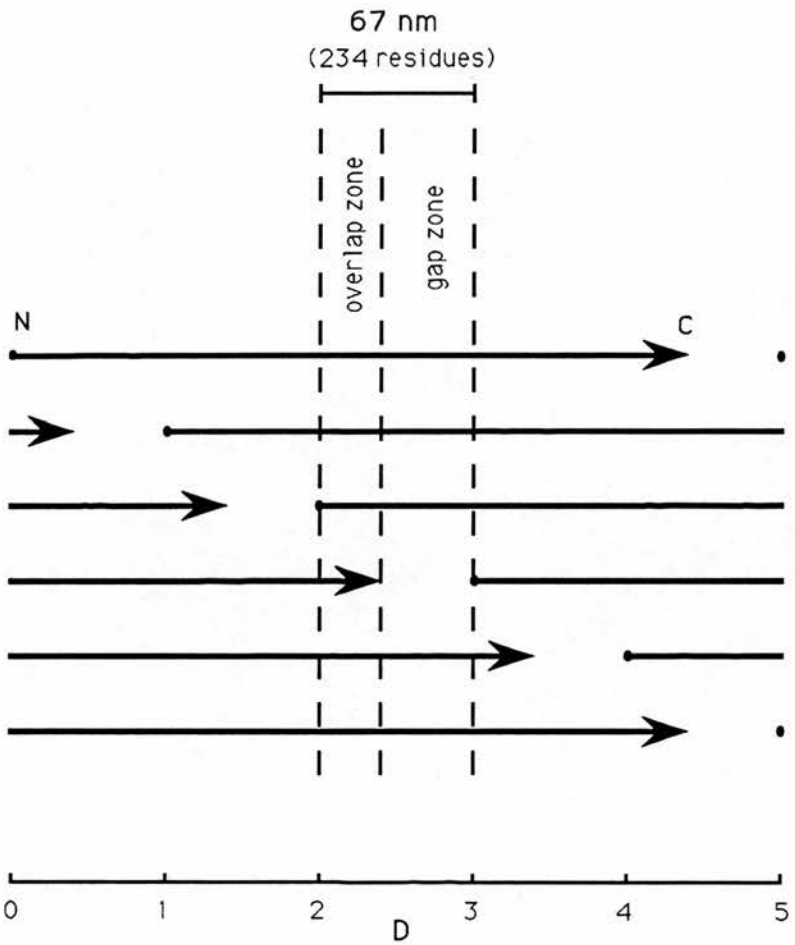


Figure 1.7 Schematic representation of the axial stagger relationship of type I collagen molecules in the fibril

(Piez, 1984).

Each molecule (approximately 4.4 D long) is represented by an arrow with the C-terminal at the arrow head. Only the 1D and 4D staggers are shown here, however 0D, 2D and 3D staggers between adjoining molecules may also exist (adapted from Chapman and Hulmes, 1984). The D-periodicity thus lies in the amino acid sequence of the α-chain.

Information about the fibril axial order has also been obtained by X-ray diffraction of moist rat tail tendon. Reflections on or near the meridian arise from axial order and indicate a periodicity of 67 nm. The sequences of the α1 (I) and α2 (I) chains have been used to predict the meridional intensities (Hulmes *et al.*, 1980). The best agreement between the predicted and observed intensities was obtained when D was set at 235 residues and the average residue spacing in the telopeptide region was assumed to be about three-quarters of the spacing in the triple helix. The latter confirms the suggestion from sequence data that the telopeptides are in a condensed conformation (Capaldi and Chapman, 1982).

The lateral arrangement of molecules in collagen fibrils is poorly understood (Hulmes *et al.*, 1985; Raspanti *et al.*, 1989; Jones and Miller, 1991). One view is that the packing is defined by a regular filamentous substructure, microfibrils. The alternative view is that the lateral packing occurs without an intermediate structure, that disorder is common but large crystalline domains are present under appropriate conditions.

Smith (1968), proposed the five-stranded microfibril model in which a molecular monolayer five molecules wide is wrapped into a cylinder (4 nm in diameter and of indefinite length) as the basic structural unit of the fibril (see also; Vies *et al.*, 1979; Piez, 1984; Scott, 1990). In this model, the molecules are staggered by D, and are in a specific helical geometry. By electron microscopy, filamentous subunits have been revealed within the collagen fibrils by negative and positive staining of dissociated material (Piez, 1984; Scott, 1990; Fleischmajer *et al.*, 1990), and freeze fracture (Ruggeri *et al.*, 1979; Marchini and Ruggeri, 1984). The filaments have been purported to be D-periodic on the basis of X-ray diffraction observations (Veis *et al.*, 1979), though a D-periodicity has not been observed by electron microscopy. Two distinct

classes of fibrils, one having helical microfibrils at an angle of about 18° to the fibril axis, the other with straight or slightly wavy microfibrils have been observed in several tissues (Ruggeri *et al.*, 1979; Marchini *et al.*, 1986). Collagen fibrils with the helical microfibrillar arrangement are characterised by a relatively small diameter.

Difficulties arose when attempts were made to use the five-stranded microfibril model to explain X-ray diffraction data. Re-interpretation of the X-ray data led to the proposal of a new model, the three dimensional crystal model (Hulmes and Miller, 1979) which has been refined (Fraser and Macrae, 1981). In this model, the fibril is a three dimensional crystal in which the collagen molecule is the basic unit without an intermediate substructure. Electron microscopic studies have shown a radially orientated periodicity of about 4 nm in collagen fibril cross-section which corresponds to the lateral periodicity detected by X-ray diffraction (Hulmes *et al.*, 1981, 1985; see Figure 1.8). The lateral periodicity has been visualised directly using image processing techniques, and the extent of crystallinity has been shown to increase towards the periphery of a fibril (Hulmes *et al.*, 1985). The electron microscopic results and the quasi-hexagonal model have been combined (Hulmes *et al.*, 1983) to give a model for fibril structure in which the molecules appear tilted by 5° when viewed from the side. In this model, the molecules in the fibril perimeter are in non-staggered axial register as in SLS assemblies which may have significance for fibrillogenesis (see section 1.8.3).

X-ray diffraction of tendon collagen suggest that the molecular segments in the gap region are more mobile than those in the overlap region, with segments in the gap region kinked to give a D-periodic molecular crimp (fold or wave) in the structure (Fraser *et al.*, 1987). The highly ordered overlap region is thought to consist of crystalline domains, whereas the gap region is less well ordered. There is a low content of aromatic residues in the gap region which would increase its mobility (Fraser *et al.*, 1987). The aromatic residues that are present are concentrated at the site of attachment of a dermatan sulphate proteoglycan (see section 1.8.3.2). The concentration of amino acids is markedly lower in those parts of the D-period where the telopeptides occur. This would permit changes in molecular direction leading to departures in the quasi-hexagonal packing and to changes in the net direction of the helical segments. The gap regions are sites within which other molecules might reside, for example, the

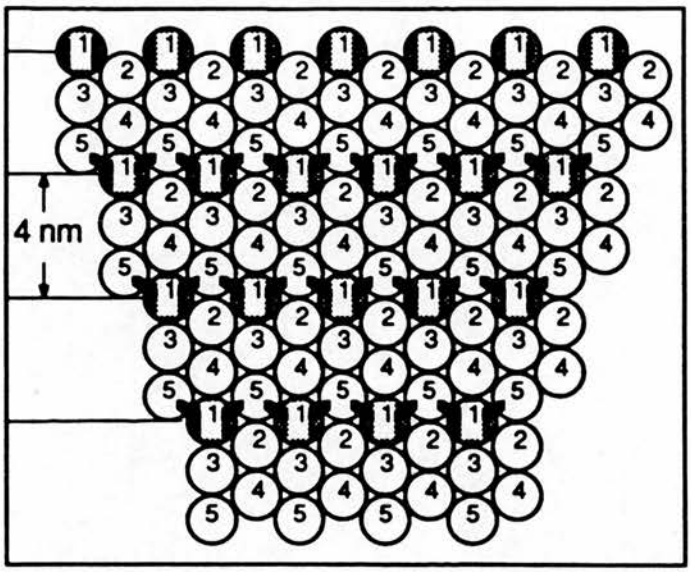


Figure 1.8 Lateral molecular packing arrangement within a crystalline domain of type I collagen fibrils

Each circle represents a collagen molecule in cross-section. Circles numbered 2,3,4, and 5 represent molecules staggered by 1D, 2D, 3D and 4D with respect to molecules represented by circles numbered 1. The model shows a radially orientated periodicity of 4 nm. Cross-links between adjacent molecules are shown by thick lines.

propeptides of procollagen (Mould *et al.*, 1990) or the side chain of type IX collagen (Vaughan *et al.*, 1988). During bone mineralisation, the initial formation of hydroxyapitite crystals has been proposed to occur within the gap region (Glimcher, 1989).

Some specimens have been shown not to exhibit three-dimensional crystallinity (Brodsky and Eikenberry, 1986), but only a diffuse equatorial intensity. Even in rat tail tendons, there was shown to be an underlying diffuse equatorial intensity in the diffraction pattern. This indicates a liquid-like disorder in the lateral molecular packing. The combination of axial order and lateral disorder is analogous to the structure of smectic A crystals (for more information, see Woodhead-Galloway and Machin, 1976; Hukins and Woodhead-Galloway, 1977).

A compromise model has been suggested for the lateral organisation of the fibril (Trus and Piez, 1980) in which microfibrils are compressed giving rise to quasi-hexagonal packing. Chapman and Hulmes (1984) proposed that fibril formation could occur via microfibrils which undergo lateral compression on assembly giving rise to quasi-hexagonal packing as growth proceeds. Alternatively, Raspanti *et al.* (1989) suggested that the fibrils may be in a smectic state in which there is a certain lateral mobility of the molecules so that the same fibril may show either the microfibril or the quasi-hexagonal molecular lattice while still maintaining the same molecular connectivity.

1.8.2 Fibrillogenesis *in vitro*

The assembly of collagen fibrils has been extensively studied by neutralisation and warming of cold acid-soluble collagen solutions (Cooper, 1970; Comper and Veis, 1977; Helseth and Veis, 1981; Suarez *et al.*, 1985; Holmes *et al.*, 1986; Na *et al.*, 1986; Na *et al.*, 1989). Electron microscopic (Williams *et al.*, 1978) and X-ray diffraction data (Eikenberry and Brodsky, 1980) suggest that these fibrils are very similar to fibrils formed *in vivo* (i.e. D-periodic) except the lateral order is generally not as good. The fibril forming process has been followed by monitoring the turbidity at 313 nm. A typical turbidity curve is characterised by a lag phase, an exponential

growth phase and a plateau. The non-helical telopeptides have been shown to play a significant role in the assembly of the fibril. Deletion of both of the telopeptides abolishes fibril formation (Capaldi and Chapman, 1982).

The mechanism for the assembly of fibrils is not fully understood (for a review, see Olsen, 1991). Electron microscopy, dynamic light scattering and X-ray scattering techniques have been used to probe the initial steps of the reaction in the lag phase (Gelman and Piez, 1980; Silver and Trelstad, 1980; Silver, 1981; Bernengo *et al.*, 1983; Suarez *et al.*, 1985; Payne *et al.*, 1986; Ward *et al.*, 1986; Na, 1989b). In the lag phase, the solution does not display any turbidity. Whether collagen forms an intermediate during the lag time is controversial. Several studies have shown the presence of 4D-dimers and trimers in the lag phase (Silver and Trelstad, 1980; Silver, 1981; Trelstad *et al.*, 1982; Ward *et al.*, 1986). Telopeptide-like interactions are greatest and contribute more to the total interaction energy when the overlap is least (Ward *et al.*, 1986). Long thin aggregates containing 5-100 monomers have also been identified in the lag phase (Gelman and Piez, 1980). These observations and others (Suarez *et al.*, 1985) have given rise to the intermediate assembly hypothesis which suggests that collagen forms intermediate aggregates which then add to each other to form higher multiples of macromolecules and ultimately fibrils. A model has been proposed for the assembly of long collagen subfibrils from 4D-trimers (for further details, see Olsen, 1991). In contrast, Bernengo *et al.* (1984) using both dynamic light scattering and electric birefringence techniques, did not detect the formation of any significant amount of collagen oligomers during the lag phase. This result was confirmed by a similar light scattering study (Payne *et al.*, 1986). Bernengo *et al.* (1984) attributed the lag phase to an intramolecular event in the form of a conformational change leading to a stiffening of the collagen molecule.

Reconstitution of disassembled fibrils no longer shows a lag phase. This phenomena is called the thermal memory effect and can be explained either by the presence of oligomers (Na *et al.*, 1986; Na *et al.*, 1989a,b) or a conformational change in the collagen molecule (Helseth and Veis, 1981).

The major driving force for fibril assembly is the increase in entropy associated with the loss of water bound to the monomer as the polymerisation occurs (Kadler *et*

al., 1987; Na *et al.*, 1989b). From kinetic studies, a cooperative nucleation and growth mechanism has been derived for the assembly of the fibrils (Cooper, 1970; Wallace and Thompson, 1983). In this mechanism, the rate limiting step is the formation of a critical nucleus, and then growth proceeds rapidly by accretion of monomers. In an equilibrium fibril reconstitution study, Na *et al* (1989b) have shown the requirement of a certain minimal concentration of collagen (less than 1 μg/ ml) for fibril assembly. Kadler *et al* (1987) have also observed a critical concentration (0.5 μg/ ml at 37 °C), in the assembly of fibrils *de novo* from processed procollagen (see below). These observation of a critical concentration are consistent with the cooperative nucleation-growth mechanism derived from the kinetic data. Critical concentrations are observed typically in strongly cooperative self-association systems such as the assembly of actin filaments and microtubules (Oosawa and Asakura, 1975), though the self assembly of type I collagen into fibrils differs somewhat, in that the final polymeric structure of collagen is formed by the addition of a rod-like monomer in both longitudinal and lateral directions. Cooperativity is recognised as one of the means used by biological systems to generate a small number of very large biomolecular assemblies rather than a large number of small aggregates. A strongly cooperative self-association exhibiting critical concentrations should not accumulate any significant amount of oligomers smaller than the nucleation centre (Oosawa and Asakura, 1975). As of yet, the nuclei have not been demonstrated or identified.

An alternative system for fibril assembly *in vitro* has been recently developed and involves generating collagen *de novo* by enzymatic processing of pC-collagen under physiological conditions (Miyahara *et al.*, 1982, 1984; Kadler *et al.*, 1987, 1988). Cleavage of pC-collagen by C-proteinase generates collagen that readily assembles into fibrils.

Kadler *et al* (1988) have shown that the kinetics of the assembly of collagen fibrils are markedly different below and above 37 °C. Increasing the temperature in the range 29-35°C decreased the lag phase and increased the rate of propagation as assayed by turbidity. In contrast, the rate of propagation decreased markedly as the temperature increased from 37 to 41 °C. A possible explanation for the abrupt change in the kinetics, is that at temperatures above 35 °C micro-unfolded forms of procollagen may

become an increasingly larger fraction of the system (for further details, see Kadler *et al.*, 1988).

Kadler *et al* (1990) used the new *in vitro* system with dark-field light microscopy to study the growth of fibrils. They demonstrated that all type I collagen fibrils formed *in vitro* have blunted and highly tapered, symmetrical, pointed tips. The pattern of cross-striations in the pointed tips indicate that all the molecules were oriented so that the N-termini were directed towards the tip. These results are consistent with observations of Birk *et al* (1989), who defined the ends of individual type I collagen fibrils by analysing electron micrographs of serial sections of leg tendons from chick embryos. Results from both studies suggest that fibrils grow unidirectionally from pointed tips, but that bidirectional growth can occur if monomers in the reverse orientation bind to a blunted end, and then form the equivalent of a new pointed tip for growth in the opposite direction (Birk *et al.*, 1989; Kadler *et al.*, 1990). The pointed tip of the fibril was suggested to be the site with the highest affinity for binding of monomers. The growth of collagen fibrils from pointed tips suggests that the molecular principles are similar to those involved in generating other large structures in nature, such as snow flakes that grow from pointed tips and frequently generate fractals (Nittmann and Stanley, 1986).

1.8.3 Fibrillogenesis *in vivo*

Studies of fibrillogenesis *in vitro* present an incomplete picture because many other factors are involved in the assembly process in living tissues. The form of procollagen,

the interaction between different genetic types of collagen (Mendler *et al.*, 1989; Linsenmayer *et al.*, 1990; Fleischmajer *et al.*, 1990b), the interactions of procollagen processing intermediates (Hulmes *et al.*, 1989), proteoglycans (Scott, 1988; Fleischmajer *et al.*, 1991) and the cell surface may play important roles in fibrillogenesis *in vivo* .

The fibril forming collagens assemble in vivo from their biosynthetic precursors,

procollagen (Prockop and Kivirikko, 1984). Several investigators have suggested that procollagen SLS-like aggregates may be intermediates in the formation of D-periodic fibrils (Bruns *et al.*, 1979; Hulmes *et al.*, 1983; Gross and Bruns, 1984). Ultrastructural studies of fibroblasts *in vivo* have demonstrated the presence of SLS-like aggregates within secretory vesicles (see section 1.6). Mould *et al* (1990) have also shown that SLS-like aggregates can form in solution at concentrations of procollagen that may be present in the extracellular matrix (for further details regarding the form of procollagen in the extracellular milieu, see section 1.6). Collagen molecules in the fibril perimeter appear to be in non-staggered axial register (Hulmes *et al.*, 1981, 1983, 1985). Fibril growth by the accretion of SLS sheets may account for the observations of Parry and Craig (1979) that fibril growth occurs in 8 nm increments (Hulmes, 1983). However, SLS-like aggregates have not been observed within Golgi vacuoles or secretory vacuoles *in vitro* (Phelps *et al.*, 1985). The abundance of collagen fibrils in the fibroblast culture system led Phelps *et al.* (1985) to suggest that cytoplasmic SLS-like aggregates are not required for procollagen secretion and fibrillogenesis.

1.8.3.1 Cell-mediated collagen fibril assembly

Many studies have suggested that the cell surface influences collagen aggregation and fibrillogenesis directly, either by determining the orientation of collagen fibrils or inducing aggregation.

Dimeric SLS-like aggregates have been observed bound to the secretory vesicle membrane (Trelstad and Hayashi, 1979). Such an interaction could dictate the alignment of the procollagen molecules and represent an early stage in fibril development. The polarised discharge of matrix components into the extracellular space has also been observed from a variety of matrix producing cells (Weinstock and Leblond, 1974; Trelstad, 1977; Trelstad and Birk, 1984). The polarised discharge of procollagen is a means by which cells can influence the organisation of the architecture of the matrix. The binding of collagenous molecules to a plasma membrane component has been proposed as an *in vivo* mechanism for initiating fibril assembly and

determining the spatial specificity of that assembly (Goldberg and Burgeson, 1982; for further information, see Chapter 4).

The study of the assembly process *in situ* using electron microscopy has indicated that the topography of the cell surface and intracellular spaces play an important role in the orientation of the assembly process (Trelstad and Hayashi, 1979; Birk and Trelstad, 1984; Birk and Trelstad, 1986; Birk *et al.*, 1989; Birk *et al.*, 1990a). Chick embryo corneal, tendon and dermal fibroblasts have been demonstrated to compartmentalise the extracellular space during the development of the extracellular matrix (Birk and Trelstad, 1984, 1986; Ploetz *et al.*, 1991). Such compartmentalisation would allow the cell to control the environment in which fibrils form. The long narrow compartments have been suggested to form from the tandem fusion of secretory vacuoles by a mechanism of compound exocytosis similar to that seen in other systems (Birk and Trelstad, 1986). D-periodic fibrils segments (approx. 10 μM in length) containing tapered ends have been shown to form within the compartments (Birk *et al.*, 1989) which are consistent with the segment-like structures formed *in vitro* (Kadler *et al.*, 1990). The removal of the propeptides must also take place in these compartments. The fibril segments have been proposed to fuse linearly and/ or laterally to form mature more continuous fibrils (Birk *et al.*, 1990). These fibrillar fusions provide a mechanism for the lateral growth observed during tendon development. Scott (1986) has also proposed that fusion of fibrils occur with possible regulation via surface decorin (see section 1.8.3.2). Three distinct extracellular compartments have been observed (Birk *et al.*, 1990; Ploetz *et al.*, 1991). These specialisations of the fibroblast surface have been suggested to be important for directing the orientation of the developing matrix.

Mechanical forces exerted by the fibroblast on the forming fibrillar matrix have also been shown to be important in establishing its architecture (Harris *et al.*, 1981; Stopak *et al.*, 1985). They showed that collagen injected into tissues or placed in culture can be aligned by fibroblasts.

Aggregation of procollagen has been shown to be strongly favoured when samples are adsorbed to a mica or carbon surface during preparation for electron microscopy (Mould and Hulmes, 1987). Large aggregates containing procollagen molecules connected via their C-propeptide were common. The formation of in-register aggregates

of procollagen molecules during adsorption to a mica surface may occur by a mechanism

suggested for the formation of parallel, in-register arrays of actin filaments at the surface of positively charged liposomes (Rioux and Gicquad, 1985). These workers suggested that the electrostatic interactions of one actin filament with the liposome surface favours the lateral interaction of other filaments in-register. Aggregation may be favoured at the cell surface and would assist both propeptide cleavage and the subsequent assembly of the processed procollagen into fibrils.

1.8.3.2 Glycosaminoglycans (GAG) and proteoglycans (PG)

Type I collagen has been shown to interact with both GAGs and PGs (for a review, see Scott, 1988). Such interactions may play a role governing the rate and extent of fibrillogenesis (Scott, 1986; Vogel and Trotter, 1987; Uldbjerg and Danielson, 1988).

Obrink (1973) has shown that dermatan sulphate, heparan sulphate and heparin interact strongly with collagen at 4 °C, whereas chondroiton sulphate and keratan sulphate show a low affinity for collagen. Using the classical reconstitution system (see section 1.8.2) the GAGs that interact strongly with collagen were shown to accelerate the lag phase. Although dermatan sulphate interacts with monomeric collagen it was not incorporated into types I and II collagen fibrils *in vitro* .

Small dermatan sulphate proteoglycans (DSPG) from tendon (Vogel *et al.*, 1984) and skin (Scott, 1986) have been shown to inhibit fibril formation of type I and II collagen. This effect was preserved following the removal of the dermatan sulphate chains suggesting that the effect of fibrillogenesis was due to the protein core. Vogel and Trotter (1987) have also shown that small DSPG decreases the final fibril diameter and suggested that it may inhibit lateral aggregation.

Small DSPG derived from aorta and cartilage have been shown to have different protein cores (Heinegard *et al.*, 1985). These PGs were demonstrated to have no effect on the rate of fibrillogenesis but were shown to increase the final absorbance by up to 90 % (Uldbjerg and Danielson, 1988). These proteoglycans have been shown to induce a lateral alignment of the collagen fibrils (Uldbjerg and Danielson, 1988).

Using Cupromeronic blue to visualise DSPG in the electron microscope, Scott and Orford (1981) showed that the PG was localised at the d and e bands[12] (in the gap zone) of collagen fibrils in tendon. The site of attachment is characterised by a high content of aromatic residues. The small DSPG appeared orthogonal to the collagen fibrils and spaced D-periodically.

Pringle and Dodd (1990) have also studied the interaction of decorin[13] with tendon fibrillar collagens, using monoclonal antibodies to the decorin protein core. They have shown that the decorin protein core associates near the d and e band in the D-period. The core protein of decorin has also been shown to interact with type I collagen fibrils in skin (Fleischmajer *et al.*, 1991). Fleischmajer *et al* (1991) speculated that the binding of decorin to the gap region may interfere with the lateral accretion of collagen molecules and stop the growth of the fibril, and thus be the regulatory mechanism for the final fibril diameter. However, the hypothesis is contradicted by the interaction of decorin with all collagen fibrils regardless of their diameter.

Microfibrils (termed protofibrils by Scott, 1990) produced by disaggregated rat tail tendon fibrils in the presence of cupromeronic blue were shown to be associated with PGs. Scott (1990) suggested that this result was due to the formation of large fibrils during development by the fusion of smaller fibrils (microfibrils) some of which had retained their associated PG. The role of PG in young tissue may be to keep the microfibrils from coalescing.

1.8.3.3 Heterotypic fibrils

Recent studies have established the existence of heterotypic fibrils assembled from more than one collagen type. Types I and III collagens codistribute in most connective tissues except for bone and cornea. Evidence that types I and III collagens are assembled in the same fibril comes from the isolation of intermolecular cross-links between $\alpha 1$ (I) and $\alpha 1$ (III) chains in human tissues (Henkel and Glanville, 1982) and immunoelectron microscopic studies demonstrating the existence of heterotypic fibrils

[12] These refer to the positively stained banding pattern of type I collagen (see section 1.8.1).

[13] There are 2 types of small DSPG, decorin (PG II) and biglycan (PG I).

in skin (Keene *et al.*, 1987; Fleischmajer *et al.*, 1990 a,b). Collagen fibrils disrupted by 8 M urea were shown to contain type I collagen throughout the fibril, except for the microfibrils at the periphery which were coated with type III collagen (Fleischmajer *et al.*, 1990a). Only type III collagen antibodies were shown to label large intact fibrils. Thin fetal collagen fibrils showed periodic labelling for type I collagen, while large adult fibrils did not. These observations suggest a specific structural organisation where type I collagen forms the bulk of the fibril surrounded at its periphery by type III collagen. The location of type III collagen at the surface of the fibril led Fleischmajer *et al* (1990) to suggest it may allow the possible interactions of type III collagen with other constituents of the extracellular matrix.

The molecular organisation of type III collagen at the periphery of the fibril is poorly understood. The type III collagen is probably linked to the underlying collagen either in a D-periodic or D-0 staggered fashion which would account for the D-periodicity of the heterotypic fibrils. The immunolabelling of regular filaments (microfibrils) formed by the disruption of dermal fibrils with urea (Fleischmajer *et al.*, 1990a) suggests that type III collagen could be in the form of microfibrils at the periphery of the fibril.

Immunoelectron microscopic studies have been used to show that fibrils from adult cornea consist of both types I and V collagen (Birk *et al.*, 1988; Fitch *et al.*, 1988; Linsenmayer *et al.*, 1985; Linsenmayer *et al.*, 1990). Both types I and V collagen have been shown to label partially disrupted collagen fibrils (Linsenmayer *et al.*, 1990). However, only type I collagen labelled intact fibrils. Collagenase treatment (specific for types I or III) confirmed the above observations and suggest that type V collagen is within the corneal collagen fibrils (Fitch *et al.*, 1988).

In vitro reconstitution studies have shown that type V collagen has a direct effect on fibril diameter (Adachi and Hayashi, 1986; Linsenmayer *et al.*, 1990). The average diameter of the heterotypic fibrils formed *in vitro* was shown to decrease when the relative proportion of type V collagen increased. Native type V collagen was shown to be more effective in producing this affect then pepsin treated material. This suggests a possible steric involvement of the type V collagen globular N-propeptide. Adachi and Hayashi (1987) suggested that the very small difference in electron density between the

gap/ overlap zone in heterotypic fibrils containing more than 50 % type V collagen was due to the presence of the globular N-terminal domain in the gap zone.

Cartilage fibrils have been shown to be heterotypically assembled from types II, IX and XI collagen (van der Rest and Mayne, 1988; Vaughan *et al.*, 1988; Mendler *et al.*, 1989). Type XI collagen has been localised in partially disrupted cartilage fibrils (Mendler *et al.*, 1989). Only type II collagen has been located on the surface of intact fibrils which suggests that type XI collagen is buried in the interior of the fibril. Type XI collagen is structurally similar to type V collagen in that both contain a large amino-terminal non-collagenous domain in addition to a 300 nm triple helix (see section 1.2.1). Both collagens also appear to be located within fibrils with uniform thin diameters (25 nm). Type XI collagen has been shown to form thin fibrils *in vitro* (Smith *et al.*, 1985) and therefore, like type V collagen may function in controlling the lateral growth of fibrils. Type XI collagen has been immunolocalised on to the surface of chondrocytes in culture (initially stripped of matrix), during the initial stages of matrix regeneration (Smith *et al.*, 1989). The nature of the association was suggested to be ionic, since 4M guanidinium chloride completely removed type XI collagen from the cells. This is consistent with a previous suggestion that type XI collagen is retained at the cell surface by interactions with a cell-associated polyanion (Smith *et al.*, 1985). The existence of type XI collagen within heterotypic fibrils has led Smith *et al* (1989) to propose the retention of this collagen at the cell surface may be a step in fibril assembly. Rotary shadowing in combination with a type IX collagen antibody has shown that type IX collagen is arranged D-periodically along chick embryo cartilage fibril fragments (Vaughan *et al.*, 1988). The projection of the amino terminal domain of type IX collagen from the fibril surface would facilitate interactions with proteoglycans in the cartilage matrix (for further details, see section 1.2.2).

The homology between the structures of types IX and XII collagen suggest that type XII may also be associated with collagen fibrils (Gordon *et al.*, 1990). Immunolocalisation studies indicate that type XII collagen is present in many tissues rich in type I collagen. However, localisation of type XII collagen on the surface of type I collagen-containing fibrils has not been demonstrated.

1.8.3.4 Intermediates of procollagen processing

According to the pathway of processing, different processing intermediates pN-collagen and pC-collagen are produced (see section 1.7.3). Many studies have suggested that pN-collagen is involved in fibrillogenesis *in vivo* (for a review, see Hulmes *et al.*, 1989a). Hulmes *et al* (1989a) studied the assembly of type I pN-collagen using a reconstitution system where the molecules were generated enzymatically from their biosynthetic precursors. Increasing the pN-collagen content was shown by electron microscopy to produce D-periodic fluted fibrils that became progressively more distorted. These fluted fibrils resembled the fibrils in dermatosparaxis, an inherited disorder in which N-propeptide processing is defective (see section 1.12).

Electron microscopic immunolocalisation studies have suggested a preferential association of the N-propeptides with small diameter fibrils (Fleischmajer *et al.*, 1983, 1985, 1987). This observation and that of Hulmes *et al* (1989a) has led to the suggestion that type I pN-collagen may coat the surface of growing collagen fibrils, thus blocking surface sites on the fibril, and hence preventing further accretion of molecules (Hulmes, 1983; Fleischmajer *et al.*, 1983; Chapman, 1989). The removal of the type I N-propeptide would allow further growth and the formation of mature fibrils.

Since the assembly of pN-collagen and collagen *in vitro* does not lead to the formation of thin cylindrical fibrils, additional factors must also operate *in vivo* to regulate normal fibril growth and form. Recent observations have suggested that under certain conditions molecules retaining the C-propeptide domain can participate in fibril formation. Fibrils from chick embryonic tibiae (Fleischmajer *et al.*, 1987), human fibroblast cultures (Phelps *et al.*, 1985), and chick embryo chondrocyte cultures (Ruggiero *et al.*, 1988) have been labelled D-periodically with antibodies to the C-propeptide. However, these studies could not show if the C-propeptides were still part of the pC-collagen. Mould *et al* (1990) demonstrated that pC-collagen can be incorporated into D-periodic procollagen assemblies. Immunolabelling studies have shown that the C-propeptide is located on the surface of D-periodic procollagen assemblies at the C-terminal gap/ overlap junction. Both pN-collagen and pC-collagen may be involved in the regulation of fibril diameters *in vivo* .

1.9 Formation of collagen fibril crosslinks

Collagen fibrils are stabilised extracellularly by the formation of covalent crosslinks (for a review, see Robins, 1982; Eyre *et al.*, 1984; Last *et al.*, 1990). In the absence of crosslinks, the mechanical stability of reconstituted collagen fibrils has been shown to be dramatically reduced compared to native tendon collagen (Kato *et al.*, 1989). The formation of crosslinks involves a complex series of spontaneous reactions that are initiated by lysyl oxidase (Kagan and Trackman, 1991). This enzyme catalyses the oxidation of specific lysine and hydroxylysine residues located in the N and C-terminal telopeptides. The resulting aldehydes react with the ε-amino groups of lysine or hydroxylysine residues in the triple helical regions on adjacent molecules to form bifunctional crosslinks. The crosslink formed from the initially oxidised hydroxylysine can undergo an Amadori rearrangement to form a more stable ketoamine crosslink (Robins, 1982). With time the bifunctional crosslinks can undergo further reactions to form a variety of mature, tissue-specific, trifunctional crosslinks (Seyedin and Rosen, 1990). The crosslinks can be present in a glycosylated form (Last *et al.*, 1990). The crosslinking sites in the triple helix are characterised by low proline and hydroxylysine content, and the presence of the sequence hyl-gly-his-arg, has been proposed to be the attachment site for lysyl oxidase (Kuhn, 1987). Aggregation of collagen is a prerequisite for lysyl oxidase activity (Siegel, 1974).

TRAMP[14] (Mr 24 kDa) is a protein that copurifies with lysyl oxidase from porcine skin which has been demonstrated to accelerate fibril formation *in vitro* (Cronshaw, Macbeath, Shackleton, Collins, Fothergill-Gilmore and Hulmes, 1992, in press). It is possible that TRAMP may influence lysyl oxidase indirectly via aggregation of the substrate.

[14] Tyrosine rich acidic matrix protein

1.10 Regulation of collagen biosynthesis

Collagen gene expression is regulated both in developmental and tissue specific manners as well as in response to a variety of growth factors, hormones, cytokines and pharmacological inducers (for a review, see Bornstein *et al.*, 1982; Raghow and Thompson, 1989; Sandell and Boyd, 1990; Vuorio and de Crombrugghe, 1990; Ramirez and Liberto, 1990). The regulation of collagen gene expression is complex utilising transcriptional, post-transcriptional and translational mechanisms.

Different collagen genes are activated in specific cells or tissues during embryogenic development. For example, fibroblasts synthesise predominantly types I and III collagens (Tolstochev *et al.*, 1981), and chondrocytes synthesise type II collagen (Mayne and Burgeson, 1987). The activation of different collagen genes in specific cells or tissues during embryogenic development, their restricted expression in specific tissues of adult organisms and the changes in expression of certain of these genes in several disease states have prompted studies aimed at identifying the regulatory segments of these genes.

Putative regulatory sequences have been deduced from sequence comparisons between collagen genes and homologous structures known to be involved in the regulation of other eukaryotic genes. The location of the initiation codon, the TATA and CAT boxes on the 5' proximal region of the gene, precise location of splicing junctions that follow Chambon's rules and multiple poly A addition sequences in the 3' proximal area of the collagen gene have been well established (de Wet *et al.*, 1987; Chu *et al.*, 1985).

Transgenic mice have provided an experimental system which has allowed the determination of the specific DNA sequences of a gene that have the ability to confer tissue specific expression in intact animals (Vuorio and de Crombrugghe, 1990). Typically, a chimeric gene in which potential regulatory sequences are fused to a marker gene (e.g. chloramphenicol acetyltransferase, CAT) is introduced into the germline of mice, and the expression of the marker gene monitored in different tissues. A 2000 base pair (bp) fragment upstream of the start of transcription of the mouse $\alpha 2$ (I) gene has been fused to the bacterial gene of CAT and introduced into the germline of mice.

The expression of the CAT transgene was found to be high only in tissues rich in type I collagen (Khillan *et al.*, 1986). Therefore, the 2000 bp segment 5' to the transcription start site was shown to be sufficient to confer tissue specificity. An α1 (II) collagen promoter-CAT chimeric gene has been demonstrated to show selective expression in tissues in which the endogenous type II collagen gene is expressed (Yamada *et al.*, 1990). Therefore, the collagen DNA sequences present in the transgenes contain the necessary cis-acting elements which by interacting with defined cellular factors (see below) determine the expression of these transgenes in specific cells and tissues of intact animals.

In the mouse α1 (I) gene a cell-specific enhancer has been located in the first intron (Rossi and de Crombrugghe, 1987). The insertion of mouse leukemia retroviral sequences in the first intron of the mouse α1 (I) collagen gene was shown to result in a block in transcription of this gene (Hartung *et al.*, 1986). Heterozygous mice that harboured the inserted sequences in one allele were shown to have a phenotype corresponding to a mild form of type I osteogenesis imperfecta (see section 1.12). This was because only 50% of the normal amount of collagen was synthesised. A possible explanation for the absence of transcription of the α1 (I) collagen gene was that the inserted viral sequence would disrupt an enhancer located in the first intron.

An 800 bp DNA sequence located in the first intron of the rat α1 (II) gene together with the promoter was shown to stimulate transcriptional activity (Horton *et al.*, 1987). The enhancer was shown to be maximally active in chondrocytes, with little response observed in chicken fibroblast or myoblasts (Horton *et al.*, 1987). The enhancer activity was shown to be lost in chondrocytes treated with retinoic acid which suppresses chondrogenesis. The mechanism by which this phenotypic-enhancer functions is not known.

Both positive and negative cis-acting elements have been identified in the first intron of the human α1 (I) collagen gene (Bornstein and Mackay, 1988; Bornstein *et al.*, 1988). Negative acting elements (silencers) share the properties of enhancers in being able to function in either orientation and being relatively independent of position, but repress rather than enhance transcription. The first intron of the human α1 (I) collagen gene has been shown to contain a negatively acting element that inhibits

transcription of the CAT gene driven by either a collagen or SV40 basal promoter (Bornstein *et al.*, 1987). This element has been shown to be flanked by sequences that both neutralise the inhibitory effect, and impart a net negative effect on transcription (Bornstein and Mackay, 1988). The presence of the intact intronic sequence was shown to stimulate transcription by a factor of 2-3 fold in comparison with intron deleted plasmids. However, the isolated negatively acting element inhibited transcription by a factor of 15-20 fold. The expression of the α1 (I) collagen gene appears to be controlled by several intronic elements that function coordinately with 5' flanking and promoter elements. However, the understanding of the regulatory elements in the first introns of the α1 (I) and α2 (I) collagen gene is still incomplete.

Several DNA-binding factors present in nuclear extracts of NIH-3T3 fibroblasts interact at approximately the same location of the promoters in the α1 (I) and α2 (I) collagen genes (Vuorio and De Crombrugghe, 1990; Ramirez and Liberto, 1990). One of the factors, a heterodimer (Mr 39 and 41 kDa) was shown to bind to a CCAAT motif in the mouse α1 (I) and α2 (I) gene (Maity *et al.*, 1988). A purified preparation of the CCAAT binding protein was shown to stimulate initiation of transcription of both type I collagen genes in a reconstituted *in-vitro* transcription system (Maity *et al.*, 1988). Two additional DNA-binding factors that bind to sequences immediately 5' to the binding site for the CCAAT-binding protein have been shown to be negative regulatory factors (Vuorio and de Crombrugghe, 1990). The two negative regulatory factors and the positive CCAAT-binding factor have been suggested to participate in the coordinate control of the type I collagen genes.

An important structural feature of the DNA of actively transcribing genes is that it is generally hypomethylated in critical sites. Investigations have shown that DNA methylation in eukaryotes is associated with the long term inactivation of genes during development (Razin and Riggs, 1980). However, the analysis of collagen genes in terms of their methylation status is somewhat controversial. The middle and the 3' regions of α2 (I) gene have been shown to contain methylated sites (McKeon *et al.*, 1982). However, no differences were found in the methylated pattern of this region between cells that actively transcribe type I collagen and cells that do not (McKeon *et al.*, 1982). In contrast, the

α1 (II) gene in chondrocytes was shown to be undermethylated compared to the gene in fibroblasts or erythrocytes (Fernandez *et al.*, 1985). However, this investigation was incomplete due to examination of only a small portion of all methylated sites, and the use of probes that corresponded only to the 3' region of α1 (II) gene. The methylation pattern of the 5' region of the gene has been shown to play the most important role in regulation of gene expression compared to the other areas of the gene .

Interestingly, high levels of α2 (I) collagen mRNA are present within chondrocytes (Olsen, 1991). However, the chondrocytes utilise a transcriptional start site within the α2 (I) gene which is different from that used in fibroblasts (Bennet and Adams, 1990). Consequently, the α2 (I) mRNA does not code for proα2 (I) chains but potentially for a non-collagenous polypeptide.

A host of biological and pharmacological agents influence collagen gene expression. Transforming growth factor β (TGF-β) has been shown to stimulate the synthesis of a number of extracellular matrix protein, including types I and III collagen (Sporn and Roberts, 1989). TGF-β has been shown to activate the chimeric α2 (I) collagen-promoter CAT gene in NIH-3T3 cells (de Crombrugghe *et al.*, 1990). A binding site for the transcription factor NF-1[15] was shown to mediate the TGF-β activation of the α2 (I) promoter. A 3-bp substitution mutation in the binding site abolished the binding of NF-1, and also prevented the induction of the promoter by TGF-β (de Crombrugghe *et al.*, 1990). NF-1 may mediate the transcriptional activation of the α2 (I) collagen promoter by TGF-β. The latter may also increase the expression of type I collagen genes by other mechanisms. Under certain conditions, the stability of α1 (I) mRNA has been shown to increase in TGF-β treated cells (Raghow *et al.*, 1987; Penttinen *et al.*, 1988). The preferential stabilisation of mRNAs specifying type I collagen may be another mechanism by which TGF-β enhances the synthesis of type I collagen in mesenchymal cells.

Steroid hormones have been shown to have profound effects on collagen biosynthesis (for a review, see Cutroneo *et al.*, 1986). Steroid hormones appear to regulate collagen biosynthesis by a complex mechanism that may involve both

[15] Nuclear factor 1 (NF1): A family of nuclear proteins exhibiting identical DNA binding specificities (see Santoro *et al.*, 1988).

transcriptional repression (Weiner *et al.*, 1987; Cockayne and Cutroneo, 1988), as well as by a mechanism utilising preferential destabilisation of procollagen mRNA's (Hamalainen *et al.*, 1985).

A conserved inverted repeat sequence has been located in the first exon of α1 (I), α2 (I), and α1 (III) genes (Yamada *et al.*, 1983). It's location around the start of translation has prompted the hypothesis that the sequence may have a role in translational control. The inverted repeat could theoretically form a stable inverted loop, thus providing a potential regulatory focus that could participate in translational discrimination of the α1 (I), α2 (I), and α1 (III) mRNAs. However, partial deletion of this segment has suggested that the sequence has no influence on translation (Bornstein *et al.*, 1988). Additional experiments are needed to understand the role of this conserved inverted repeat sequence.

Lichtenstein *et al* (1973) observed that cells from patients with Ehlers-Danlos VII syndrome (see section 1.12) where removal of N-propeptides is defective, showed an elevated rate of collagen synthesis. They raised the hypothesis that cleavage of N-propeptides may regulate the synthesis of collagen by feedback inhibition. Wiestner *et al* (1979) demonstrated that the intact N-propeptide of type I or III procollagen could selectively inhibit collagen biosynthesis by human fibroblasts without affecting total protein synthesis, procollagen degradation or procollagen hydroxylation. The effect was collagen type specific since the addition of type I procollagen N-propeptide to the media of cultured fetal calf chondrocytes or chick sternal chondrocytes was shown not to inhibit collagen II synthesis (Paglia *et al*, 1981). As mentioned above, the mRNAs for α1 (I), α2 (I), and α1 (III) contain inverted repeats at the 5' end capable of forming a hairpin structure. The hairpin cannot be formed in the α1 (II) mRNA (Kohno *et al.*, 1985). Whether this observation has implications for the mechanisms of N- and C-propeptide feedback inhibition remains to be established

The intact propeptide and certain subfragments derived from the propeptide have been shown to selectively inhibit collagen synthesis by inhibiting translation of the collagen mRNA (Horlein *et al.*, 1981; McPherson *et al.*, 1982; Perlish *et al.*, 1985). Different fragments of the N-propeptide were found to inhibit synthesis with different degrees of selectivity and its various steps of translation. A synthetic copy of a highly

conserved portion of the C-propeptide (22 amino acids) of human proα2 (I) procollagen has been shown to specifically inhibit collagen synthesis in human fibroblast (Aycock *et al.*, 1986). Since there was no significant change in the steady state level of collagen mRNAs, a post-transcriptional regulatory mechanism was proposed (Aycock *et al.*, 1986). The minimum sequence necessary to inhibit collagen synthesis by the N-propeptide (McPherson *et al.*, 1982) is not found in the highly conserved portion of the C-propeptide. In contrast with Aycock *et al* (1986), both the C- and N-propeptide were shown to decrease steady state levels of α2 (I) mRNA in human fibroblasts (Wu *et al.*, 1986). Although they found a good correlation between mRNA levels and collagen production in cells treated with the C-propeptide, such correlation was much weaker in fibroblasts treated with the N-propeptide. They suggested the predominant effect of the C-propeptide was at a pretranslational site. The propeptides may be acting by decreasing the rate of transcription or increasing the rate of mRNA degradation, or both.

Ascorbic acid acts as a cofactor for the enzymic hydroxylation of specific prolyl and lysyl residues in procollagen during biosynthesis (see section 1.5.2). This post-translational modification is essential for efficient procollagen helix formation and subsequent secretion from the cell (Prockop and Kivirikko, 1984). Ascorbic acid was shown to specifically stimulate type I and III collagen synthesis in human skin fibroblasts (Chan *et al.*, 1990). The increased level of collagen synthesis after different ascorbic acid exposure times was also shown to be achieved by a brief treatment (10 hours) of scorbutic cultures (ascorbic acid deficient) with ascorbic acid (Chan *et al.*, 1990). Exposure to ascorbic acid did not alter the steady state levels of collagen mRNA levels, and therefore the increase in collagen synthesis was due to post-transcriptional mechanisms.

1.11 Collagen degradation

The intact triple helix of type I collagen is resistant to proteolytic attack, and

crosslinking of fibrils further increases resistance to proteases. Collagenase is the only enzyme active at neutral pH that can cleave the interstitial collagens (for reviews, see Harris *et al.*, 1984; Jeffrey, 1986). It contains Zn^{2+} at its active site and requires Ca^{2+} for maximal activity. Collagenase cleaves each α1 (I) chain at a specific site between gly 775-ile 776 (leu in the α2 (I) chain) generating two fragments (Gross and Nagai, 1965). The low content of proline and hydroxyproline at the cleavage site produces a relaxation in the triple helical conformation which has been suggested to facilitate the collagenase action (Kuhn, 1987). The two resulting fragments denature at physiological temperatures and are then either degraded extracellularly by other proteinases such as gelatinase, stromelysin and plasmin (Murphy *et al.*, 1990) or pinocytosed and degraded intracellularly in lysosomes.

Collagen degradation is precisely regulated at several levels which include the synthesis and secretion of procollagenase, conversion of procollagenase to its active form, substrate effects and the effect of tissue inhibitors (Harris *et al.*, 1984; Mainardi, 1985). Cytokines such as interleukin 1 and tumour necrosis factor-α (TNF-α) produced by mononuclear cells adjacent to fibroblasts and chondrocytes have been shown to activate the transcription of the procollagenase gene (Krane *et al.*, 1990). Other cytokines surpress collagenase activity (see below).

Following secretion, procollagenase (54kDa) is converted to its active form (collagenase) by proteolytic cleavage with serine proteinases such as plasmin, kallikrein, trypsin or by mercurial compounds (Harris *et al.*, 1984). Stromelysin is a metalloproteinase that has been shown to activate procollagenase (Murphy *et al.*, 1988), and also degrade non-collagenous components of the ECM such as fibronectin, laminin and the core proteins of PG (Alexander and Werb, 1989). In concert with collagenase it can digest nearly all connective tissue macromolecules.

Activated collagenase will continue to degrade substrate unless it is inhibited. A potent inhibitor of collagenase is α_2 macroglobulin (Harris *et al.*, 1984). TIMPs (tissue inhibitors of metalloproteinases) are a group of related proteinase inhibitors isolated from a variety of tissues (Hay, 1991). They are glycoproteins (28 kDa) which like α_2 macroglobulin react with only the active enzyme. Other inhibitors of collagenase include ovastatin and

β_1 anti-collagenase. TGF-β and interferon–γ have been shown to decrease the levels of collagenase activity in synovial fibroblasts (Krane *et al.*, 1990). TGF-β acts primarily by increasing the production of TIMP (Krane *et al.*, 1990). The balance between the amounts of metalloproteinase inhibitor and active collagenase determines the rate of collagen degradation.

In heterotypic fibrils of cartilage and matrices rich in type I collagen, the FACIT collagens (IX,XII) that coat the fibrils may protect the 'internal' fibril from attack by collagenase. In this case, degradation may be a multiphasic process, involving the initial attack of the FACIT collagens by enzymes like stromelysin (Eyre *et al.*, 1991).

1.12 Collagen related diseases

The use of nucleated growth in the folding of the collagen triple helix and in the assembly of collagen monomers into fibril makes the biosynthesis of collagen fibrils highly sensitive to mutations that alter the primary structure of the protein. Several mutations have been identified in human procollagen genes that lead to the abnormal biosynthesis of procollagen (for reviews, see Byers, 1990; Prockop *et al.*, 1990; Kuivaniemi *et al.*, 1991).

Osteogenesis imperfecta (OI) is a group of heritable disorders characerised by brittleness of bone. The disease is also associated with changes in other tissues rich in type I collagen, such as skin and the sclerae of the eyes. There is large variation in the OI phenotype, from lethal in the perinatal period, crippling but not lethal to a mild form of the disease where the tendency for bone fractures to occur increases only marginally. Over 70 OI mutations have been found in type I procollagen genes (Kuivaniemi *et al.*, 1991). The mutations include deletions, insertions and RNA-splicing mutations. The most common mutations are single base substitutions that convert a codon for glycine for a codon for an amino acid with a bulkier side chain (Byers, 1990; Prockop *et al.*, 1990; Kuivaniemi *et al.*, 1991). Such mutations would interfere with the folding of the triple helix because only glycine can be accomodated in the centre of the triple helix.

The nature and position of the glycine substitution determines the severity of the OI phenotype (Byers, 1990). The phenotype becomes progressively more severe (mild to lethal) as the mutation approaches the C-terminus. Triple helix formation proceeds in a zipper-like manner from the C- to the N-terminus (see section 1.5.3). Therefore, mutations that delay the folding of the triple helix will cause regions N-terminal to the mutation to spend longer in the non-triple helical conformation. Since, the post-translational enzymes are only active on non-triple helical α-chains, these regions will undergo more post-translational modifications (overmodification).The extent of overmodification is inversely correlated with the distance of the mutation from the C-terminus. However, there are exceptions, and the correlation in the α2 (I) chain is much weaker than that in the α1 (I) chain (see Sykes, 1990). Substitutions of serine for glycine at positions 832 and 844 in the α-chain should have been lethal but were described from surviving patients (Sykes, 1990). The type of amino acid that substitutes the glycine can also effect the OI phenotype. The substitution of aspartate for glycine is lethal wherever it occurs in the collagen chain, whereas arginine and cysteine are comparatively mild towards the N-terminus.

Most of the mutants decrease the thermal stability of the triple helix enough to unfold the triple helix (Bachinger and Davis, 1991). These unfolded procollagen molecules are degraded intracellularly. The effects of the mutation are amplified in a process referred to as procollagen suicide (Prockop and Kivirikko, 1984; Prockop, 1990). The mutated procollagen chains are able to associate with normal procollagen chains via the C-propeptide. However, the trimer cannot form into a stable helix at physiological temperature and is degraded. Kuivaniemi *et al* (1991) suggested that procollagen suicide was probably the reason why most of the mutations cause dominant lethal forms of OI.

Glycine substitutions that do not markedly affect the folding of the triple helix have been found in lethal variants of OI. The substitution of a cysteine for glycine has been observed by rotary shadowing to result in a kink in the procollagen molecule synthesised (Vogel *et al.*, 1988). The molecules with the flexible kink were found to form co-polymers with normal collagen synthesised by the same fibroblasts (Kadler *et al.*, 1991). The formation of co-polymers was shown to delay fibril formation and

generate abnormally branched fibrils (Kadler *et al.*, 1988; Kadler *et al.*, 1991). Byers (1990) suggested that only a small number of abnormal molecules will be required to produce abnormal fibrils, because of the molecular constraints needed to pack molecules into fibrils.

Procollagen N-proteinase requires its substrate to be in a native conformation (see section 1.7.1). A mutation that alters the structure of the proα chains at a site 500 or more amino acids away from the N-proteinase cleavage site has been shown to inhibit N-proteinase activity (Dombrowski *et al.*, 1989). Lateral displacement of the hairpin structure which is necessary to stabilise the conformation of the cleavage site may account for these observations (for further information, see section 1.7.1), an effect that is further amplified by the fact that a mutation in only one proα chain can interfere with the processing of all three proα chains. The persistence of the N-propeptide on the collagen molecule prevents normal packing in the fibrils and leads to the formation of abnormal fibrils.

The type VII variant of Ehlers-Danlos syndrome (EDS VII) is characterised by laxity of joints, and usually results in dislocation of the hip and knee (Prockop and Kivirikko, 1984). Mutations in the intervening sequence that flanks exon 6 have been shown to cause the skipping of this exon during the processing of the RNA for the proα chains (Ramirez *et al.*, 1990). Since exon 6 contains the cleavage site for the N-proteinase, such a mutation leads to the persistence of the N-propeptide on the collagen. The fibrils that are found in the tissues are highly irregular in cross-section and appear like twisted ribbons.

Dermatosparaxis, an autosomal recessively inherited disease of connective tissue in calf and sheep is characterised by extreme fragility of the skin (O'Hara *et al.*, 1970; Fjolstad and Helle, 1974). Electron microscopy of the skin of animals affected with dermatospraxis show abnormal collagen fibrils that are similar to those in EDS VII (Pierard *et al.*, 1986). Other tissues rich in type I collagen appear normal or only slightly affected. A deficiency in N-proteinase activity has been found to be responsible for the accumulation of pN-collagen in the dermis (for further information, see chapter 4).

1.13 Introduction to the project

Cellular involvement in extracellular matrix organisation is a poorly understood phenomenon. In this chapter, many examples have been cited where connective tissue cells have been shown to influence collagen fibril assembly. The mechanisms involved in the interaction of the cell membrane with collagenous molecules are poorly understood.

The aim of the work described in subsequent chapters is to investigate the role of cell-matrix interactions in the assembly of the extracellular matrix. The focus of the research is the potential interaction of procollagen with cell membranes and its influence on the initiation, control and spatial organisation of fibril assembly. The specific questions posed by the project are as follows:

- How does procollagen/collagen bind to the surface of membranes ?
- What is the specificity of this binding ?
- What types of non-covalent interaction are involved in the binding ?
- Does the binding induce aggregation of procollagen ?
- Does the interaction have a role in the spatial organisation of fibril assembly ?
- Is the processing of procollagen enhanced at the cell surface ?
- What factors are involved in the regulation of procollagen processing ?

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Procollagen preparation

2.1.1 Materials

Fertilised hen eggs were supplied by Ross Breeders Ltd.(Newbridge, Midlothian). Trypsin solution (2.5 % w/ v), penicillin/ streptomycin (PS; 10,000 units penicillin/ ml, 10,000 μg streptomycin/ ml) Minimal Essential Medium (MEM), foetal calf serum (FCS), Hanks Balanced Salt Solution (HBSS) were all obtained from GIBCO, Paisley, Strathclyde, UK. L-[5-^{3}H]proline (30 Ci/ mmol) was obtained from Amersham, Aylesbury, Bucks, UK. Bacterial collagenase (isolated from Clostridium histolyticum) was supplied by Boehringer Mannheim, Lewes, Sussex, UK. Phenylmethylsulphonylfluoride (PMSF), N-ethylmaleimide (NEM), β-aminoproprionitrile (BAPN, fumarate salt), bovine serum albumin (BSA) and Tris (Trizma base) were from Sigma, Poole, Dorset, UK. DEAE-Sephacel was supplied by Pharmacia Ltd., Bucks, UK. Mixed bed ion-exchange resin (AG 50-X8, wet bed size 300-1180 μm) was from BioRad, Herts., UK. All other chemicals (analytical grade) were obtained from BDH Chemicals, Poole, Dorset, UK.

2.1.2 Preparation and culture of chick embryo tendon fibroblasts

The preparation of procollagen was a modification of that of Dehm and Prockop (1971). Prior to dissection, instruments, filters and flasks were autoclaved, and Hanks balanced salt solution (HBSS) was sterilised by filtering with a 0.2 μm bottle filter (Costar). Semi sterile techniques were used throughout the dissection.

The metatarsal tendons of 30 dozen 17 day chick embryos were removed and placed into sterile HBSS/ 1% penicillin/ streptomycin (PS). The tendons were washed three times in HBSS to remove blood, etc. Trypsin and collagenase were used to digest the collagenous matrix of the tendon to produce a cell suspension. The wet weight of the tendons was determined, and for each gram of tendon, 3 ml Minimal Essential Medium (MEM), 0.5 ml Trypsin (2.5 % w/v) and 22 mg of

bacterial collagenase (from Clostridium histolyticum; Boehringer Mannheim) was added to an Erlenmyer flask (Nalgene). The flask was gassed with 95 % air/ 5 % CO_2 for about 30 seconds and sealed firmly. The tendons were digested at 37 °C in a shaking water bath (Grant) for 1 hour until only small pieces of intact tendon remained. Care was taken to avoid over digestion since this can lead to cell rupturing.

The digest was filtered using 2 layers of lens paper in a Swinnex filter (Millipore). An equal volume of modified Krebs II[1]/ 10 % fetal calf serum (FCS)/ 1% PS was added to the filtrate. The FCS contained inhibitors which prevent further action of the trypsin and collagenase. The digestion enzymes were removed from the filtrate by centrifugation (MSE centaur bench centrifuge) for 6 minutes at 1800 r.p.m., and aspiration of the supernatant. The cells were resuspended in 30 ml of modified Krebs II/ 10% FCS/ 1% PS, using a sterile 10 ml pipette. The resuspended cells were centrifuged at 1800 r.p.m. for 3 minutes, and the supernatant aspirated. The cell pellet was washed a further two times in this way, and finally resuspended in serum-free krebs II/ 1% PS. The cell number was determined using a haemocytometer (Weber). Typically, 2 x 10^9 cells were obtained from 19 day chick embryos. The cell density was adjusted to 10^7 cells/ ml with krebs II/ 1% PS.

The tendon fibroblasts were cultured as described by Mould and Hulmes (1987) in the presence of about 2 µCi/ ml of L-[5-^{3}H] proline and freshly prepared ascorbate at a final concentration of 50 µg/ ml, in an Erlenmyer flask. The flask was gassed as previously described, sealed and incubated on an orbital shaker (Lab Therm Shaker, Adolf Kuhner, Basel) at 37 °C for 4-5 hours.

2.1.3 Medium collection and polyethylene glycol precipitation

After the incubation period, the cell suspension was transferred to centrifuge tubes and centrifuged for 3 minutes at 1800 r.p.m. The supernatant was collected and centrifuged for a further 10 minutes at 3000 r.p.m. to remove cell debris. Bovine serum albumin (BSA) was added to the supernatant to a final concentration of 5 mg/

[1] 111.2 mM NaCl/ 5.4 mM KCl/ 1.3 mM KH_2PO_4/ 1.3 mM $MgCl_2$/ 4 mM $NaHCO_3$/ 12.5 mM Na_2HPO_4/ 3.1 mM NaH_2PO_4/ 13 mM glucose, pH 7.3.

ml. The BSA was added to bind contaminating proteases which might otherwise degrade the procollagen. A stock solution of inhibitors (10x) was added to the supernatant (stirring on ice), to final concentrations of 25 mM EDTA/ 10 mM N-ethylmaleimide (NEM)/ 1 mM phenylmethylsulphonylfluoride (PMSF)/ 0.1 M Tris-HCl, pH 7.4. Polyethylene glycol (PEG) 4000, 25% (w/v) in 0.15 M NaCl, was added to give a final concentration of 5 % PEG. PEG 4000 at a final concentration of 5-10 % has been shown to precipitate virtually all the procollagen in solution in tissue culture medium (Ramshaw *et al.*, 1984). The solution was left unstirred in the cold room at 4 °C overnight. All subsequent procedures were undertaken at 4 °C.

The solution containing precipitated procollagen was centrifuged for 30 minutes at 15,000 g in a J2-20 centrifuge (Beckman). The supernatant was discarded and the procollagen pellet washed in 100 ml 10 mM Tris-HCl pH 7.4. After a further centrifugation for 30 minutes at 15,000 g, the supernatant was discarded and the pellet dissolved by stirring in 30 ml 0.4 M NaCl/ 0.1 M Tris-HCl, pH 7.4 for approximately 4 hours. The redissolved pellet was centrifuged for 45 minutes at 27,000 g and the supernatant collected. The supernatant was then dialysed against 1 litre DEAE starting buffer (2 M urea/ 10 mM NaCl/ 2.5 mM EDTA/ 50 mM Tris-HCl, pH 7.8 (20 °C)). An 8M urea stock solution was deionised on the day of use by stirring for two hours with mixed-bed ion-exchange resin (BioRad AG 50-X8; wet bead size 300-1180 μm).

2.1.4 Ion exchange chromatography

The procedures used were based on those described by Gerard and Mitchell (1979). DEAE Sephacel (Pharmacia, supplied as a 50 % slurry in 20 % aqueous ethanol) was equilibrated with degassed starting buffer. 40 ml of the slurry was placed into a measuring cylinder and an equal volume of starting buffer added. The mixture was gently shaken, and the resin allowed to settle. The supernatant was then poured off, and the procedure repeated several times. The DEAE Sephacel was packed into a column (1.6 x 8 cm) at a flow rate of 100 ml/ hour. After the column

was equilibrated with starting buffer (150 ml), the dialysate was applied at a flow rate of 20 ml/ hour. The column was then washed with two column volumes of the starting buffer to remove unbound material. A linear salt gradient (400 ml total, 10-300 mM NaCl) formed using a gradient former (Bethesda Research Laboratories, Inc.), was applied to the column. Fractions of 8 ml were collected from the time of loading the dialysate. 50 µl aliquots were assayed for ^{3}H by scintillation counting. To each aliquot, 350 µl water (to give a 10% aqueous phase) and 3.6 ml cocktail T (BDH) were added in an insert vial, and counts were measured with a liquid scintillation analyser (Packard 1900A). Procollagen peak fractions were pooled and dialysed against storage buffer (0.4 M NaCl/ 0.1 M Tris-HCl, pH 7.4 (20 °C)), with two changes. The pooled and dialysed procollagen was concentrated at least 40-fold using an ultrafiltration cell (10 ml capacity Amicon cell) with a YM30 membrane (molecular weight exclusion limit 30,000 Dalton). The filter was washed under atmospheric pressure by stirring with 0.25 ml of the final dialysis buffer for at least 20 minutes. The wash was added to the concentrate procollagen solution to give a final volume of 1.5 ml. The purified procollagen was divided into 0.5 ml aliquots and stored at -20 °C.

2.2 Characterisation of procollagen

2.2.1 Hydroxyproline assay

Large amounts of hydroxyproline occur almost exclusively in collagens. An assay for hydroxyproline provides a means of determining the concentration of collagenous molecules. Free hydroxyproline produced by the complete hydrolysis of peptide bonds was assayed using a method described by Woessner (1961) which involves the conversion of the hydroxyproline to pyrrole by chloramine T, and then the formation of a chromophore (which can be spectrophotometrically assayed at 557 nm) by reaction with p-dimethylaminobenzaldehyde (for assay protocol, see Figure

2.1).

2.2.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate the proα1 (I) and proα2 (I) chains and any degradation products of the chains. The gel matrix consists of random chains of polyacrylamide incorporating a small proportion of N',N methylene-bis-acrylamide (BIS) molecules which forms crosslinks with other chains resulting in a three dimensional network. In the gel media, the passage of any particle is hindered by the structure of the matrix, and the extent depends upon the relative sizes of the particles and the pores in the gel network. The pore size is altered by changing the acrylamide concentration.

In SDS-PAGE, all components migrate solely according to size. Sodium dodecyl sulphate (SDS) is a strongly denaturing detergent and gives rise to essentially random coil configurations. Dodecyl sulphate binds strongly to proteins (Reynolds and Tanford, 1970), and 2 % SDS is sufficient to saturate the polypeptide with approximately one detergent molecule per two amino acid residues. This amount of highly charged detergent molecule is sufficient to overwhelm effectively the intrinsic charges on the polypeptide chain, so that the net charge per unit mass becomes approximately constant.

Collagenous proteins migrate anomalously on SDS gels in comparison with most globular proteins (Furthmayr and Timpl, 1971). The α-chains of collagen migrate slower than globular proteins of comparable molecular weight, and additionally a difference in mobility between the equally sized α1 and α2-chain is observed. A possible explanation for the behaviour of the collagen molecule in SDS-PAGE may be the existence of a certain rigidity of the polypeptide structure, even in the denatured state. The restriction in the flexibility of the random coil would be imposed by the imino acid residues, which make up a high proportion of the collagen sequence (Furthmayr and Timpl, 1971). Although the collagen α-chains are

1. Place duplicate samples (containing approximately 2.5 μg/ ml hydroxyproline) in Pyrex tubes with screw on tops lined with teflon. Add distilled water to give sample volume of 1 ml. Add 1.1 ml concentrated HCl to give final concentration of 6 M. Screw top tightly to prevent evaporation of solvent. Hydrolyse duplicate samples for 16 hours at 116°C in an oven.
2. Evaporate hydrolysate to dryness using Speedivac concentrator (Savant instrument Inc.). Dissolve residue in 2 ml distilled water.
3. Label Pyrex tubes (duplicate samples): 0, 1, 2, 3, 4, 5 μg. Add appropriate amount of hydroxyproline (stock solution 10 μg/ ml) to each tube, and distilled water to give 2 ml 'standard' volume. Treat standards in the same way as sample.
4. Add 1 ml chloramine T solution (0.05 M chloramine T in 20 % dH_2O, 30 % 2-methoxyethanol, 50 % buffer) to each tube in a predetermined order. The buffer is 0.24 M citric acid/ 0.21 M acetic acid/ 0.9 M sodium acetate trihydrate/ 0.85 M sodium hydroxide pH 6.0, stored under toluene. Mix tubes content thoroughly using vortex. Allow to stand for 20 minutes at room temperature.
5. Add 1 ml perchloric acid (3.15 M) to each tube in the same predetermined order. Mix tubes content with vortex. Allow to stand for 5 minutes.
6. Add 1 ml Ehrlich's reagent (20 % 4-dimethylaminobenzaldehyde in 2-methoxyethanol) to each tube in the same order as before. Mix contents of each tube until schlieren pattern is no longer observed.
7. Incubate at 60°C (water bath) for 20 minutes. Place tubes in cold water for 5 minutes. Measure absorbance at 557 nm.

Figure 2.1 A protocol for the hydroxyproline assay

identical in molecular weight, the α2-chain has a higher binding capacity for SDS and so migrates further than the α1-chain (Kubo and Takayi, 1984).

Discontinuous polyacrylamide gels (Laemmli, 1970), consisting of 4.5 % stackinggels and 6 % separating gels were used in SDS-PAGE to analyse the procollagen samples (for details, see Figure 2.2).

2.2.3 Fluorography

The [^{3}H]proα-chains in the polyacrylamide gel were detected by fluorography, using X-ray film. [^{3}H] cannot be detected by autoradiography because of the low energy of the β-particle emission, which is almost entirely absorbed within the sample itself. Fluorography overcomes this difficulty by impregnating the gel with a scintillator before exposing the gel to X-ray film. The film is not exposed directly by the β-particle radiation, but indirectly by light generated by the interaction of β-particles with the scintillator molecules. The fluor used in the experiment was 2,5-diphenyloxazole (PPO; Bonner and Laskey, 1974).

The gels were fixed for 30 minutes in glacial acetic acid (Skinner and Griswold, 1983), and then transferred into a solution of 20 % PPO in glacial acetic acid, and agitated gently on a reciprocal shaker (Denley) for 60 minutes. 15 % PPO gives the optimal sensitivity, however a 20 % solution was used to avoid a decrease in detection efficiency due to any losses of PPO (Skinner and Griswold, 1983). Excess PPO was then removed, and water was added to precipitate out the PPO in the gel. The precipitate does not reduce fluorographic efficiency. The gel was then washed a few times in water, and then placed into 200 ml 0.25 % (v/ v) glycerol solution. The glycerol prevents the gel from cracking whilst being dried. After 60 minutes of gentle shaking (reciprocal shaker), the gels were covered with cling film, and dried on a gel dryer at 70 °C for 90 minutes. The gels were then exposed to pre-fogged X-ray film (XAR 5 X-OMAT, Kodak) at -70 °C. The efficiency of fluorography is increased at low temperatures. Each photon of visible light produces a single atom. However, a single silver atom in a silver halide crystal is unstable,

1. Add 20 % (w/v) SDS to each sample to give final concentration of 2 % SDS.
2. Add one-quarter the volume of 5 x reducing sample buffer (50 % (v/v) glycerol/ 2 % SDS/ 15 % (v/v) β-mercaptoethanol/ 0.05 % (w/v) bromophenol blue/ 0.125 M Tris-HCl, pH 6.8) to each sample.
3. Heat at 100 °C in boiling water bath for 3 minutes.
4. Immediately load samples on to discontinuous gels consisting of 6 % separating (6 % (w/v) acrylamide/ 0.16 % (w/v) BIS/ 0.2 % SDS/ 0.05 % (w/v) ammonium persulphate/ 0.125 M Tris-HCl, pH 8.8, with 0.0005 % (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED)) and 4.5% stacking gels (4.5 % acrylamide/ 0.12 % BIS/ 0.2 % SDS/ 0.05 % ammonium persulphate/ 0.0005 % TEMED/ 0.125 M Tris-HCl, pH 6.8), using Hamilton syringe.
5. Carry out electrophoresis with water cooled LKB vertical electrophoresis unit for approximately 4 hours at constant current of 40 mA per gel in running buffer (0.192 M glycine/ 0.1 % SDS/ 0.025 M Tris, pH 8.3). Turn current off when dye front is 1 cm from bottom of gel.

Figure 2.2 Protocol for SDS-polyacrylamide gel electrophoresis

undergoing thermal decomposition back to a silver ion. At room temperature, the half life of the back reaction is 1 second. The production of a second silver atom in the same crystal within the half-life of the back reaction stabilises the first and virtually eliminates the chance of a back reaction occurring. The increase in efficiency of light detection at low temperatures results from a decreased rate of thermal reversion of the first silver atom (Lasky and Mills, 1975).

The absorbance of the fluorographic image is not proportional to the concentration of radioactivity in the gel. The absorbance is dependent not only on the number of disintegrations which occur, but also at the rate at which they occur. By preexposing the film to a very short (less then 1second) flash of light, a stable pair of silver atoms are preformed. An approximate light intensity is selected to produce more than one silver atom in each silver halide crystal, but not enough to render most crystals developable. After pre-fogging, the absorbance of the image is proportional to the amount of radioactivity, since all photons produced by the sample should have an equal chance of contributing to the growth of the latent image. Also, sensitivity to small amounts of radioactivity should be increased, since the initiation of image formation is no longer dependent on the rare coincidence of photons within a fixed time interval.

Pre-fogging was performed by exposing film to a single flash from an electronic photographic flash unit (Kodak). Two filters were taped to the window of the flash unit, a Kodak Wratten no. 21 to reduce the light output, and a Whatman no.1 filter paper to diffuse the light. Adjustments to the illumination intensity could be made by varying the distance between the flash unit and the film. Positioning the flash unit at a distance of 35 cm above the film gave an increase in background 'fog' of 0.15 OD units above the background level of unexposed film. For fluorographic exposures at -70 °C, the fog absorbance of 0.15 results in a linear relationship between fluorographic image absorbance and the amount of radioactivity (Laskey and Mills, 1975). The film was backed by a Whatman no.1 filter paper during pre-fogging, and the surface which had been nearest the light source was applied to the gel. After the fluorographic exposure, the film was developed at 20 °C.

2.2.4 Densitometry

Fluorograms were scanned using the Chromoscan 3 (Joyce Loebl) linked to an IBM-compatible DCS microcomputer. The Chromoscan 3 was operated in transmission mode with an aperture of 0.3 x 5 mm. The light source was a 100 W tungsten lamp, and measurements were made at a wavelength of 530 nm. Before calculating integrated peak intensities, a background correction was made.

2.3 Preparation of C-proteinase

The method used for the purification of the C-proteinase was a modification of that of Hojima *et al* (1985). Prior to dissection, instruments, filters and flasks were autoclaved. Hanks balanced salt solution (HBSS) was sterilised by filtering with a 0.2 μm bottle filter (Costar). Semi sterile techniques were used throughout the dissection.

2.3.1 Materials

Fertilised hen eggs were supplied by Ross Breeders Ltd., Newbridge, Midlothian. Penicillin/ streptomycin (PS; 10,000 units penicillin/ ml, 10,000 μg streptomycin/ ml) Minimal Essential Medium (MEM), and Hanks Balanced Salt Solution (HBSS) were all obtained from GIBCO, Paisley, Strathclyde, UK. Green A dye matrix gel was obtained from Amicon. Concanavalin A Sepharose 4B was from Pharmacia Ltd., Bucks, UK. Methyl α-D-mannopyranoside was from Sigma, Dorset, UK. All other chemicals (analytical grade) were supplied by BDH, Poole, Dorset, UK.

2.3.2 Preparation of culture medium

In total, 120 dozen 17 day chick embryo metatarsal tendons (30 dozen per week for a month) were used for the preparation. Each week, the metatarsal tendons of 30 dozen chick embryos were incubated in 200 ml Minimal Essential Medium (MEM)/ 1 % PS/ 50 μg/ml ascorbate/ 64 μg/ml β-aminopropionitrile (BAPN), for 9 hours at 37 °C under 95 % air/ 5 % CO_2, with gentle shaking. The cultured tendons were centrifuged at 1800 g for 3 minutes (MSE bench centrifuge), and the supernatant collected and stored at -20 °C. The tendons were resuspended in a further 200 ml MEM/ 1 % PS/ 50 μg/ml ascorbate/ 64 μg/ml BAPN, and incubated for 12 hours at 37 °C. The culture medium was collected by centrifugation as above and stored at -20 °C. The tendons were again resuspended in 100 ml of the MEM solution, incubated for 8 hours, the culture medium collected by centrifugation, and stored at -20 °C.

The frozen culture media harvested from a total of 120 dozen chick embryos were then pooled (about 2 litres). The pH of the pooled media was then adjusted to pH 7.5 with concentrated HCl. Tris, NaCl, and NaN_3 were then added to the media to give final concentrations of 0.05 M Tris-HCl/ 0.3 M NaCl/ 0.001 % NaN_3, and the pH adjusted to pH 8.0. The media was then centrifuged at 10,400 g for 30 minutes at 4 °C. The specific activity in the culture medium was determined to be 0.9 units/ mg.[2]

2.3.3 Chromatography on Green A Dye Matrix gel

The Green A Dye Matrix gel is used in dye-ligand chromatography, a variant of affinity chromatography. Synthetic textile dyes are used in place of the natural substrates or cofactors commonly employed as immobilised ligands. The mechanism of the interaction between the dye ligands and proteins is not fully understood. Circular dichroism spectral data (Edwards and Woody, 1977), and enzyme inhibition

[2] 1 unit is the amount of enzyme which cleaves 1 μg of type I procollagen in 1 hour at 35 °C in the electrophoretic assay (see section 4.2). 1 absorbance unit was assumed to be 1 mg protein.

studies (Dean and Watson, 1978) suggest that the dyes interact directly with the specific substrate and cofactor binding sites. The interaction of the dyes with proteins is strongly anionic in nature (anionic dye with cationic protein sites), but is in many cases potentiated by apolar (hydrophobic) effects. The precise nature of the binding mechanism of the dyes varies significantly from protein to protein, and from dye to dye.

The Green A Matrix Gel (Amicon) was packed into a column (2.5 x 17 cm) and washed with 6 M urea/ 0.5 M NaOH, until the dye was no longer visible in the eluate. The wash removed sodium azide (an enzyme inhibitor used as a preservative) and free dye. The column was equilibrated with 0.3 M NaCl/ 0.05 M Tris-HCl, pH 7.5. After application of the enzyme solution, the column was washed with equilibration buffer at aflow rate of 40 ml/ hour until the first peak of protein had been eluted. The column was then washed with 1M NaCl/ 0.05 M Tris-HCl, pH 7.5 at a rate of 40 ml/ hour, in order to remove impurities bound to the column. The C-proteinase was eluted from the column with 3 M NaCl/ 2 M urea/ 0.05 M Tris-HCl, pH 7.5 at 20 ml/ hour (see Figure 2.3). Proteinase fractions were pooled (about 40 ml) and concentrated by pressure ultrafiltration in an Amicon 10 ml cell fitted with a YM30 membrane. The concentrated pooled solution was then diluted 1:1 with 2 mM $CaCl_2$/ 0.15 M Tris-HCl, pH 7.5 to give final concentrations of 1.5 M NaCl/ 1 M urea/ 1 mM $CaCl_2$/ 0.1 M Tris-HCl, pH 7.5. The solution was clarified by centrifugation at 17,300 g for 20 minutes. The C-proteinase was purified by 8-fold and the specific activity was determined to be 6.9 units/ mg.

2.3.4 Con A Sepharose chromatography

Concanavalin A (con A) is a lectin that binds molecules which contain α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related molecules. Unmodified hydroxyl groups at C-3, C-4 and C-5 of the binding sugar are required for reaction with con A (Goldstein et al., 1965). Con A Sepharose consists of con A coupled to Sepharose 4B, a bead-formed agarose gel. The open pore structure and the exclusion

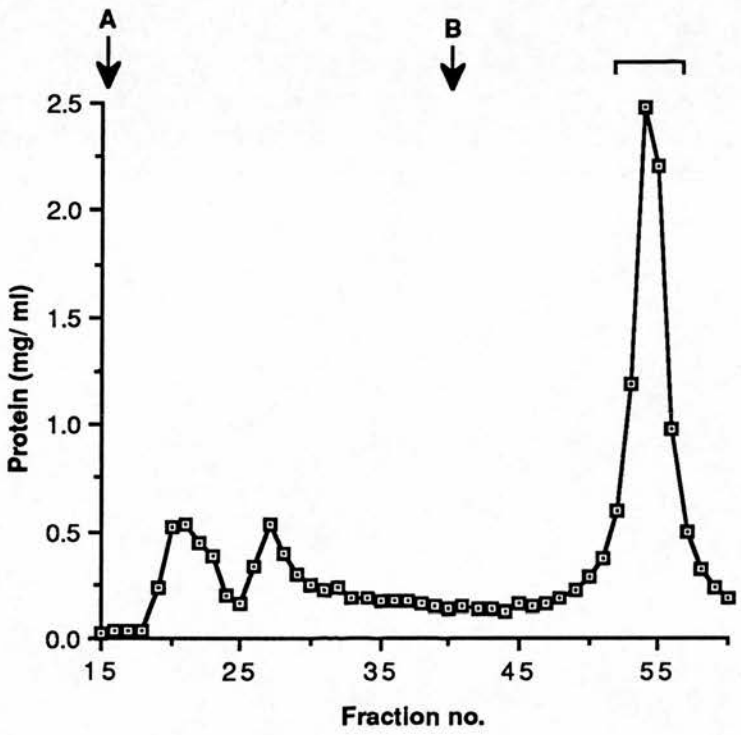


Figure 2.3 Green A Dye Matrix chromatography of C-proteinase from chick embryo tendon Culture medium obtained by organ culture of 120 dozen chick embryo tendons was applied to a (2.5 x 17 cm) Green A Dye Matrix column equilibrated with 0.05 M Tris-HCl pH 7.5/ 0.3 M NaCl. The column was washed with equilibration buffer; 0.05 M Tris-HCl pH 7.5/ 1.0 M NaCl (A) at 40 ml/ hour; and 0.05 M Tris-HCl pH 7.5/ 3 M NaCl/ 2 M Urea (B) at 20 ml/ hour. The fraction was 10 ml. Bracketed fractions were pooled.

limit of Sepharose 4B (2 x 10^7) makes the interior of the matrix available for ligand attachment, and ensures good binding capacities even for large molecules. The matrix also exhibits extremely low non-specific adsorption which is essential because the power of affinity chromatography relies on specific interactions.

The con A Sepharose 4B (Pharmacia) was packed into a column (1.5 x 15 cm) and washed with 1.5 M NaCl/ 1 M urea/ 1 mM $CaCl_2$/ 1 mM $MnCl_2$/ 1 M methyl α-D-mannopyranoside/ 0.1 M Tris-HCl, pH 7.5 (con A elution buffer) at 10 ml/ hour to remove any 'free' con A. The column was then equilibrated with 1.5 M NaCl/ 1 M urea/ 1 mM $CaCl_2$/ 1 mM $MnCl_2$/ 0.1 M Tris, pH 7.5 (equilibration buffer) at 5 ml/ hr. The enzyme solution was then applied to the column at 10 ml/ hour. After washing the column with 30 ml equilibration buffer, the C-proteinase was eluted with 1.5 M NaCl/ 1M urea/ 1 mM $CaCl_2$/ 1 M α-methyl-D-mannoside/ 0.1 M Tris-HCl, pH 7.0 at 5 ml/ hour (Figure 2.4). Proteinase fractions were pooled (120 ml) and concentrated by pressure ultrafiltration, (10 ml capacity Amicon cell with a YM30 membrane). The concentrated pooled C-proteinase was dialysed against 1.5 M NaCl/ 5 mM $CaCl_2$ / 0.01 % NaN_3/ 0.05 M Tris-HCl, pH 7.5 and then stored at -20 °C. The C-proteinase was purified by 43-fold and the specific activity was determined to be 36.8 units/ mg (see section 4.2).

2.4 Preparation of pepsinised collagen

The method used for the pepsinisation of collagen was based on that of Miller (1972). Lathyritic rat skin type I collagen was a generous gift of Mr J.R.E. MacBeath. An aliquot of collagen was dialysed against 0.5 M acetic acid at 4 °C. Pepsin (from porcine gastric mucosa, Boehringer Mannheim) was added to the collagen solution in an enzyme:substrate weight ratio of 1:10. The pepsin digestion was carried out for 18 hours at 4 °C. Following the incubation, the digestion mixture was clarified by centrifugation at 50,000 g for 1 hour at 4°C. Collagen was then precipitated from the clarified solution by the addition of crystalline NaCl to a

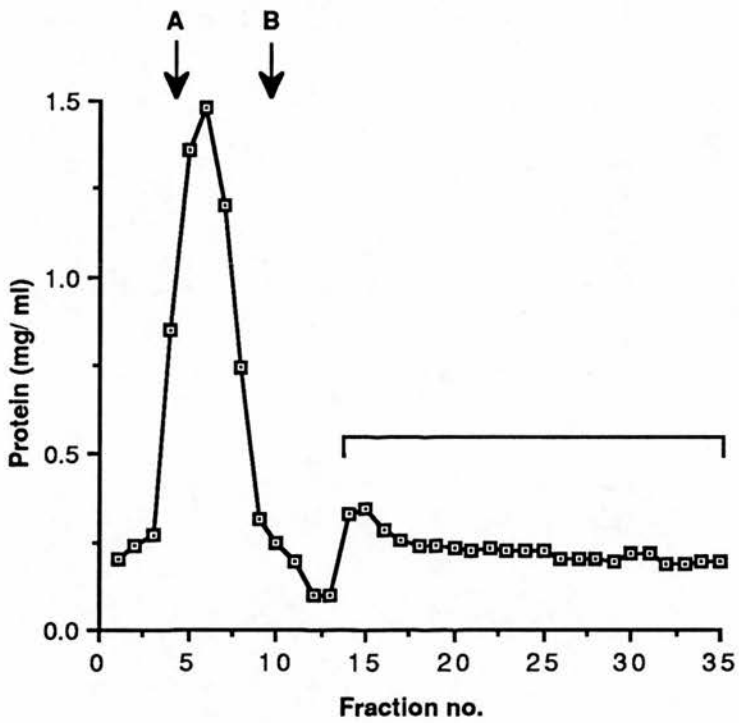


Figure 2.4 Con A Sepharose chromatography of C-proteinase from chick embryo tendons The enzyme solution was applied to a Con A column (1.5 x 15 cm) equilibrated with 0.1 M Tris-HCl pH 7.5/ 1.5 M NaCl/ 1M urea/ 1 mM $CaCl_2$/ 1 mM $MnCl_2$ (A). The column was washed with equilibration buffer; 1 M α-methyl D-mannoside. The fraction size was 6 ml and the flow rate 5 ml/ hour. Bracketed fractions were pooled.

concentration of 2.0 M. The resulting precipitate was retrieved by centrifugation at 50,000 g for 1 hour at 4 °C and redissolved in 1 M NaCl/ Tris-HCl, pH 7.5. The collagen solution was then concentrated and transferred into 0.15 M NaCl/ 8.1 mM Na_2HPO_4/ 1.9 mM NaH_2PO_4, pH 7.4 using an Amicon microconcentrator. The concentration of the pepsinised collagen was typically 400 µg/ ml determined using the hydroxyproline assay (see Figure 2.1).

[^{3}H]collagen was produced by the pepsinisation of [^{3}H]procollagen using the above method. SDS-PAGE and fluorography showed the procollagen propeptides to be absent (see Figure 3.2).

2.5 Cross-linking of procollagen

The cross-linking reagent glutaraldehyde (25 % w/ v) was added to the [^{3}H]procollagen solution to a final concentration of 1 % at 20 °C. The cross-linking reaction was stopped after 2 minutes by the addition of 100 µl of 2 M $NaBH_4$ (in 0.1 M NaOH). Excess $NaBH_4$ was removed by excessive dialysis against 10 mM phosphate buffer pH 7.4/ 0.15 M NaCl.

2.6 Gel filtration of procollagen

The gel filtration procedure used was similar to that described by Mould *et al* (1990). A Sephacryl S500 column[3] (Pharmacia,1.5 x 55 cm) was equilibrated with 10 mM phosphate buffer pH 7.4, 0.15 M NaCl, 0.5 % (w/ v) Brij 35 at a flow rate of 5 ml/ hour at 20 °C. 1.0 ml of [^{3}H]procollagen solution (glutaraldehyde fixed) in equilibration buffer was loaded on to the column at 5 ml/ hour. The column was then eluted with the equilibration buffer. Fractions of 0.8 ml were collected from the time of loading the sample. For each fraction (50 µl aliquot), ^{3}H radioactivity was detected by liquid scintillation counting using Cocktail T.

[3] The column was packed by L.Vuillard.

2.7 Preparation and characterisation of liposomes

The value of liposomes as model membrane systems derives from the fact that liposomes can be constructed of natural constituents such that the liposome membrane forms a bilayer structure which is in principal identical to the lipid portion of natural cell membranes.

2.7.1 Materials

Bovine brain sphingomyelin (SPM) and dimyristoylphosphatidylcholine (PC) were purchased from Avanti Polar Lipids, Pelham, Alabama. Bovine spinal cord phosphatidylserine, wheat germ phosphatidylinositol and egg phosphatidylserine were supplied by lipid products, UK. L-3-phosphatidylcholine, 1-2-di[1-^{14}C]oleoyl (105 mCi/ mmol) was purchased from Amersham, Bucks, UK. BioGel P6-DG and n-octyl-β-D-glucoside was obtained from Sigma, Poole, UK. All other reagents were obtained from BDH Chemicals, Poole, Dorset, UK.

2.7.2 Preparation of liposomes

Phospholipid membranes form spontaneously as a result of unfavourable interactions between phospholipids and water (New, 1990). The emphasis in making liposomes is not towards assembling the membranes (which happens of its own accord), but towards getting the membrane to form vesicles of the right size and structure. The main difference between the various methods of manufacturing liposomes is in the way in which the membrane components are dispersed in aqueous media, before being allowed to coalesce in the form of bilayer sheets. The mode of dispersion used in this study was detergent solubilisation (detergent removal method).

2.7.3 Detergent removal method

In this method, phospholipids are brought into intimate contact with the aqueous phase via the intermediary of detergents, which associate with phospholipid molecules and serve to screen the hydrophobic portions of the molecule from water. The structures which form as a result of this association are known as micelles, their shape and size depending on the chemical nature of the detergent. The removal of detergent (using gel filtration or dialysis) from preformed mixed micelles containing phospholipids leads to a spontaneous formation of unilamellar vesicles (Houslay and Stanley, 1982).

The detergent used in this method was n-octyl β-D-glucoside (Mimms *et al.*, 1981; Jackson *et al.*, 1982). In contrast to phospholipids, detergents are highly soluble in both aqueous and organic media, and there is an equilibrium between the detergent molecules in the water phase and in the lipid environment of the micelle. The critical micelle concentration (CMC) is the concentration of detergent in water at which micelles just start to form, and can give an indication of the position of the equilibrium. The advantage of n-octyl β-D-glucoside is that it has a high CMC (23.2 mM, New, 1990) which indicates that the equilibrium is strongly shifted towards the aqueous phase, so that removal from the mixed micelle upon lowering the concentration of detergent in the aqueous phase by gel filtration (Perez-Castineira, 1990) or dialysis (Baron and Thompson, 1975) is relatively easy.

The liposomes were prepared fresh prior to use. Lipids used for the formation of liposomes were initially obtained in a chloroform/ methanol (2:1 v/v) solvent. Latterly, bovine brain sphingomyelin (SPM) and dimyristoylphosphatidylcholine (PC) were obtained lyophilised to ensure no solvent contamination. The lipids (total concentration 10 mM) were dried down to a thin film in a glass vial, using a stream of nitrogen.The dried film or lyophilised lipid was then dissolved by stirring overnight in 1 ml of 50 mM n-octyl-β-D-glucoside (previously purged by bubbling with a stream of nitrogen). The detergent/ lipid ratio was 5:1 which is sufficient for

effective solubilisation of lipids, and thus avoids the formation of lipid aggregates (Mimms *et al.*, 1981).

Removal of detergent from the mixed micelle was as described by Perez-Castineira and Apps (1990). Desalting gel (Bio-gel P6-DG; exclusion limit 7000) was allowed to swell for four hours in 0.15 M NaCl/ 8.1 mM Na_2HPO_4/ 1.9 mM NaH_2PO_4, pH 7.4, and then packed into a microcentrifuge tube (supported by glass wool) by centrifugation (MSE centaur bench centrifuge) for 1 minute at 1200 r.p.m. Detergent dissolved lipid (200 µl) was then applied dropwise to each microcentrifuge tube[4] containing 1 ml of dried desalting gel. After centrifugation (as above), a turbid solution was collected. The liposome solution was diluted 1:1 with 0.15 M NaCl/ 8.1 mM Na_2HPO_4/ 1.9 mM NaH_2PO_4, pH 7.4, and then stored at 4 °C in an atmosphere of nitrogen. The liposomes were used within 8 hours of preparation.

The recovery of lipid during liposome preparation was determined by the incorporation of L-3-phosphatidylcholine, 1-2-di[1-^{14}C]oleoyl[5] (Amersham, 105 mCi/ mmol) into the liposomes. 10 µl of [^{14}C]PC in chloroform/ methanol was dried to a thin film in a glass vial. Lyophilised SPM (10 mM) was added to the vial and dissolved in 1 ml of 50 mM n-octyl-β-D-glucoside (see above). 100 µl of the dissolved lipid solution was assayed for ^{3}H by scintillation counting. After liposome preparation (see above), a 100 µl aliquot was assayed for ^{3}H. The recovery of SPM was determined to be 8.9 % ± 0.8 % (standard error of the mean, n=6). The concentration of lipid recovered after the liposome preparation was assumed to be 0.9 mM.

2.7.4 Characterisation of the liposomes

To ensure the liposomes were capable of providing an adequate surface for the interaction, the size distribution of the liposome preparation was determined. Two techniques were used to determine the size and polydispersity of the liposome preparations, quasi-elastic light scattering and electron microscopy.

[4] The microcentrifuge tube with a hole in its base was supported on a 10 ml centrifuge tube.

[5] Abbreviated as [^{14}C]PC.

2.7.4.1 Electron microscopy

Negative stain electron microscopy is a useful method for determining the size distribution of liposomes and also whether the liposomes are multi or unilamellar. The technique of negative staining is based on embedding the particulates in a thin film of an electron dense 'glass'. When examined by electron microscopy, the relatively electron transparent particulates appear as bright areas around a dark background (for details of specimen preparation, see section 2.8.1). Liposomes were also examined by electron microscopy after rotary shadowing with platinum (see section 3.5.1).

2.7.4.2 Photon correlation spectroscopy (PCS)

Liposomes were analysed by photon correlation spectroscopy (PCS, Pusey, 1989) to determine their translational diffusion coefficient, and hence their diameter. PCS first described by Berne and Pecora (1976) is the analysis of the time dependence of intensity fluctuations in scattered laser light due to brownian motion of particles in solution/ suspension. Since small particles diffuse more rapidly than large particles, the rate of fluctuations of scattered light varies accordingly. Thus, the translation diffusion coefficient (D) can be measured, which in turn can be used to determine the mean hydrodynamic radius (R_h) of the particles using the Stokes-Einstein relation (see below). Once the signal has been recorded in terms of a series of photomultiplier bursts over a period of time, a mathematical process called correlation is carried out in which the similarity between the signal and itself separated from the original by a time delay is measured. This is performed by multiplying the amplitudes of the signal and its time delayed copy together at different time points to give a correlation function. As the signals become more and more out of phase with each other (i.e. time separation is increased), their randomness with respect to each other results in a decay of the correlation function to a constant value.

The correlation function at any given time separation is described mathematically as;

$$G_{(t)} = < N >^2 (1+Be^{-\Gamma t})$$

in which N is the intensity of the signal averaged over many sample times, B is a constant determined by mechanical constraints of the apparatus and the sampling procedure, and Γ, the decay constant, is $2DK^2$ where $K = 4\pi/ \lambda n \sin(\theta/ 2)$ and n is the refractive index and D is the diffusion coefficient of the particles causing the fluctuation. Having obtained a value for the diffusion coefficient, the particle radius can then be determined by inserting D in the Stokes-Einstein relation;

$$D = k T/ 6 \pi \eta R_H$$

Where k= Boltzmans constant, T= absolute temperature, η = solvent viscosity and R_H = mean hydrodynamic radius.

PCS measurements were made on a Malvern 4700c system with an argon-ion laser (Innova 4). The argon ion laser was set at 514.5 nm with a power range from approximately 100 to 250 mW. This was focussed into the centre of the cell (1 cm diameter cylindrical quartz (ALV lasers F.R.G.)). To remove dust or contamination, cells were stored in sulphuric acid. Prior to use, cells were thoroughly rinsed with distilled water to remove traces of acid, and then with filtered sample buffer. Both buffer and sample were filtered using 0.45 μm nucleopore filters. Drops of the liposome preparation were transferred on to the side of the cells. The cuvette containing the filtered liposome solution was placed into the water bath (25 + 0.1°C) of the spectrometer. Careful control of the temperature minimised any potential errors due to variation in fluid viscosity, and also minimised random convection currents superimposed on the brownian movement which would lead to substantial errors in particle size measurement. The experimental time was set to 10 seconds, and each measure was made up of several runs (typically 10). The software calculated the

mean value of the integration of counts for each run and rejected any run with a significant difference in integration. The photomultiplier assembly collected the scattered light (at 90 °) and transmitted signals to the correlator.

2.8 Electron microscopy

The intrinsic contrast of biological material in the electron microscope is poor because the scattering of the carbon atoms in the support film is of approximately the same magnitude as that of all the principal atoms (carbon, nitrogen, oxygen, phosphorous and sulphur) of the material. Therefore, heavy metals of very high scattering power are deposited in such a way that the pattern of the metal indicates the features of the sample.

Negative staining and rotary shadowing can be used to visualise macromolecules in the electron microscope. Negative staining offers the advantages of higher resolution, ease of preparation and gentle conditions, thus minimising artifacts. However, for procollagen, a long rod shaped structural protein, the contrast provided by negative staining is inadequate to obtain good images. Rotary shadowing allows procollagen molecules to be visualised in transmission electron microscopy (TEM).

2.8.1 Negative staining

Negative staining as a method of contrast enhancement was developed by Hall, (1955). It is a simple and rapid method for studying the morphology and structure of particulate specimens. This method offers the advantages of higher resolution than rotary shadowing (Walker *et al.*, 1985), ease of preparation, and gentler conditions with fewer experimental steps, thus minimising artifacts. Staining is achieved by using heavy metal salts which upon drying, form a glassy cast of the embedded objects. The electron density difference between the specimen and the surrounding

heavy metal atoms produces the contrast. The specimen substructure is revealed by the penetration of the stains into its holes and crevices. Uranyl acetate and sodium phosphotungstate (PTA) are the most commonly used negative stains.

The drop method of Huxley and Zubay (1960) was used for the preparation of specimens. A carbon, collodion coated copper grid was clamped in a pair of anticapillary forceps. A 10 µl drop of specimen suspended in buffer was placed onto the coated grid. 1 minute was allowed for the specimen to attach to the collodion film. Excess buffer was drawn off using the edge of a piece of filter paper (Whatman no. 1), held at 90° to the plane of the grid. The grid was then washed by applying and drawing off several drops of assay buffer. A drop of 1 % phosphotungstic acid (PTA) pH 7.4 was then placed on to the grid for 30 seconds. The bulk of the PTA solution was then drawn off using filter paper, to leave behind a thin film of stain. The grid was then allowed to dry thoroughly before being viewed in a Philips CM12 electron microscope. To ensure adequate spreading and good staining many grids were prepared each with a different specimen dilution. The grids were viewed and photographed within 1 or 2 days of preparation (recrystallisation of the negative stain can occur on storage).

2.8.2 Rotary shadowing

This technique involves the shadowing of a rotating specimen with a heavy metal from an oblique angle, in vacuo. The observation of single molecules with the electron microscope after shadow casting with metals dates back to the early forties (Williams and Wyckoff, 1945). One of the critical improvements of this technique was made by Hall using freshly cleaved mica which is flat to atomic dimensions, as the inert surface (Hall, 1956). Later, advances came in terms of resolution with the introduction of cryoprotective agents (glycerol or ethylene glycol) and a rotating stage on which the samples are mounted during shadow casting. The technique has been successfully used to elucidate the structures of myosin (Elliot and offer, 1978); spectrin (Shotten *et al.*, 1979); and procollagen (Bachinger *et al.*, 1982). The rotary

shadowing of connective tissue molecules has been reviewed by Furthmayer and Madri (1982).

The low resolution mapping of protein-protein and protein-nucleic acid association sites has been determined by rotary shadowing. The association of spectrin with actin via its end (Glenney *et al.*, 1981), and a complex between E.coli RNA polymerase haloenzyme and T7 DNA (Koller *et al.*, 1978) have been visualised by rotary shadowing. This is the first demonstration of the use of rotary shadowing in examining protein-lipid interactions. Potential artifacts are inherent in the use of electron microscopy. Each step in the rotary shadowing procedure has the potential for creating artifacts. Therefore, information collected on procollagen-lipid interaction using rotary shadowing was interpreted in conjunction with data from the density gradient centrifugation binding studies (see Chapter 3).

2.8.3 Specimen preparation for rotary shadowing

The preservation of biological material prepared for rotary shadowing depends strongly on the dehydration procedure used. Simple air drying is harmful, since surface energy phenomena associated with the liquid-gas interface may alter the original features of a structure (Anderson, 1952). Two widely used methods to help preserve the structure for subsequent shadowing are glycerol drying and freeze drying.

2.8.3.1 Glycerol drying

The introduction of glycerol as a protective agent during drying at low and room temperature has vastly improved the quality of the images observed (Elliot and Offer, 1978; Shotten *et al.*, 1979). The exact reason for the effect of glycerol is not known. The exchange of glycerol with intramolecular water may slow the drying process or modify the interfacial tension at the surface of the specimen. Drying in its absence may lead to the collapse of domains within the molecules, or to a more

compact structure of the molecule. Glycerol drying has proved effective in the presence of physiological concentrations of non-volatile salts. Tyler and Branton (1980), surmised that as the glycerol containing solvent evaporates, the retreating edge of the liquid droplet sweeps the surface and carries with it molecules that do not adhere to the substrate.

2.8.3.2 Freeze drying

Freeze drying is an established technique for the preservation of biological structure (Williams, 1953). The biological structures are adsorbed or sprayed onto mica, and are then rapidly frozen with liquid nitrogen. The specimen is then dried by vacuum sublimation of the frozen water. Fowler and Aebi (1983) have shown that freeze drying has significant advantages over glycerol drying for preserving structural details in a range of supramolecular assemblies. Since volatile molecules pass directly into the vapour phase, the specimen is not exposed to potentially damaging surface tensions. Freeze drying has been shown to preserve the structural details of crystalline actin sheets, actin filaments and keratin filaments that are often lost during drying at room temperature in the presence of glycerol (Fowler and Aebi, 1983).

2.8.4 Deposition of the sample on to the mica substrate

2.8.4.1 Spraying

The protein solution in 70 % glycerol is usually sprayed at room temperature to form a cloud of droplets, some of which are caught onto the freshly cleaved mica substrate (Shotten et al., 1979). Droplets have also been sprayed onto mica cooled to the temperature of liquid nitrogen (Elliot and Offer, 1978; for details of the specimen preparation, see Figure 2.5).

Damage to supramolecular assemblies and to some larger macromolecules can

(a)

1. Connect glass Pasteur pipette to nitrogen supply with flexible tubing. Clamp pipette about 30 cm from vertical screen (perspex sheet covered with absorbent paper).
2. Insert point of tweezer (Dumont) into side of $4cm^2$ mica sheet (Agar Aids). Use another tweezer to pries mica pieces apart.
3. Attach freshly cleaved mica to vertical screen using small piece of double-sided adhesive tape (3 mm^2). Direct nitrogen flow on to mica sheet.
4. Mix sample (protein concentration typically 200 μg/ ml) by gentle pipetting with glycerol to give final concentration of 65 % (v/v) glycerol.
5. Use pipette with fine plastic tip (TI-FLEX, Scotlab) to take up 50 μl of glycerol diluted sample. Remove tip from pipette. Place narrow end in front of nitrogen source (0.5 bar) to expel sample as fine spray. Stop nitrogen flow.
6. Attach mica to rotary stage of vacuum coating unit (Edwards E306A) with small piece of double sided adhesive tape. Evacuate coating unit to 0.1 Torr with the rotary pump. Evacuate to 2×10^{-5} with the diffusion pump.

(b)

1. Submerge brass block (diameter 50 mm; depth 15 mm) into liquid nitrogen for several minutes. Immediately prior to spraying, clamp frozen brass block 30 cm from nitrogen supply.
2. To prevent 'frost' formation on the brass block, obstruct nitrogen flow (0.5 bar).
3. Place freshly cleaved $4cm^2$ mica sheet onto frozen brass block. Remove obstruction to nitrogen flow. Spray sample as above.
4. Place brass block immediately into coating unit. Insert thermocouple into brass block. Evacuate coating unit to 2×10^{-5} Torr.
5. After 60 minutes remove high vacuum. Use flexible tubing to connect liquid nitrogen supply to closed aperture of coating unit. Open aperture to allow dry N_2 into unit.

Figure 2.5 A protocol for the spraying of specimen on to mica. (a) Glycerol dried. (b) freeze dried

readily occur by this technique. Actin filaments are abnormally short after spraying,indicating a shearing of filaments during droplet formation (Tyler and Branton, 1980; Mould *et al.*, 1985). Spraying a solution of macromolecules onto a mica substrate, creates velocity gradients in the flowing liquid, generating viscous forces in the solution, and subjecting the molecules to varying degrees of shear stress. Mould *et al* (1985) have shown that this stress can be great enough to bring about the shear rupture of some elongated macromolecules and supramolecular structures.

2.8.4.2 Mica-sandwich technique

Mould *et al* (1985) developed a gentler spreading procedure than spraying, the mica-sandwich technique. The sample is sandwiched between two freshly cleaved mica sheets and allowed to adsorb to the surface. The sandwiching procedure can be employed in conjunction with either glycerol drying or with freeze drying (for details of specimen preparation, see Figure 2.6). This technique has several advantages over spraying. The observations of Mould *et al* (1985) on collagen and F-actin show it is less likely to cause fragmentation, yields a more uniform distribution of the specimen and only a very small sample volume at low concentrations is required.

The metal chosen for this study was platinum which has been shown to give good resolution and is easily evaporated by resistive heating (Abermann et al, 1982; for details of the rotary shadowing procedure, see Figure 2.7).

2.9 Immunoblotting

Immunoblotting is a method of detecting or identifying proteins on the basis of their immunogenecity. Proteins are fractionated by SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane (Towbin *et al.*, 1979). Specific proteins can be identified on the membrane by their ability to bind a particular antibody.

(a)

1. Cleave 4 cm^2 mica sheet by inserting point of tweezer into its side. Use another tweezer to pries mica pieces apart.
2. Use a micropipette to place a 5 µl drop of sample (protein concentration typically 200 µg/ ml) in 0.2 M ammonium acetate to newly formed surface. Reappose other newly formed surface on top causing drop to spread between the two.
3. Place sandwich in a humid atmosphere. Allow 4-5 minutes for the specimen to adsorb to the mica substrate.
4. Submerge brass block in liquid nitrogen (15 x 50 mm). Plunge mica sandwich into liquid nitrogen. Separate mica pieces under liquid nitrogen by fracturing ice layer.
5. Quickly place frozen mica sheet with ice-layer uppermost on to submerged brass block. Immediately place brass block into vacuum coating unit (Edwards E306A). Attach thermocouple to brass block, reduce pressure to 2×10^{-5}.
6. After 60 minutes slowly admit dry N_2 into coating chamber.

(b)

1. Mix sample with glycerol to give a final concentration of 65 % (v/v) glycerol.
2. Sandwich sample between two freshly cleaved mica sheets as described above.
3. Separate mica pieces. Attach mica with glycerol-layer uppermost to rotating stage (coating unit) with adhesive tape. Reduce pressure of coating unit to 2×10^{-5} Torr.

Figure 2.6 A protocol for the deposition of specimen on to mica using the mica-sandwich technique. (a) Freeze dried. (b) Glycerol dried

1. **Pre-clean tungsten wire**

   Cut 10 cm of 0.7 mm diameter tungsten wire. Clamp in filament holder of Edwards coating unit. Pass current of 60 Amps (A) for 10 seconds through tungsten wire.

2. **Set up coating unit**

   Using tweezers (Dumont) tightly coil 8 cm of 0.1 mm diameter platinum (Agar Aids) around centre of cleaned tungsten wire. Clamp in filament holder of coating unit. Position rotary stage 10 cm from tungsten/ platinum wire, and adjust shadowing angle to 5°. Attach prepared mica substrate to rotary stage, using small piece of double-sided adhesive tape. Cover specimen with shutter (to avoid heating it). Evacuate coating unit to 2×10^{-5} Torr.

3. **Shadowing mica substrate**

   Rotate stage at maximum speed. Pass current of 60 A through tungsten/ platinum wire. Observe the process through darkened film. Slowly increase current, remove shutter when platinum melts. Increase current until platinum evaporates (about 90 A). Turn power off. Bring system to air pressure.

4. **Carbon coating**

   Using tweezers, clamp 3 cm carbon fibre (Bio Rad) between appropriate electrodes in coating unit. Close automatic shutter. Position shadowed mica substrate 9 cm from carbon source. Evacuate coating unit to 10^{-4} Torr. Outgas carbon by increasing power to between 3 and 4.5 Volts (V) for 1 minute. Increase power to about 13 V. Open shutter when brightness of heated carbon fibre fades.

5. **Transfer of replica to copper grids**

   Fill glass dish with distilled water. Clean surface with veline tissue (General Paper and Box). Slowly submerging mica at about 30° to clean water surface to float off replica. Pick up replicas from below water surface with 400-mesh copper grids (Agar Aids). Leave grids to dry

Figure 2.7 A protocol for rotary shadowing on a mica substrate

2.9.1 Transfer of proteins

After SDS-PAGE (see section 2.2.2), the acrylamide gel was equilibrated in transfer buffer (20 mM Na_2HPO_4, 20 % methanol). Nitrocellulose (EC corporation, St Petersburg; pore size 0.45 μm) of the same size of the gel was touched to the buffer and allowed to wet by capillary action. The transfer sheet was placed on to the gel, and then carefully rubbed to remove air bubbles and excess liquid. The gel and nitrocellulose were sandwiched between Whatman filter paper (3 mm) and fiber pads. The whole 'sandwich' assembly was enclosed in a perforated support which ensured the gel was firmly and evenly pressed against the nitrocellulose. Protein transfer was carried out in a BioRad Trans-Blot tank for approximately 16 hours at a constant current of 250 mA in prechilled transfer buffer. The proteins were transferred electrically from a negative to a positive direction onto the nitrocellulose (Towbin *et al.*, 1979). The extent of transfer was determined by staining of the nitrocellulose (after detection, see below) with amido black for 1 minute. The nitrocellulose was destained with 90 ml methanol/ 20 ml acetic acid/ 90 ml distilled water.

2.9.2 Detection of the protein

The nitrocellulose was immersed into TBST (50 mM Tris-HCl, pH 7.9/ 0.15 M NaCl/ 1% Tween 20) containing 5 % dried milk (Marvel) for 1 hour at room temperature. Sufficient solution was used to cover the membrane and the container was agitated gently (Lab Therm Shaker, Adolf Kuhner, Basel). The role of the 5 % dried milk and 1 % Tween 20 was to block non-specific binding sites. The solution was then poured off and the blot washed in TBST for 15 minutes followed by 2 more washes each of 5 minutes duration. The blot was then incubated in primary antibody[6] (diluted 500 fold with TBST) for 1 hour. The solution was poured off and the blot washed for 15 minutes in TBST followed by 3 more washes each of 5

[6] The primary antibody was anti-anchorin CII for the anchorin CII preparation (see section 4.3).

minutes. The horseradish peroxidase (HRP)-labelled second antibody[7] was diluted by 500 fold in TBST. The blot was incubated in the second antibody for 30 minutes and then washed (1 x 15, 4 x 5 minutes) in fresh changes of TBST.

The HRP-second antibody was detected with the enhanced chemiluminescence system (ECL, Amersham). The ECL method is based on the HRP catalysis of the oxidation of luminol (cyclic diacylhydrazide) in the presence of H_2O_2. The oxidised luminol (excited state) decays to a ground state via a light emitting pathway. The following steps were carried out in a dark. The blot was incubated in the detection reagent (ECL, Amersham) for 1 minute and then excess reagent was drained off. The blot was then wrapped in cling film and exposed to X-ray film (XAR 5 X-OMAT) for 3 minutes. The film was developed immediately.

[7] HRP-conjugated anti-rabbit IgG for the anchorin CII preparation.

CHAPTER 3

PROCOLLAGEN-LIPID INTERACTION

3.1 Introduction

3.1.1 Collagen fibre-lipid associations

There have been numerous observations of the association of collagen fibres with lipids (Tall et al, 1978; le Lous et al, 1982; Barnes, 1985; Özgünes and Artvinli, 1988). Glycerides, cholesterols and phospholipids have been found in purified collagen preparations of rat skin and tendon (le Lous *et al.*, 1982), and bovine skin (Rabinowitz and Shapiro, 1972). In addition to these lipids, Ozgunes and Artvinli (1988) have also observed the presence of plasmalogens and glycolipids, including gangliosides in rat tail tendon preparations. Gangliosides are important components of specific receptor sites on the cell surface, and therefore may be involved in cell binding to collagen fibrils.

The process of collagen-lipoprotein lipid exchange has been examined *in vitro* (le Lous *et al.*, 1982). Collagen was shown to become associated with phospholipids and cholesterol when delipidated type I collagen fibrils were incubated with low density lipoprotein (LDL) at neutral pH (le Lous *et al.*, 1982). The types of phospholipids associated with the collagen were not determined. The interaction of LDL with the extracellular matrix by means of collagen may be relevant for the development of atherosclerotic lesions (Eskenasy *et al.*, 1984).

3.1.2 Liposome drug carriers

The use of liposomes as drug carriers has been limited by poor stability in biological media (Pajean *et al.*, 1991). Phosphatidyl choline (PC) liposomes are considerably more stable when mixed within a collagen gel (Weiner *et al.*, 1985). The stability of the liposomes was suggested to be partly due to specific interactions with the collagen molecules. Liposome stability has also been analysed in a collagen solution (Pajean *et al.*, 1991). Solubilised collagen (telopeptides removed chemically) has been shown to decrease the permeability of egg lecithin[1] liposomes at physiological pH,

[1] composed mainly of phosphatidyl choline

whereas albumin and γ-globulin have no effect (Pajean *et al.*, 1991). Pajean *et al* (1991) suggested the different effect of collagen on liposome permeability compared to albumin and γ-globulin was due to a collagen-PC interaction (see section 3.1.4).

3.1.3 Collagen-like proteins

Surfactant protein-A (SP-A) is the major component of pulmonary surfactant (lipid-protein complex) which promotes alveolar stability by lowering the surface tension at the air-fluid interface in the peripheral air-spaces (Kuroki and Akino, 1991). SP-A is characterised by a collagen-like domain (see section 1.2.2.6). Purified delipidated SP-A has been shown to bind to phospholipid vesicles (SM)[2] in a non-saturable manner with the use of an ELISA assay (Ross *et al.*, 1986), and density gradient centrifugation (sucrose gradient) to separate the protein-lipid complex from unbound constituents (King *et al.*, 1983). In contrast, the non-collagenous fragment of SP-A (generated by collagenase digestion) binds to SM vesicles weakly. Therefore, the N-terminal collagen-like fragment appears to be important in the interaction with phospholipids. The non-collagenous fragment has also be shown to bind to type II alveolar cells to a lesser extent compared to SP-A (Wright *et al.*, 1989), which suggests the collagen-like region may be involved in an interaction with the cell surface.

At neutral pH, all forms of acetycholinesterase having a collagen-like tail bind to sphingomyelin (SPM) liposomes with almost no binding to PC (Watkins *et al.*, 1977; Cohen and Barenholz, 1984). The observation that collagenase-treated enzyme does not bind to SPM liposomes localises the site of interaction in the collagen-like tail. Cohen and Barenholz (1984) suggested the interaction may involve hydrogen bonding between the hydroxylysine and/ or hydroxyproline residues and the interface region of the SPM molecule (see section 3.4.3).

[2] SM liposomes consist of 65% DMPC, 20% egg PC, 7.5% egg Phosphatidyl glycerol, and 7.5% egg PI. This composition is reflective of lung surfactant phospholipids.

3.1.4 Collagen-lipid interaction

Monomeric type I collagen has been shown to bind to dipalmitoyl- and dimyristoyl- phosphatidylcholine vesicles at acidic pH (Martinez del Pozo *et al.*, 1988). The interaction results in a protein-lipid complex which could be isolated by ultracentrifugation on a sucrose gradient. With the use of differential scanning calorimetry, the collagen-lipid interaction was shown to cause a decrease in the enthalpy of the phase transition (from a gel crystalline to a liquid crystalline phase) of the PC vesicles, whereas the transition temperature was unaffected. Similar results were obtained with pepsin and carboxypeptidase Y treated collagen which consists of only the triple helical region. These results agree with a simple surface binding of the triple helical region of collagen on the lipid vesicle.

At neutral pH, collagen fibrils were said to interact with PC and phosphatidyl glycerol (PG) vesicles on the basis of differential scanning calorimetry and fluorescence polarisation studies (Martinez del Pozo *et al.*, 1989). The perturbation on the lipid bilayer produced by collagen fibrils was shown to be lower than that produced by monomeric collagen at acidic pH. However, the formation of a collagen-lipid complex was not demonstrated (for further analysis of these results, see section 3.4.7).

3.1.5 Research strategy

The strategy of the project was to characterise the interaction of procollagens with liposomes of defined lipid compostion. Ionic strength, temperature and procollagen concentration were varied to determine the nature of the interaction, and various structural domains of procollagen were analysed separately for their role in binding.

3.2 Methods

3.2.1 Density gradient centrifugation binding assay

Discontinuous sucrose gradient centrifugation was developed for the isolation of plasma membranes (Schimmel *et al.*, 1973; Cates and Holland, 1978). Plasma membranes were enriched at the 17/ 40 % (w/ v) interface of a 8.5/ 17/ 40 % sucrose gradient. Such a sucrose gradient has also been used in a sedimentation-type binding assay to examine the interaction of collagen with lipids (Martinez del Pozo *et al.*, 1988) and lipid associated collagen-binding proteins (Mollenhauer and von der Mark, 1983; Mauch *et al.*, 1988). In these assays, the binding mixture was layered on to the top of the gradient. The liposomes migrated to the 8.5/ 17 % interface, whereas collagen either remained at the top of the gradient (Martinez del Pozo *et al.*, 1988) or migrated to the 17/ 40 % interface (Mollenhauer and von der Mark, 1983; for an analysis of these results, see section 3.10). This sedimentation assay was initially used to investigate procollagen-lipid interactions. However, a total separation of bound procollagen from unbound could not be achieved.

A flotation-type binding assay has also been used to examine the interactions of collagenous molecules with lipids (Watkins *et al.*, 1977; Cohen and Barenholz, 1984). In such an assay, the binding mixture is incorporated into a region of the sucrose gradient where the density is much greater than that of the liposomes but less than that of the protein. Therefore, liposomes float to the surface, whereas free protein migrate further down the gradient.

In this study, the flotation-type binding assay was used to examine the interaction of procollagen with liposomes of defined lipid composition. The sucrose gradient was constructed so that unbound procollagen would be completely separated from bound procollagen. The binding mixture was present in the 40 % component of a 0/ 30/ 40/ 60 % sucrose gradient (see Figure 3.1). A 30 % sucrose region (density is 1.123 g/ ml at 30 °C) was chosen to be placed above the incubation mixture because its density is.

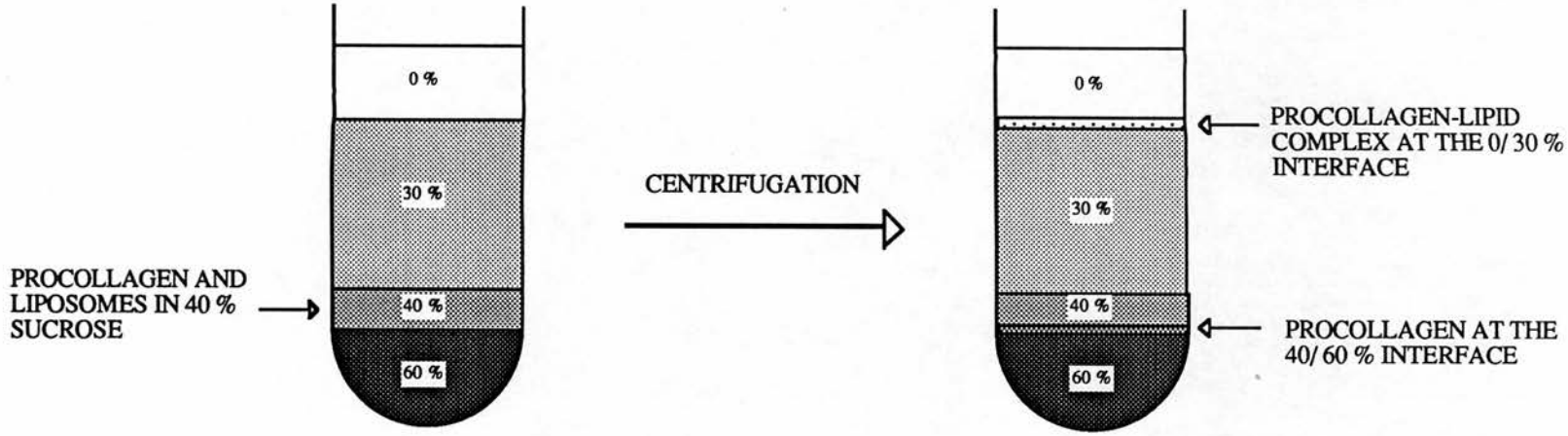


Figure 3.1 Density gradient centriufugation. Separation of procollagen from the procollagen-lipid complex.

much greater than that of liposomes[3], and yet it will still form a sharp interface with the 40 % region. The liposomes and protein-liposome complex[4] (density less than 30 % sucrose) migrate to the 0/ 30 % interface. A 60 % sucrose region was placed beneath the incubation mixture so that procollagen would sediment at the 40/ 60 % interface.

Tendon procollagen in 100 mM 0.4 M NaCl/ Tris-HCl, pH 7.4 was diluted with an equal volume of distilled water to reduce both the Tris and NaCl concentrations (for details of the procollagen preparation, see section 2.1). The procollagen was further diluted with 8.1 mM Na_2HPO_4/ 1.9 mM NaH_2PO_4/ 0.15 M NaCl, pH 7.4 (assay buffer), so that the final Tris concentration was below 0.1 mM. The phosphate buffer was chosen for the study because it is recognised as being closely physiological. A 50 µl aliquot of the diluted procollagen (typically 9 µg/ ml or 20 nM) was added to 100 µl of the liposome solution in assay buffer (lipid concentration 0.45 mM) freshly prepared by gel filtration (for details, see section 2.7.3). 300 µl of 60 % sucrose (in assay buffer) was then mixed into the solution by gentle pipetting to give a final concentration of 40 % sucrose. The mixture was then incubated in a water bath (Grant) at 37 °C for 1 hour before being incorporated into the stepwise sucrose gradient.

The stepwise sucrose gradients were formed in 5 ml centrifuge tubes (Beckman) by upwards displacement with increasing sucrose concentrations, 0 % (1 ml), 30 % (2 ml), 40 % (450 µl) and 60 % (600 µl). Sharp interfaces were observed between the sucrose solutions. The centrifuge tubes were placed into SW50.1 swinging buckets (Beckman), and centrifuged at 41000 r.p.m. (200,000 g) for 4 hours at the selected temperature, usually 35 °C. After centrifugation, liposomes were always present at the 0/ 30 % sucrose interface, as shown by the visible turbidity A bung was inserted in the top of each tube, with tubing connected to a peristaltic pump. A hole was made in the base of the tube and its contents pumped out at a convenient rate. Approximately 150 µl fractions were collected in microcentrifuge tubes. As the sucrose concentration decreased, the drop size increased slightly. For each fraction, ^3H or ^{14}C radioactivity was detected by liquid scintillation using Cocktail T. The flotation assay was also used

[3] The density of egg PC liposomes has been reported to be 1.013 g/ ml (Johnson and Buttress, 1973), and that of bovine brain SPM liposomes to be 0.998 g/ ml (Cohen and Barenholz, 1984).

[4] The density of acetylcholinesterase-associated SPM liposomes (1.030 g/ ml; Cohen and Barenholz, 1984) is less than that for 30 % sucrose.

to examine the interaction of [^{125}I]C-propeptide and [^{3}H]collagen with SPM liposomes. ^{125}I c.p.m were measured in a gamma counter

SDS-PAGE and fluorography were used to visualise the bound and unbound procollagen. 50 µl of each fraction was counted to determine the position of the bound and unbound procollagen. The remainder of the fraction were applied to discontinuous gels. Following electrophoresis (for details, see Figure 2.2), gels were processed for fluorography and exposed to pre-flashed Kodak XAR-5 film at -70 °C for a few days. Rotary shadowing was also used to investigate procollagen-lipid interactions (see section 3.3.7).

3.3 Results

3.3.1 Characterisation of type I procollagen

Procollagen was characterised by SDS-PAGE (section 2.22). The procollagen was electrophoresed on a 6 % acrylamide gel under reducing conditions (Figure 3.2). The majority of the protein migrated as two bands which were identified as proα1 (I) and proα2 (I) by comparison with known intermediates (Butkowski *et al.*, 1982; Prockop and Tuderman, 1982). A minor component identified as pCα1 (I) (Butkowski *et al.*, 1982; Prockop and Tuderman, 1982) migrated between proα1 (I) and proα2 (I) (see Figure 3.2). This minor contaminant was present in all procollagen preparations which by densitometry accounted for about 10 % of the collagenous proteins in tendon procollagen.

The yield of procollagen was determined by measuring the levels of hydroxyproline in the sample (see section 2.2.1). As there are 85 residues/ 1000 in type I procollagen (Fielder-Nagy *et al.*, 1981) it can be assumed that 7.6 % (by weight) of type I procollagen is hydroxyproline, and therefore, it is possible to determine the amount of procollagen in the sample, and the specific activity. A typical procollagen yield from 30 dozen embryos was 1.5 mg. The specific activity was typically about

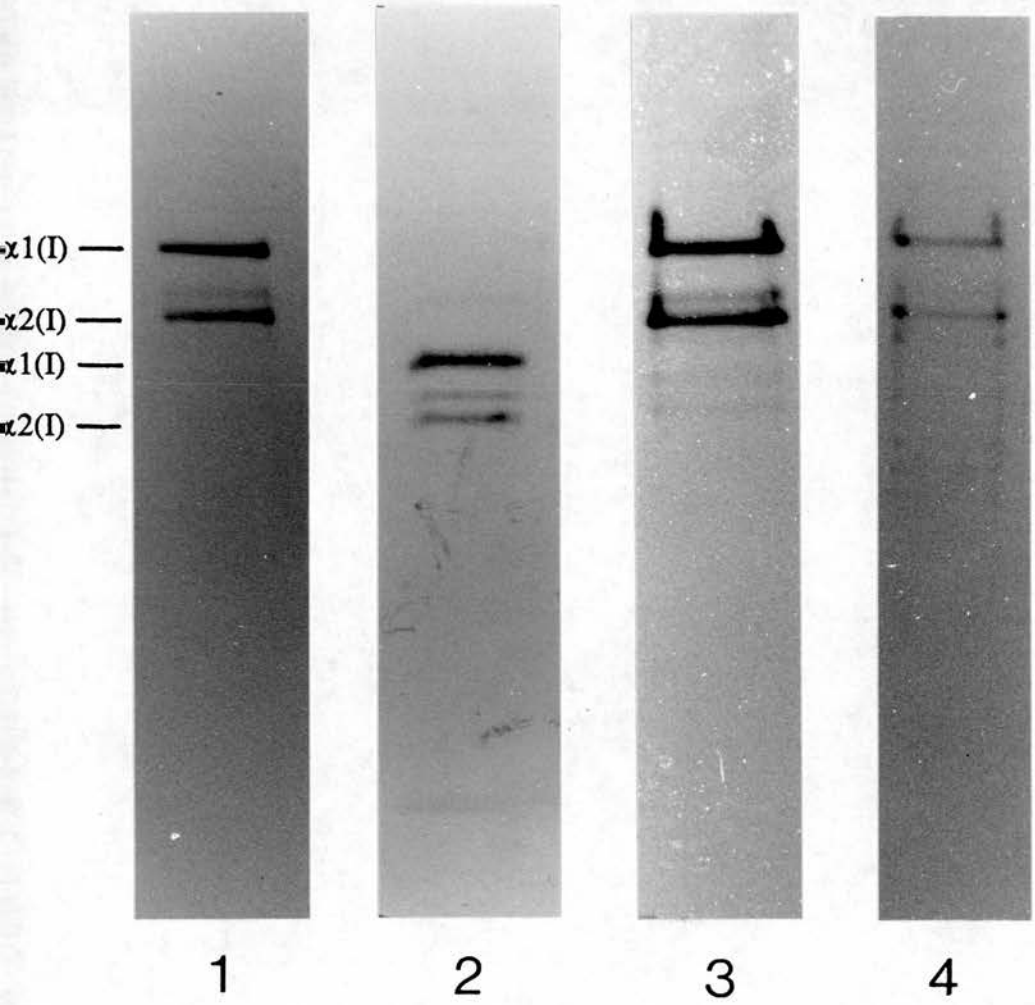


Figure 3.2 Fluorogram showing type I procollagen and collagen preparations

[^{3}H]collagen (lane 2) was prepared by the pepsinisation of [^{3}H]procollagen (lane 1). Both procollagen bound to SPM (lane 3) and unbound procollagen (lane 4) remain intact.

9000 cpm/ μg and was used to estimate all subsequent concentrations. Coomassie Blue staining showed that the purified procollagen was essentially free of BSA and other proteins (see Figure 3.3).

3.3.2 Characterisation of liposomes

Two techniques were used to determine the size of the liposome preparation, electron microscopy and quasi-elastic light scattering.

3.3.2.1 Electron microscopy

Liposomes of SPM (0.45 mM) were prepared using the detergent removal method (see section 2.6.2). Electron microscopy after rotary shadowing[5] was used to determine the diameter of the SPM liposomes. The SPM liposome diameter was determined to be 116 nm ± 43 nm (standard deviation; n=70). This value was used to determine the number of moles of liposomes (see section 3.6.11). The liposomes were also charactersied by negative staining (see section 3.8.4).

3.3.2.2 Photon correlation spectroscopy

PCS measurements were determined with liposomes of SPM and other lipids (0.45 mM) at 25 °C (for details of the method, see section 2.6.3.2). To determine the accuracy of the method, measurements of latex beads of known size (91 mm) were also made. The z-average mean of the liposome diameter for each lipid type is shown in Table 3.1. The diameter of SPM liposomes was determined to be 175 nm ± 3 nm (n=3) which is considerably greater than that obtained from rotary shadowing. This may be explained by the fact that the z-average mean is a weight average whereas the diameter determined by rotary shadowing is a number average.

[5] For details of the specimen preparation (freeze dried/ mica sandwich technique) see section 2.7.3.

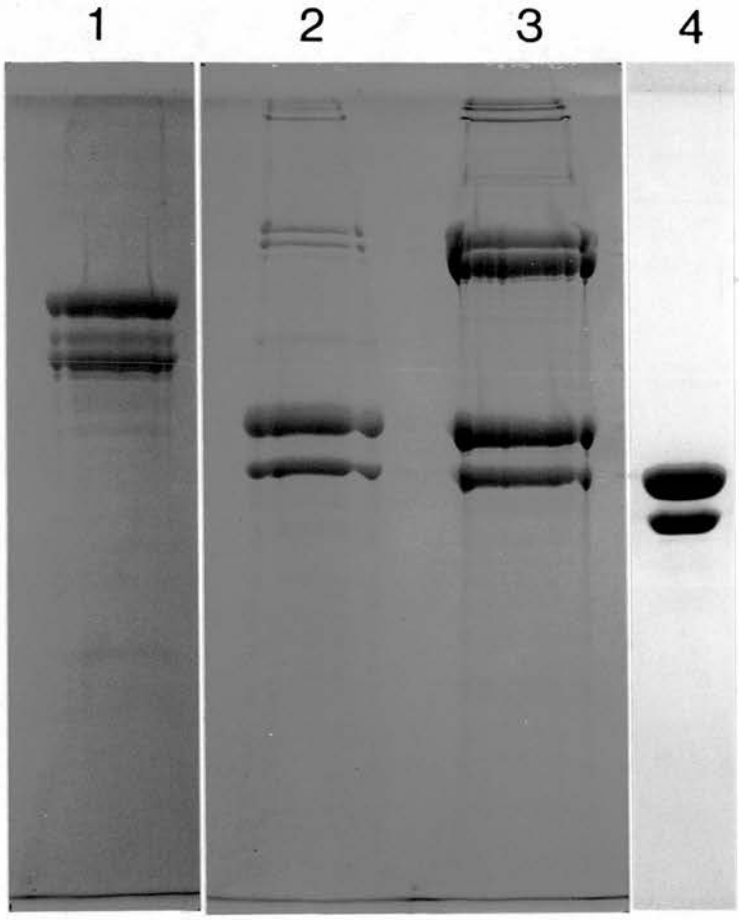


Figure 3.3 SDS-PAGE of procollagen, collagen and C-propeptide preparations.

Lane 1 = unlabelled type I procollagen. Lane 2 = pepsinised collagen. Lane 3 = rat tail tendon collagen. Lane 4 = type I C-propeptide (courtesy of Mr I.F. Purdom). The type I procollagen is essentially free of BSA and other proteins. Gels were run in reducing conditions with 6 % acrylamide (lanes 1 to 3) or 10 % acrylamide (lane 4) in the separating gel, followed by Coomassie Blue staining.

Table 3.1 Liposome diameters determined from photon correlation spectroscopy measurements

Lipid type	Z-average mean (nm)	error (nm) (n=3)	Polydispersity	error (n=3)
Phosphatidylcholine	156	4	0.39	0.04
Phosphatidylserine	132	2	0.17	0.02
Phosphatidylinositol	62	5	0.58	0.03
Sphingomyelin	175	3	0.28	0.01
91 nm Latex beads[6]	91	–	0.04	–

[6] Only a single measurement was made.

3.3.3 Procollagen-lipid interaction

The flotation-type binding assay was used to examine the interaction of [^{3}H]procollagen with lipids. [^{3}H]procollagen alone, in the absence of lipid, was found to sediment to the 40/ 60 % interface of a 0/ 30/ 40/ 60 % sucrose gradient (see Figure 3.4), while only 9.1 % ± 0.4 % (standard error of the mean[7]; n=6) of total cpm were recovered from the top half of the gradient. L-3-phosphatidylcholine, 1-2-di[1-^{14}C]oleoyl incorporated into unlabelled-PC[8] liposomes was found to migrate to the 0/ 30 % interface (see Figure 3.5). In determining the % of total procollagen bound, the fractions collected were divided into two groups. Those from the bottom of the gradient were said to represent unbound procollagen, whilst those from the top, bound procollagen. The total c.p.m. in each group were calculated with the background subtracted. The number of moles of procollagen in each assay was determined from the total c.p.m. using the specific activity of procollagen. The concentration of procollagen (nM) was calculated for the original incubation mixture (450 µl).

A disadvantage of the flotation-type binding assay is that the SPM-procollagen complex is separated from the unbound procollagen during the centrifugation. Therefore, the procollagen in the bound fraction is probably an underestimate.

3.3.4 Procollagen-SPM interaction

In physiological conditions of pH, ionic strength and temperature, there was noevidence for procollagen binding to phosphatidylcholine, phosphatidyethanolamine, phosphatidyinositol or phosphatidyserine, as illustrated for phosphatidylcholine in Figure.3.6. In contrast, procollagen bound strongly to sphingomyelin liposomes (see Figure 3.7). In standard assay conditions (typically 2.0 nM procollagen, 0.1 mM phospholipid, 35 °C), 85.6 % ± 0.8 % (n=6) of the procollagen recovered from the gradient was found in the bound fractions. The overall recovery of procollagen

[7] Errors are described as the standard error of the mean, unless otherwise stated.

[8] PC is used as an abbreviation for dimyristoylphosphatidylcholine.

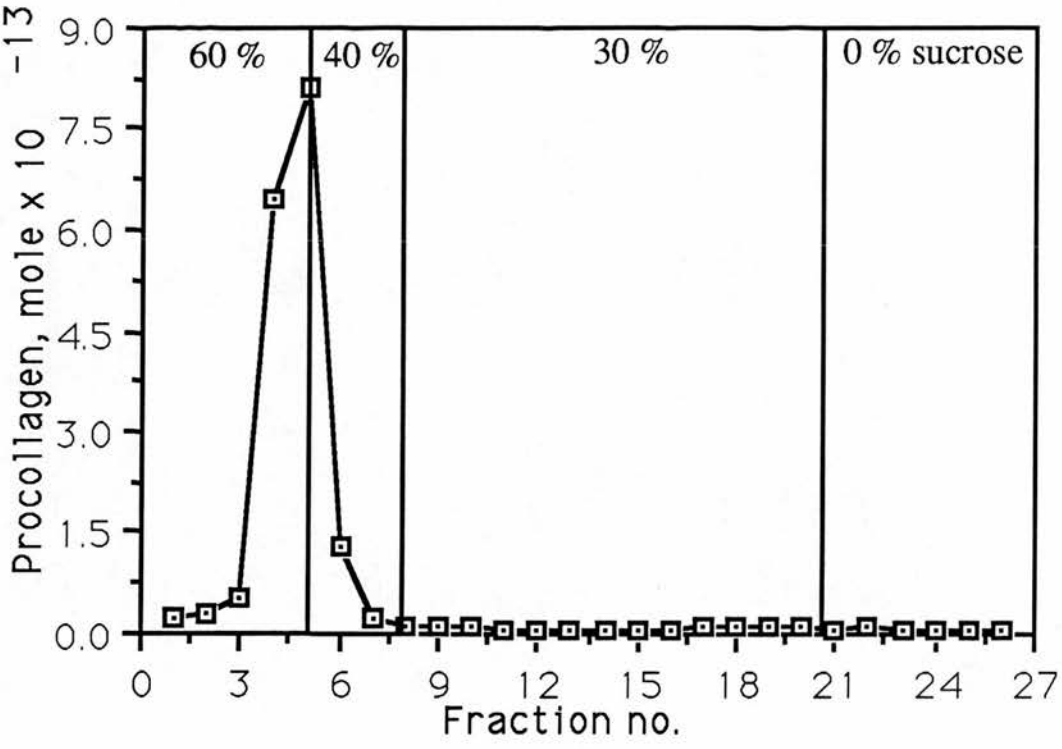


Figure 3.4 Density gradient centrifugation of type I procollagen

[^{3}H]procollagen migrates to the 40/ 60 % interface of a stepwise sucrose gradient consisting of 0 % (1 ml), 30 % (2 ml), 40 % (0.45 ml) and 60 % (0.6 ml) sucrose.

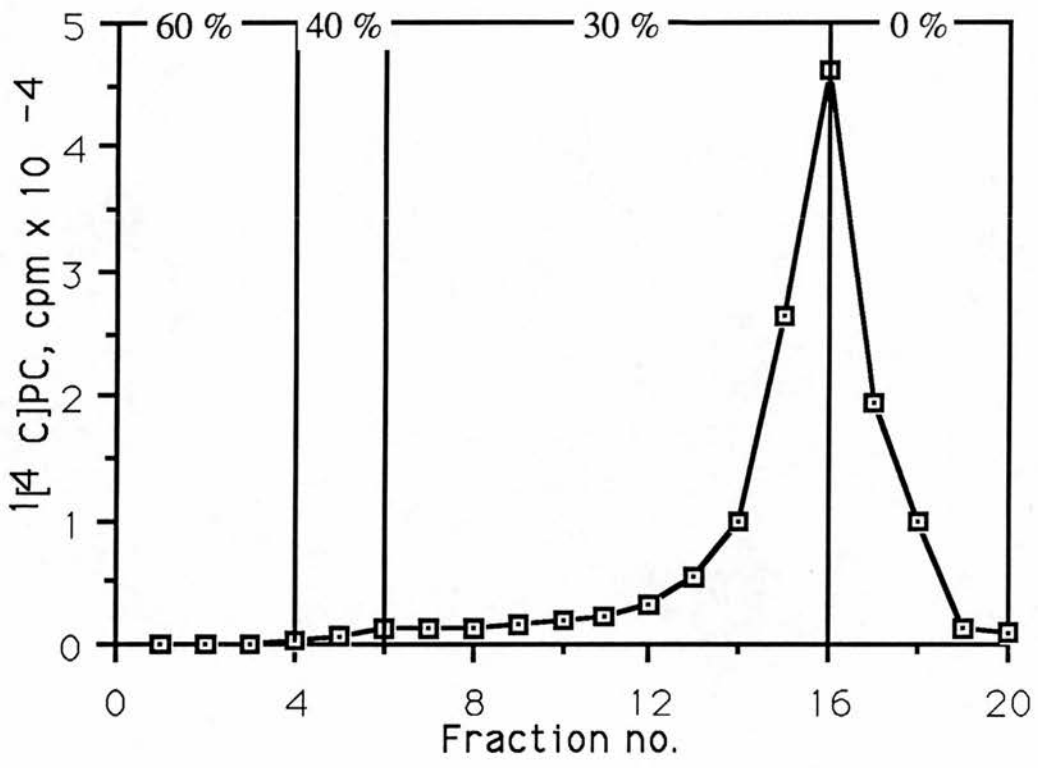


Figure 3.5 Density gradient centrifugation of phosphatidylcholine liposomes

[^{14}C]phosphatidylcholine liposomes migrate to the 0/ 30 % interface of a stepwise sucrose gradient.

throughout all fractions was determined to be 82.5 % ± 1.8 % (n=6). SDS-PAGE and fluorography showed the procollagen chains remained intact in both the bound and unbound fractions (see Figure 3.2).

To study the reversibility of the protein-lipid interaction, the procollagen-SPM complex was isolated from the sucrose gradient (bound fractions), incubated for 1 hour in 40 % sucrose and then re-centrifuged (see Figure 3.8). Only 70 % of the procollagen in the procollagen-SPM complex remained bound. This suggests that the procollagen is in equilibrium with the procollagen-SPM complex.

3.3.4.1 Concentration dependence of the procollagen-SPM interaction

For a fixed concentration of SPM (0.1 mM), the amount of procollagen bound increased with concentration, approaching saturation at approximately 5 nM (see Figure 3.9). The standard procollagen concentration is well below saturation.

A liposome diameter of 116 nM (see section 3.3.2.1) and a surface area for each molecule of 0.58 nm^2 for SPM in the gel-crystalline phase (Barenholz and Thompson, 1980; see section 3.4.4.1) were used to calculate the number of molecules in each liposome. This enabled the moles of liposomes to be determined. The liposomes were assumed to be unilamellar (see section 3.4.4.6). Direct non-linear least squares fitting to the binding data (courtesy of Dr G L Atkins) gave an apparent dissociation constant of 2.55 +/- 0.14 nm, and the number of binding sites on each SPM liposome was determined to be 7.36.

For a fixed procollagen concentration (3.5 nM), the amount of procollagen bound was shown to increase as the SPM concentration was increased from 12.5 μM to 100 μM (see Figure 3.10).

3.3.4.2 Nature of the interaction

The nature of the procollagen-SPM interaction was studied by varying the buffer conditions during pre-incubation and in the subsequent density gradient centrifugation. The binding of procollagen to SPM was examined over a range of NaCl concentrations

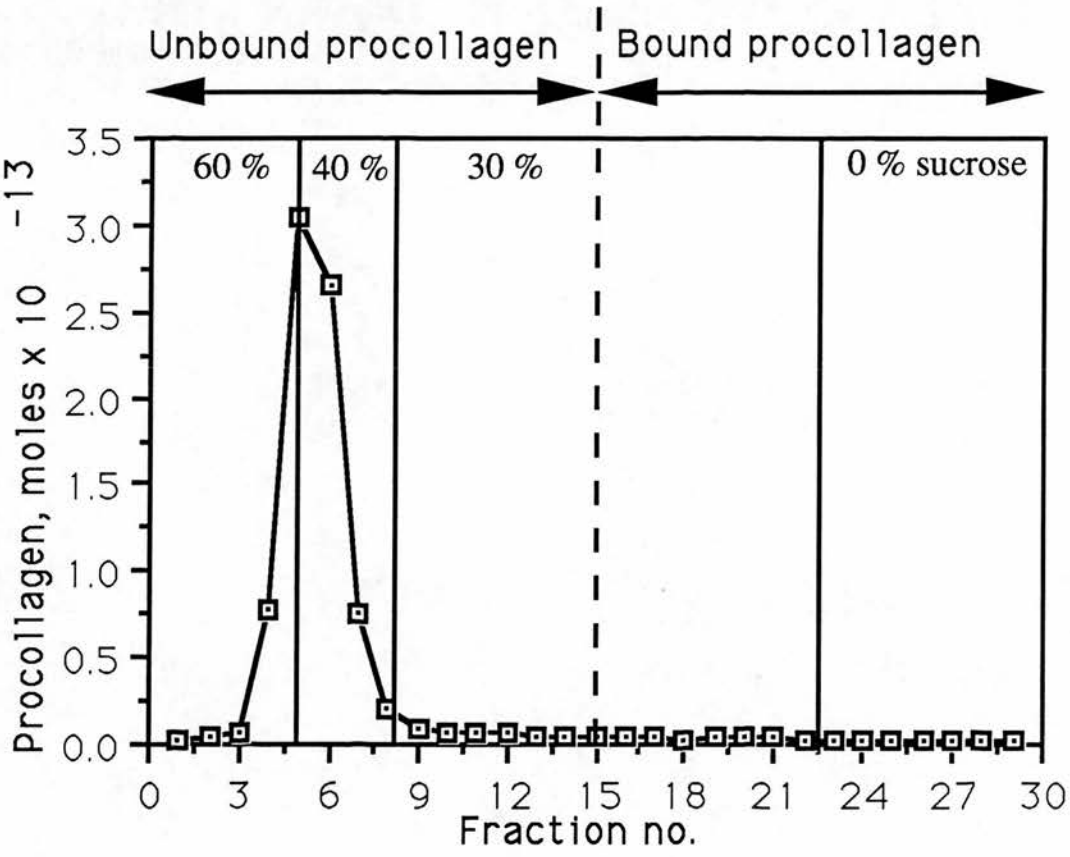


Figure 3.6 Type I procollagen does not interact with phosphatidylcholine liposomes

Density gradient centrifugation of [^{3}H]procollagen with phosphatidylcholine liposomes in 10 mM sodium phosphate pH 7.4 at 35 °C.

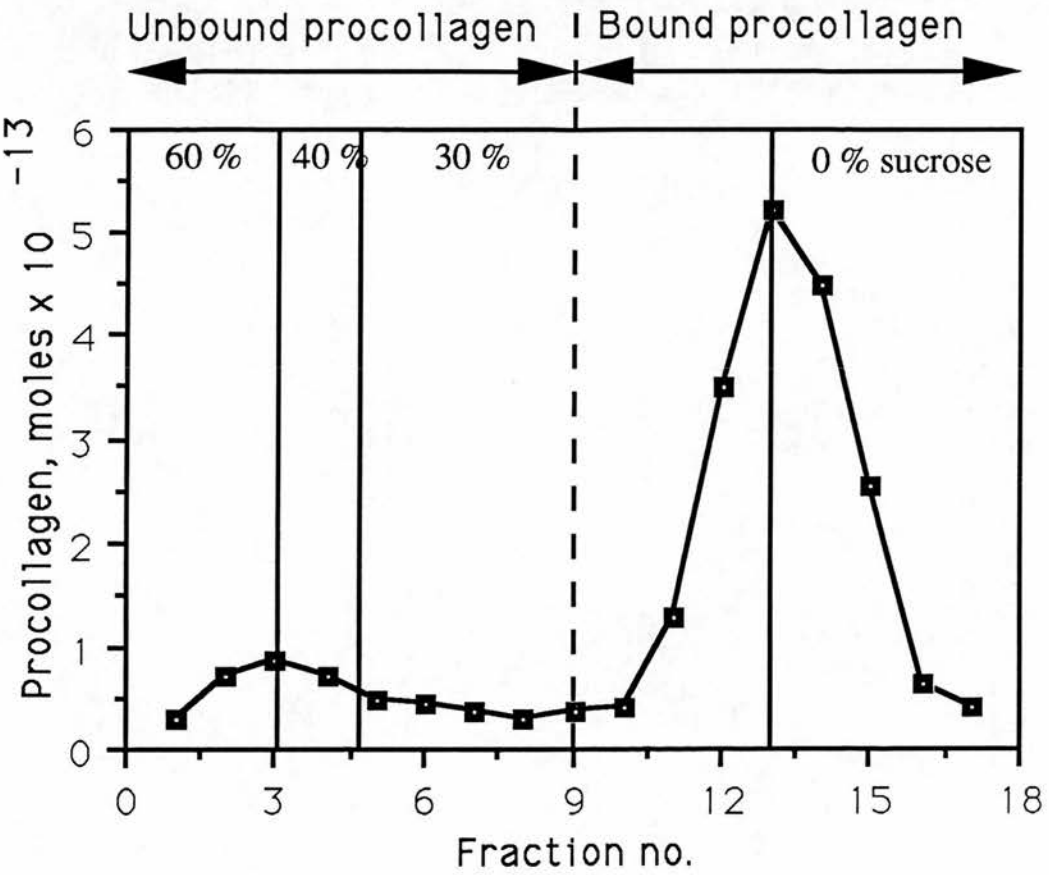


Figure 3.7 Type I procollagen does interact with sphingomyelin liposomes

Density gradient centrifugation of [^{3}H]procollagen with sphingomyelin liposomes in 10 mM sodium phosphate pH 7.4 at 35 °C.

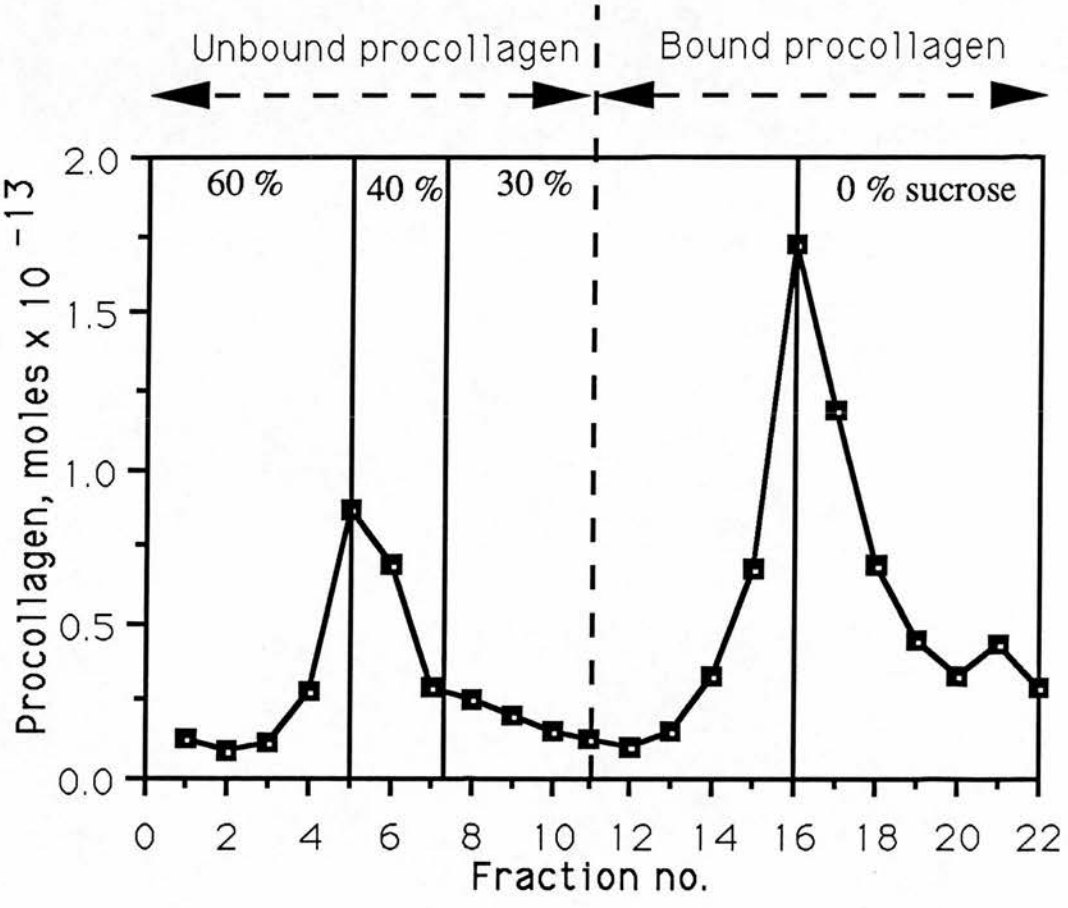


Figure 3.8 Procollagen is in equilibrium with the procollagen-sphingomyelin complex

Density gradient centrifugation of the procollagen-sphingomyelin complex in the stepwise sucrose gradient at 35 °C.

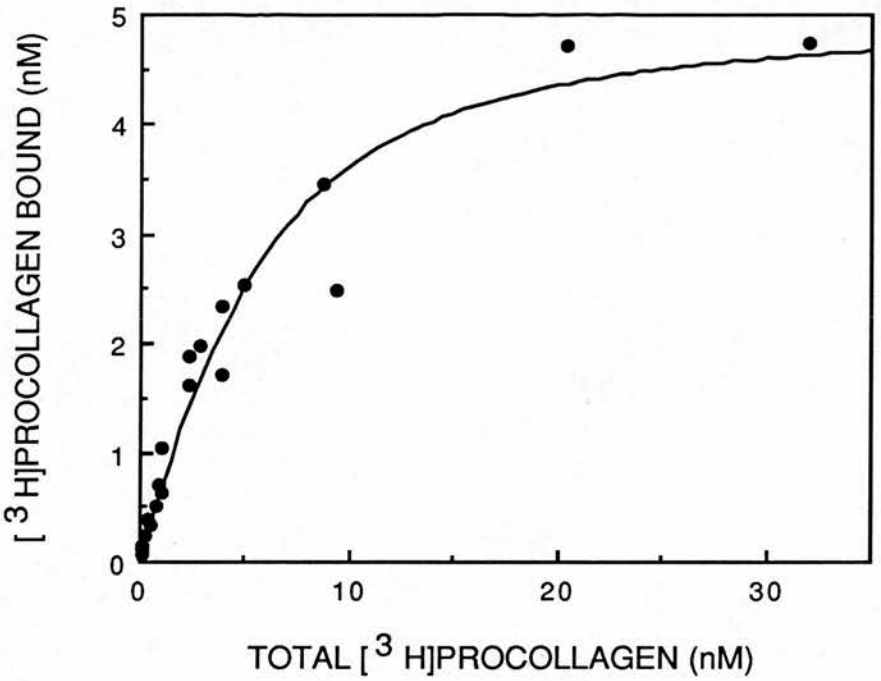


Figure 3.9 The procollagen-sphingomyelin interaction is concentration-dependent and saturable Increasing concentrations of [^{3}H]procollagen were incubated with 0.1 mM SPM and the amount of procollagen in lipid bound fractions determined by discontinuous sucrose gradient centrifugation. The curve shows the non-linear least squares fit to the data using a dissociation constant (K_d) 0f 2.55 nM and a satuation limit of 5.04 nM.

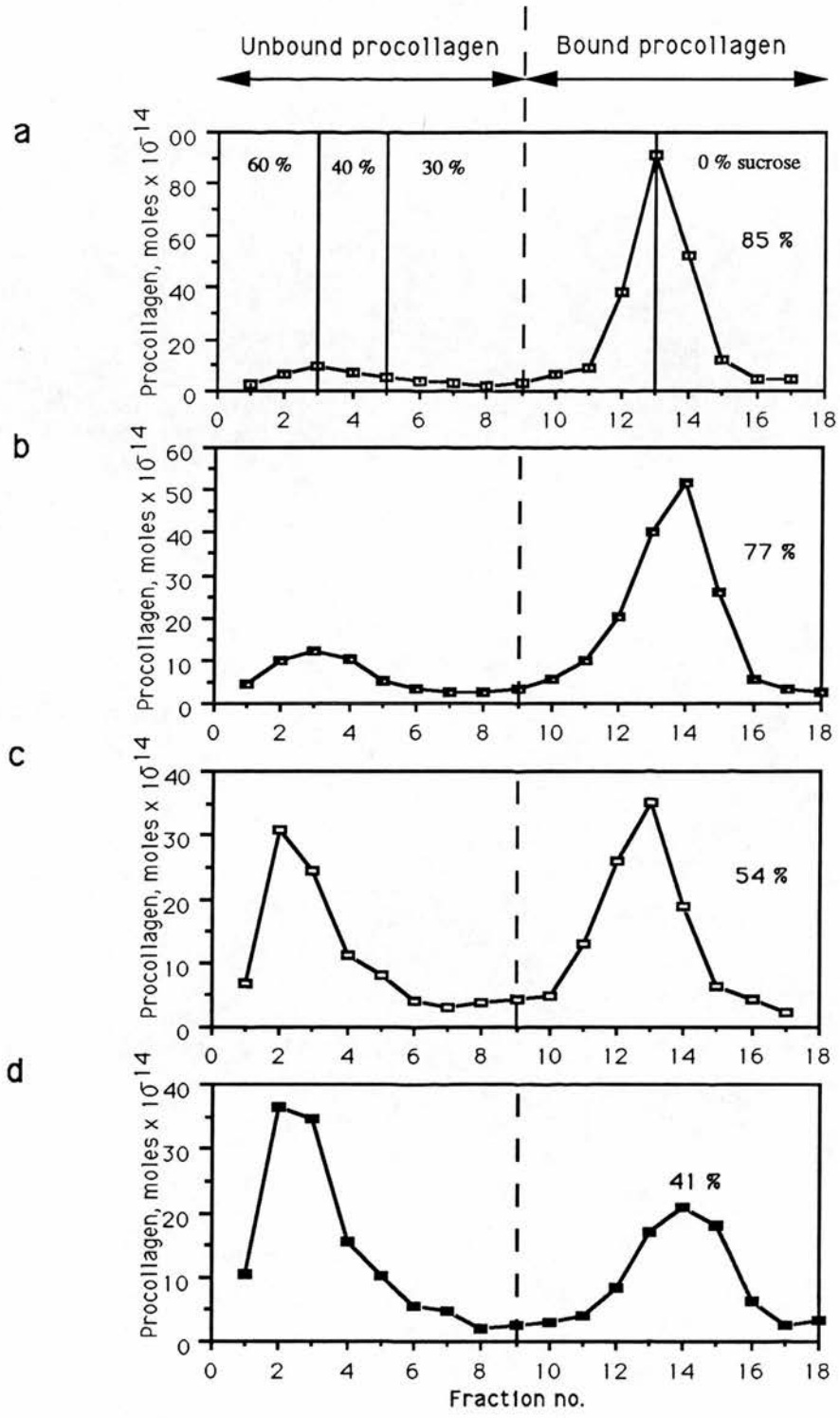


Figure 3.10 Concentration dependence of the procollagen-sphingomyelin interaction

[^{3}H]procollagen was incubated with the following concentrations of SPM (a) 100 μM (b) 50 μM (c) 25 μM (d) 12.5 μM in 10 mM sodium phosphate buffer pH 7.4 at 35 °C, and then centrifuged in discontinuous 0/ 30/ 40/ 60 % sucrose gradients.

in order to determine whether the interaction was electrostatic in nature. The SPM liposomes were prepared in the absence of salt, and procollagen was diluted with phosphate buffer containing no salt. When present, salt was added to the incubation mixture with the 60 % sucrose to give the desired sucrose concentration in the 40 % sucrose part of the gradient, and the salt concentration throughout the rest of the gradient was adjusted accordingly. Most of the procollagen remained bound to SPM up to a salt concentration of 1.2 M (see Figure 3.11). This suggests the interaction is not electrostatic in nature. In fact, the amount of procollagen bound was found to increase, although very slightly, from 0-1.2 M (for a discussion, see section 3.4.2).

The procollagen-SPM interaction was also examined over a range of temperatures (4 °C-39 °C). If hydrophobic interactions are involved, then the amount of procollagen bound would decrease with decreasing temperature. The amount of procollagen bound at 4 °C was similar to that observed at the higher temperatures (see Figure 3.12). This suggests there is no obvious hydrophobic interaction involved. In fact, 93.5 % ± 0.9 % (n=10) was found to be bound at 4 °C which is slightly larger than the 85.6 % ± 0.8 % (n=6) observed at 35 °C (for a discussion, see section 3.4.2).

At 39 °C, only 17.5 % ± 3.5 % (n=2) of [^{3}H]procollagen was found to bind to SPM liposomes (see Figure 3.12). Procollagen would begin to melt at this temperature. However, this is not the cause of the poor binding since denatured procollagen was shown to interact with SPM (see Figure 3.12; also, see section 3.3.4.4).

3.3.4.3 Lipid-phase dependent interaction

The phase transition temperature of bovine brain SPM is about 41 °C (Untracht and Shipley, 1977). At 39 °C, one would expect the phase transition to have begun (for details of the phase transition, see section 3.4.4). The interaction of procollagen with SPM in only the gel-crystalline phase would explain the observation at 39 °C.

Since SPM has a relatively high transition temperature, SPM/ PC mixtures were prepared which enabled the phase dependence of the interaction to be studied at a much lower temperature. Increasing the mol % PC of a SPM/ PC mixture results in a

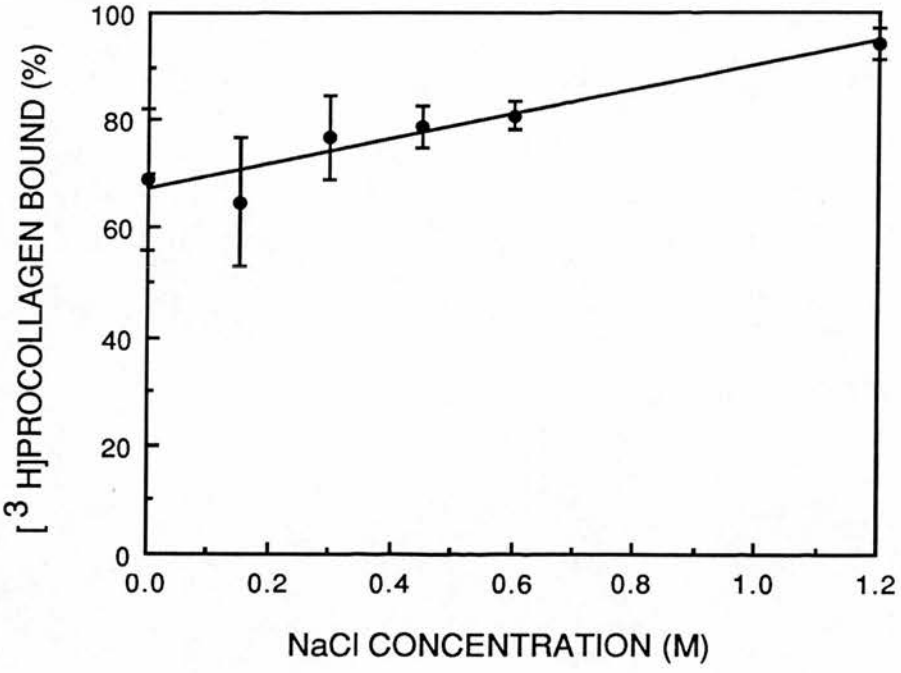


Figure 3.11 Effect of ionic strength on procollagen binding to SPM liposomes [^{3}H]procollagen (2 nM) was incubated with SPM liposomes (0.1 mM) at 35 °C in phosphate buffer in a range of NaCl concentrations, and the incubation mixtures were centrifuged in discontinuous sucrose gradients with the NaCl concentrations adjusted accordingly. Binding is expressed as the percentage of total recovered ^{3}H cpm in the top half of the gradient. Error bars represent the standard error of the mean (n=3).

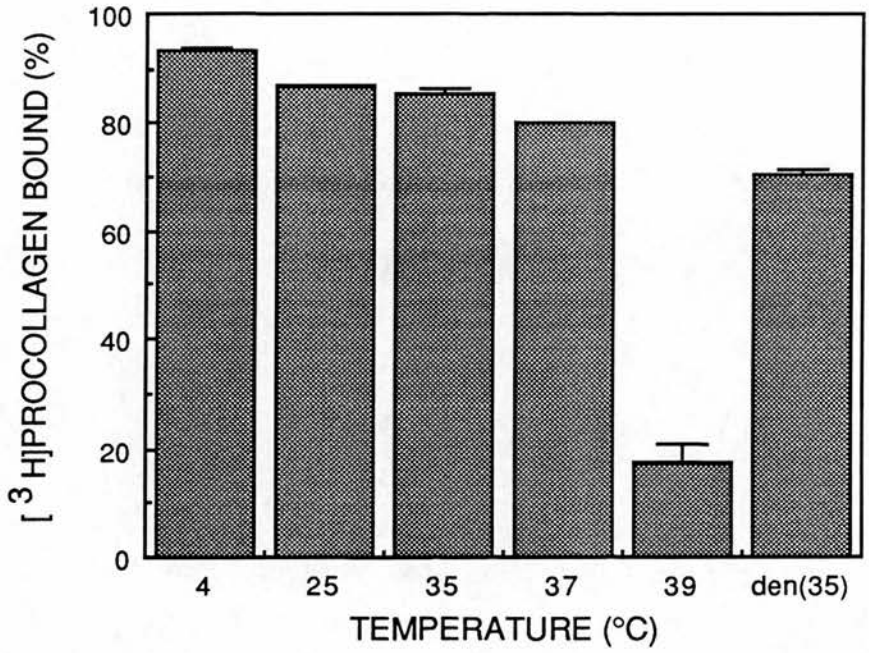


Figure 3.12 Effect of temperature on procollagen binding to SPM liposomes
Binding was assayed by discontinuous sucrose gradient centrifugation in standard assay conditions, and is expressed as the percentage of total ^{3}H in the top half of the gradient. The binding of denatured [^{3}H]procollagen at 35 °C is indicated by den(35). Error bars represent the standard error of the mean.

decrease in its phase transition temperature (Lentz *et al.*, 1981). The total concentration of lipid in each assay was 0.1 mM. The presence of small amounts of PC greatly reduced the amount of procollagen bound (see Figure 3.13). For example, only 14 % binding was observed when the lipid mixture contained 14 mol % PC at 35 °C. No interaction was observed up to a concentration of 25 nM procollagen with 14 mol % PC. The binding was restored when the temperature (4 °C) was reduced below the phase transition of the mixture. Therefore, procollagen appears to only interact with the gel-crystalline phase of SPM (for a discussion, see section 3.4.4.2). However, even at 4 °C, significant binding was only observed with mixtures containing less than 30 mol % PC (see Figure 3.14). The % procollagen bound was shown to decrease rapidly between 30 and 40 mol % PC (for a discussion, see section 3.4.4.5).

3.3.4.4 Other features of the interaction

Binding was unaffected by denaturation of the procollagen (by heating to 60 °C for 5 minutes) prior to incubation with SPM and density gradient centrifugation at 35 °C in standard buffer conditions (see Figure 3.12), when 70.5.% ± 0.9 % (n=3) of the denatured procollagen was found to bind to SPM (for further information, see section 3.3.5.1)..

SPM binding was also observed with ^{3}H-labelled type II procollagen (a generous gift from D.J.S. Hulmes). 85.1 % ± 2.3 % (n=4) of [^{3}H]type II procollagen (3 nM) was shown to interact with SPM (0.45 mM) at 35 °C in standard buffer conditions.

The interaction was abolished in the presence of both Tris (50 mM Tris-HCl pH 7.4, 0.15 M NaCl) and Hepes (20 mM Hepes pH 7.4, 0.15 M NaCl) at 35 °C. However, procollagen did interact at a much lower concentration of Tris (0.5 mM) present in the incubation mixture, and also in 0.2 M ammonium acetate.

3.3.5 Regions of procollagen involved in the interaction with SPM

In order to determine which region(s) of the procollagen molecule were important for interactions with SPM, binding of [^{3}H]type I procollagen was investigated in the

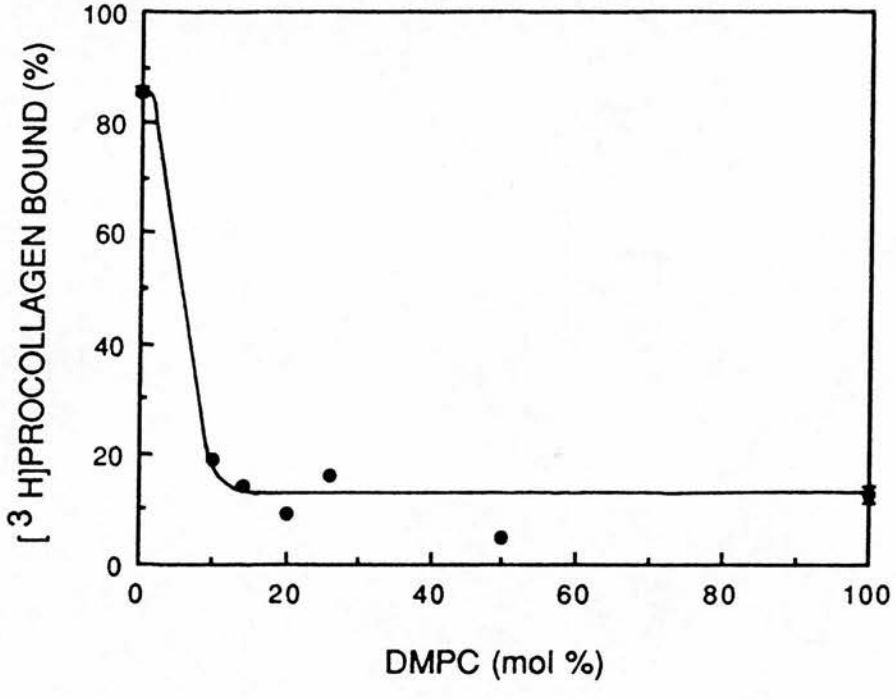


Figure 3.13 Binding of procollagen to liposomes made from mixtures of SPM and PC at 35 °C The percentage of ^{3}H cpm in the bound fractions was measured in standard assay buffer as a function of PC content using 2 nM [^{3}H]procollagen and 0.1 mM total lipid concentration in the incubation mixture. Errors are the standard error of the mean (n=2 or 3)

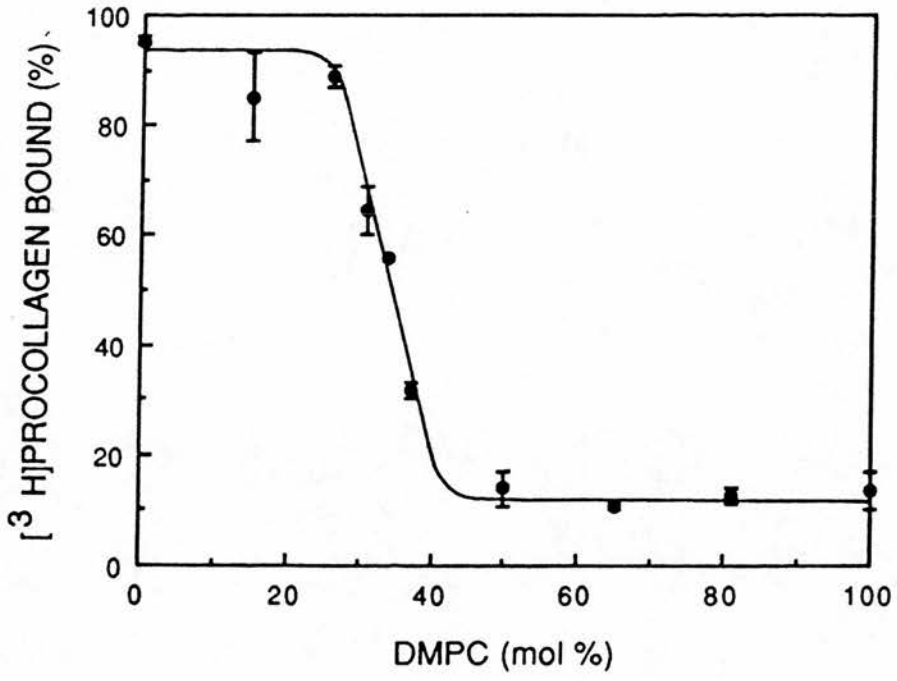


Figure 3.14 Binding of procollagen to liposomes made from mixtures of SPM and PC at 4 °C The percentage of ^3H cpm in the bound fractions was measured in standard assay buffer as a function of PC content using 2 nM [^3H]procollagen and 0.1 mM total lipid concentration in the incubation mixture. Errors are the standard error of the mean (n=2 or 3).

presence of unlabelled procollagen or procollagen domains (inhibitors). In each competitive inhibition binding assay, 50 µl of inhibitor was incubated with the standard [^{3}H]procollagen and SPM concentration in 40 % sucrose at 35 °C for 1 hour. The flotation-type binding assay was used to determine the % [^{3}H]procollagen bound.

As expected, unlabelled procollagen successfully competed for binding of [^{3}H]procollagen to SPM (see Figure 3.15). Only 27 % of the maximum [^{3}H]procollagen bound was observed at a 29 fold molar excess of unlabelled procollagen.

In contrast, no inhibition was observed in the presence of highly purified lathyritic rat type I collagen (a generous gift of Mr. J.R.E. MacBeth) in physiological conditions. At a 29 fold molar excess of unlabelled collagen, all the [^{3}H]procollagen remained bound to SPM. A temperature of 4 °C was used for this competitive inhibition binding assay to minimise collagen aggregation. The interaction was also unaffected in the presence of pepsin treated collagen (i.e. with shortened non-triple helical telopeptides) up to a molar excess of 30 fold. Only a slight inhibition (71 % bound) was observed in the presence of bovine serum albumin at a molar excess of up to 100 fold.

When unlabelled purified procollagen C-propeptides (a generous gift from I. Purdom; see Figure 3.3) were present in the competitive inhibition binding assay, inhibition was observed that appeared to be equivalent up to a 15 fold molar excess to the effect of added unlabelled procollagen. This suggests the possibility that type I procollagen binds to SPM via its C-propeptide, which was confirmed with the use of [^{125}I]C-propeptide (see section 3.3.5.1).

The presence of C-propeptide at a molar excess greater than 15 fold was shown to have little further effect on the inhibition of procollagen binding. At a 54 fold molar excess, 40 % of [^{3}H]procollagen remained bound to SPM. This suggests an additional weaker binding site exists on the procollagen molecule, remote from the C-propeptide. Although unlabelled collagen does not inhibit binding (up to a 40 fold molar excess), studies with [^{3}H]collagen do suggest a possible interaction (see section 3.3.5.2).

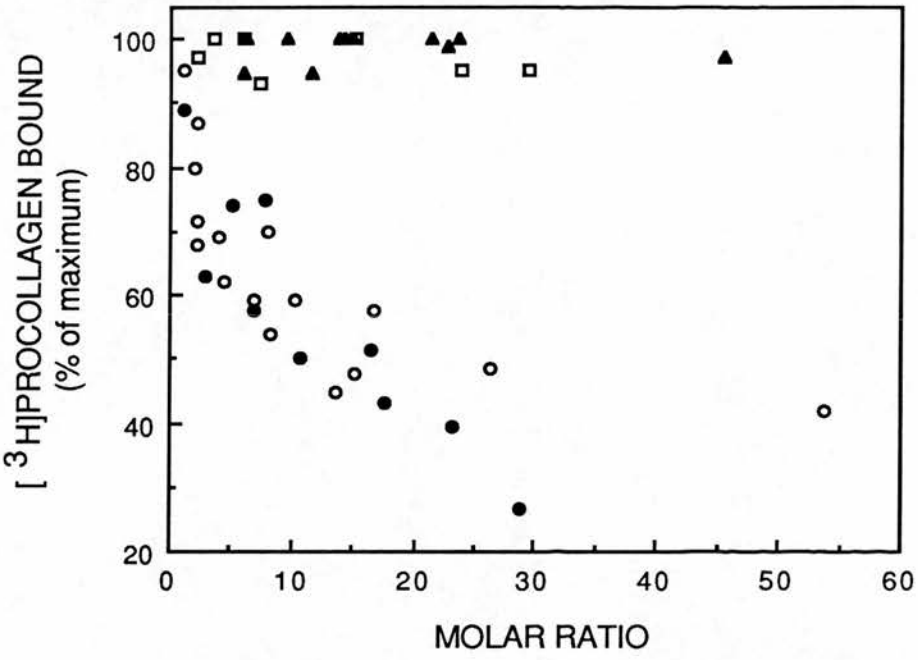


Figure 3.15 The effect of unlabelled procollagen and its various structural domains on the binding of [^{3}H]procollagen to SPM Binding was measured in standard assay conditions at 35 °C in the presence of added procollagen (●), collagen (▲), pepsinised collagen (□) or procollagen C-propeptides (○), and is expressed as the percentage of maximum binding in the absence of competitive inhibitors, with the molar ratio of inhibitor to [^{3}H]procollagen on the horizontal axis.

3.3.5.1 C-propeptides.

Procollagen C-propeptides were ^{125}I-iodinated (courtesy of Dr. D.K. Apps using the Iodogen method) to investigate its possible binding to SPM. The specific activity of the [^{125}]C-propeptide was 564,700 c.p.m/ μg. When incubated with SPM liposomes (0.1 mM) in standard buffer conditions, [^{125}I]C-propeptide was shown to bind in a saturable manner (see Figure 3.16)[9]. The apparent dissociation constant was determined (by Dr G L Atkins) to be 2.32 +/- 0.53 nM. Therefore, both the procollagen and C-propeptide molecules bind with similar affinity to SPM (for a discussion, see section 3.4.5.1). The number of binding sites for the C-propeptides was determined to be about 37 which is 5 fold greater than with procollagen (see section 3.3.4.1).

Binding was unaffected by denaturation of the procollagen (see Figure 3.12). One would usually conclude from such an observation that the interaction is not dependent upon the conformation of the molecule. However, interchain disulphide bridges exist within the C-propeptide, and therefore, the C-propeptide may remain intact whilst the rest of the procollagen is denatured. In a preliminary study, the C-propeptide was both denatured (by heating to 60 °C for 5 minutes) and reduced with (15 %) β-mercaptoethanol to determine whether its conformation was important for the interaction of procollagen with SPM. 14.1 % ± 0.7 % (n=2) of denatured/ reduced procollagen was found to be bound compared to 70.5 % ± 0.9 % (n=3) when unreduced, while 50 % of native/ reduced procollagen was found to interact with SPM. These observations suggest that the conformation of the C-propeptide is important for the interaction (see discussion).

3.3.5.2 Collagen domain

The lack of inhibition of [^{3}H]procollagen binding to SPM by unlabelled collagen suggested that the binding mechanism was independent of the mature collagen domain.

[9] Due to the short half-life of the [^{125}I]label, the binding studies were carried out soon after iodination of the C-propeptide.

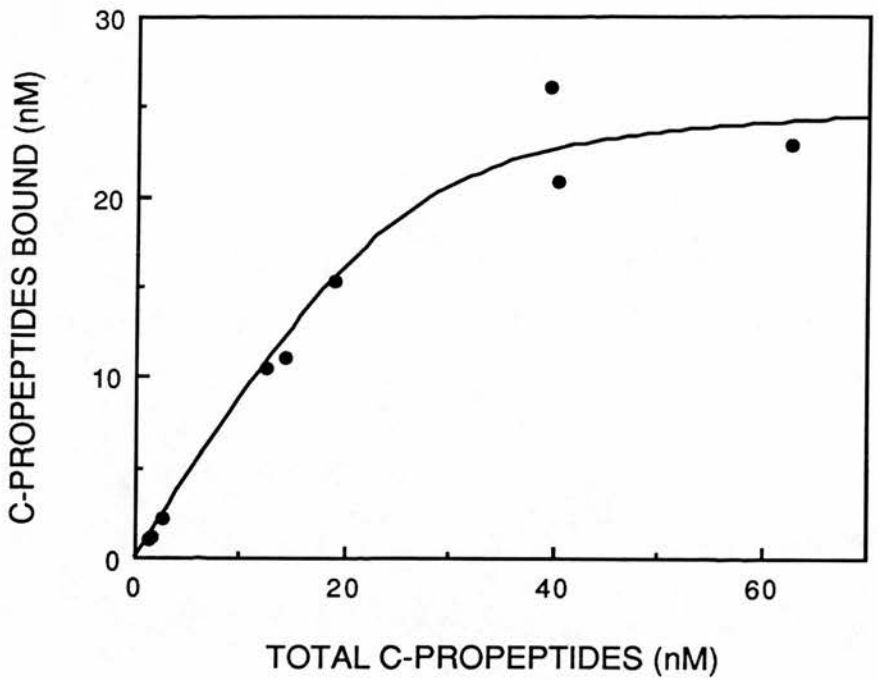


Figure 3.16 Concentration dependence of the binding of [^{125}I]procollagen C-propeptides to SPM liposomes Increasing concentrations of [^{125}I]C-propeptides were incubated with 0.1 mM SPM and the extent of binding was determined by discontinuous sucrose gradient centrifugation. The curve shows the non-linear least squares fit to the data using a dissociation constant (K_d) of 2.32 nM and a saturation limit of 25.5 nM.

To investigate this, [^{3}H]collagen was prepared by the pepsinisation of [^{3}H]procollagen (see section 2.5). The binding of [^{3}H]collagen to SPM was studied in standard assay buffer at 4 °C (to minimise collagen aggregation).

At a [^{3}H]collagen concentration of about 2 nM (standard concentration)[10] 29.1 % ± 0.7 % (n=4) was found to interact with SPM at 4 °C. Thus, there was limited binding of the collagen domain to SPM, approximately 3-fold less than that for intact procollagen in equivalent conditions.

The weak interaction of [^{3}H]collagen with SPM may account for the lack of inhibition of [^{3}H]procollagen by unlabelled collagen. The presence of different binding sites for the collagen domain and the C-propeptide may also explain this lack of inhibition assuming procollagen binds mainly via the C-propeptide (for a discussion, see section 3.4.5.2).

3.3.6 The interaction of procollagen with PC

In physiological conditions of pH, ionic strength and temperature, there was no evidence of procollagen binding to PC (see Figure 3.5).

3.3.6.1 Interaction in 0.1 M acetic acid

Martinez del Pozo *et al* (1988) demonstrated the interaction of collagen with both DMPC and DPPC in 0.1 M acetic acid using a sedimentation-type binding assay (see section 3.10). The interaction of [^{3}H]procollagen with PC and SPM was also studied in 0.1 M acetic acid. No interaction was observed when procollagen was incubated with PC (0.1 mM) at 4 °C. Only 10.7 % ± 0.9 % (n=2) of the procollagen recovered from the gradient was found in the bound fractions. In contrast, 90 % of the procollagen was bound to SPM in 0.1 M acetic acid.

[10] The specific activity of pepsinised collagen was determined from that for procollagen using a correction factor that had taken into account the % of proline and hydroxyproline residues in the N- and C-propeptides (12.5 %).

3.3.6.2 Interaction at neutral pH

Procollagen was shown to interact with PC (standard concentrations) in 10 mM phosphate buffer, pH 7.4, only in the absence of salt, where 54 % ± 8 % (n=4) of procollagen was observed in the bound fractions. Since no binding was observed in the presence of salt, this suggests an electrostatic nature to the interaction (for further discussion, see section 3.4.7).

3.3.7 Rotary shadowing

Electron microscopy after rotary shadowing was used to visualise procollagen molecules in the presence of SPM and PC.

3.3.7.1 Appearance of procollagen

The appearance of procollagen monomers deposited on to the mica using the mica-sandwich technique, and prepared by glycerol drying is shown in Figure 3.17. The procollagen molecules were similar to those prepared by freeze drying from 0.2 M ammonium acetate. The collagen triple helix appeared as a central thread-like domain approximately 290 nm in length[11]. The C-propeptide appeared as a globular structure about 10 nm in diameter. A kink was sometimes observed at the junction of the N-propeptide (about 15 nm in length) and the triple helix (see Figure 3.17).

Approximately 69 % (n=177) of the procollagen at a concentration of 25 nM remained in the monomeric form. Of the large % of aggregates, some had no clearly defined structure. However, aggregates with common characteristic features were observed. In-register dimers, both separate and part of larger aggregates were frequently observed (see Figure 3.18). Dimers of procollagen molecules bound via their ends (i.e. end to end dimers) and larger aggregates also consisting of molecules bound via their ends (similar to those described by Mould and Hulmes, 1987) were

[11] The dimensions of the molecules are increased by the shadowing metal.

Figure 3.17 Procollagen monomers prepared by glycerol-drying The collagen triple helix appears as a central thread-like domain approximately 290 nm in length. The C-propeptide is visualised as a globular structure about 10 nm in diameter. The junction between the N-propeptide and the collagen triple helix is sometimes defined by a sharp kink (arrow). Magnification 112,500 x.

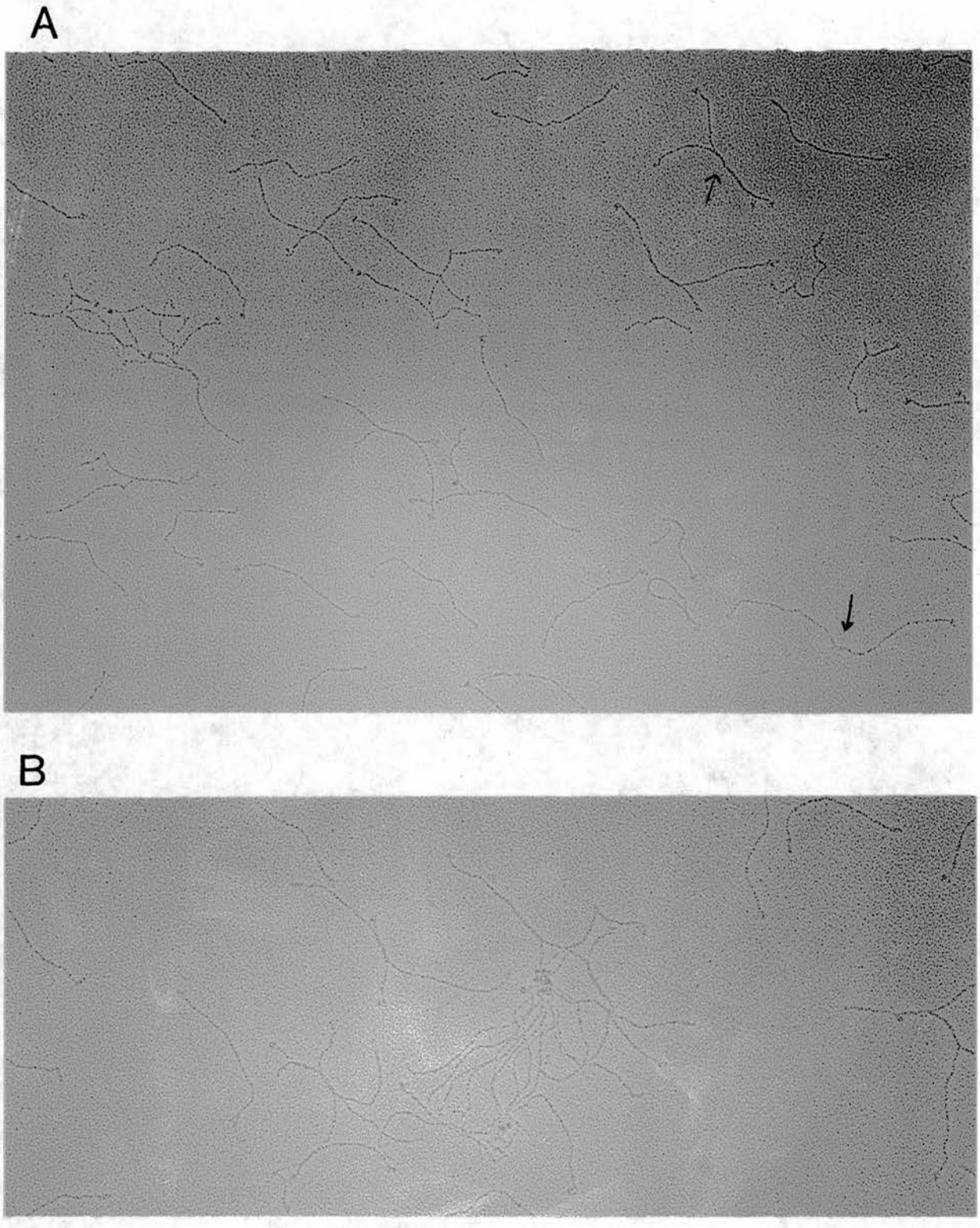


Figure **3.18** Procollagen aggregates (A) In register dimers (small arrow) and dimers of procollagen molecules bound via their ends (large arrow) are observed. Magnification 87,500 x. (B) Spider-like aggregates of procollagen are also visualised. Magnification 112,500 x.

also observed (see Figure 3.18).

3.3.7.2 Sphingomyelin

Rotary shadowed SPM liposomes prepared by freeze drying are shown in Figure 3.19. A 'stacking' effect is observed within most liposomes. This is possibly caused by the collapsing of the liposomes during drying of the specimen. Large aggregates of SPM were also observed, particularly when specimens were deposited with the mica-sandwich technique.

3.3.7.3 Procollagen-lipid interaction

Both freeze drying (mica sandwich technique) and spraying on to pre-cooled mica were used to prepare specimens to investigate procollagen-lipid interactions. Rotary shadowing in the presence of glycerol[12] was found to be inappropriate since binding studies showed it interfered with the procollagen-SPM interaction.

Procollagen was incubated with SPM (standard concentrations) in 0.2 M ammonium acetate at 35 °C for 1 hour. The interaction was not affected by ammonium acetate (see section 3.3.4.4). The procollagen-SPM complex was visualised using rotary shadowing (see Figure 3.20). In most cases, procollagen was observed to interact with SPM via its ends (for comparison with binding studies, see section 3.3.4.1). However, procollagen molecules interacting with the triple helical domain would be difficult to observe using this technique. In most cases, no more than 2 molecules were observed interacting with the SPM molecules. However, in a few exceptions, 3 to 4 molecules were found to bind to SPM (see Figure 3.20). At the concentration of procollagen and SPM used in this study, one would have expected most of the procollagen to have been bound to SPM (see section 3.6.1.1). However, a large number of unbound procollagen molecules and free liposomes were observed. Quantitation was made difficult because of the disruptiveness of the technique.

[12] Glycerol drying was used in the preparation of specimens to investigate the interaction of dextran sulphate with procollagen (see section 4.2.1.2).

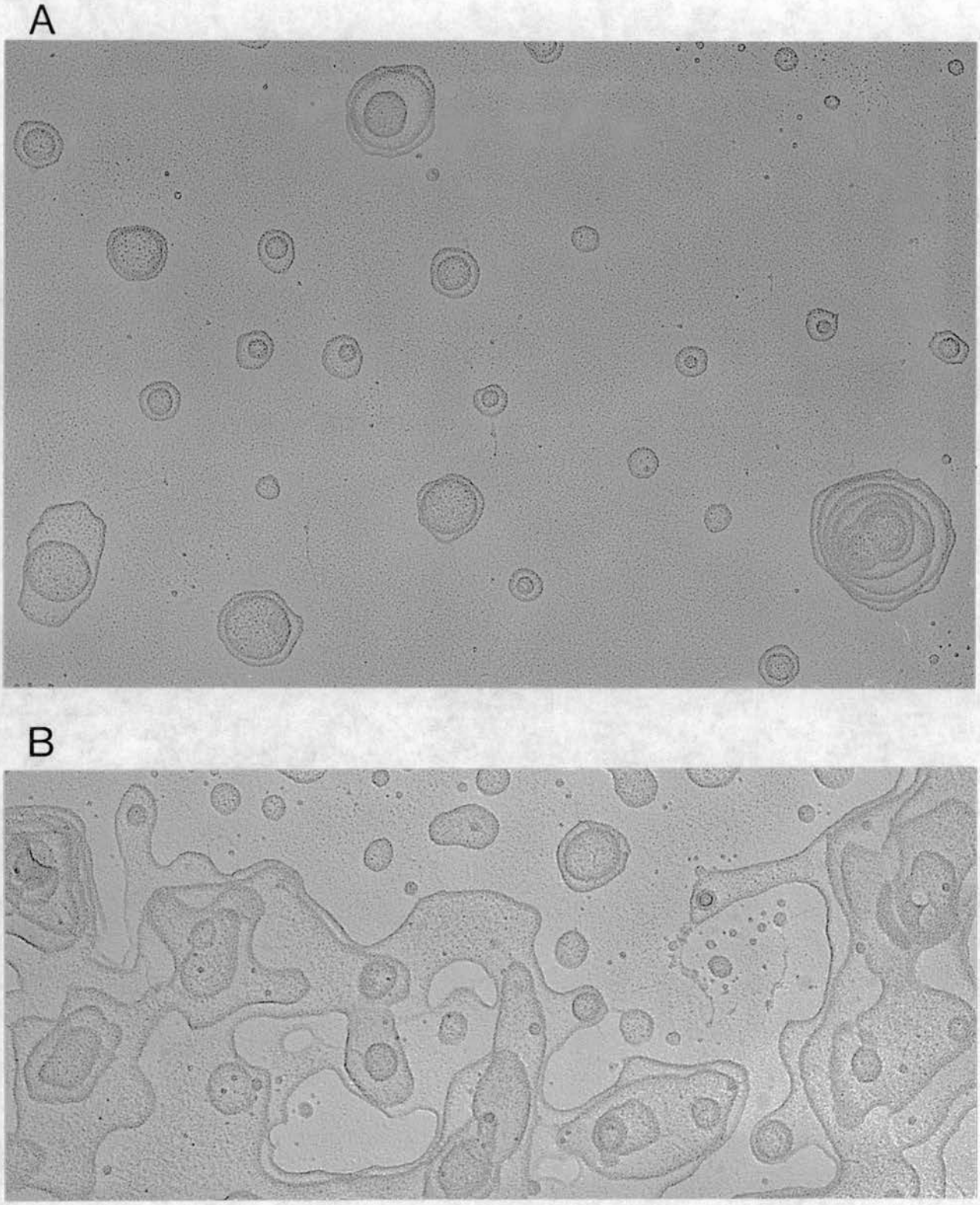


Figure 3.19 Rotary shadowed SPM liposomes prepared by freeze-drying (A) A stacking effect is visualised within most SPM liposomes. Magnification 87,500 x. (B) Large aggregates of SPM are also observed. Magnification 112,500 x.

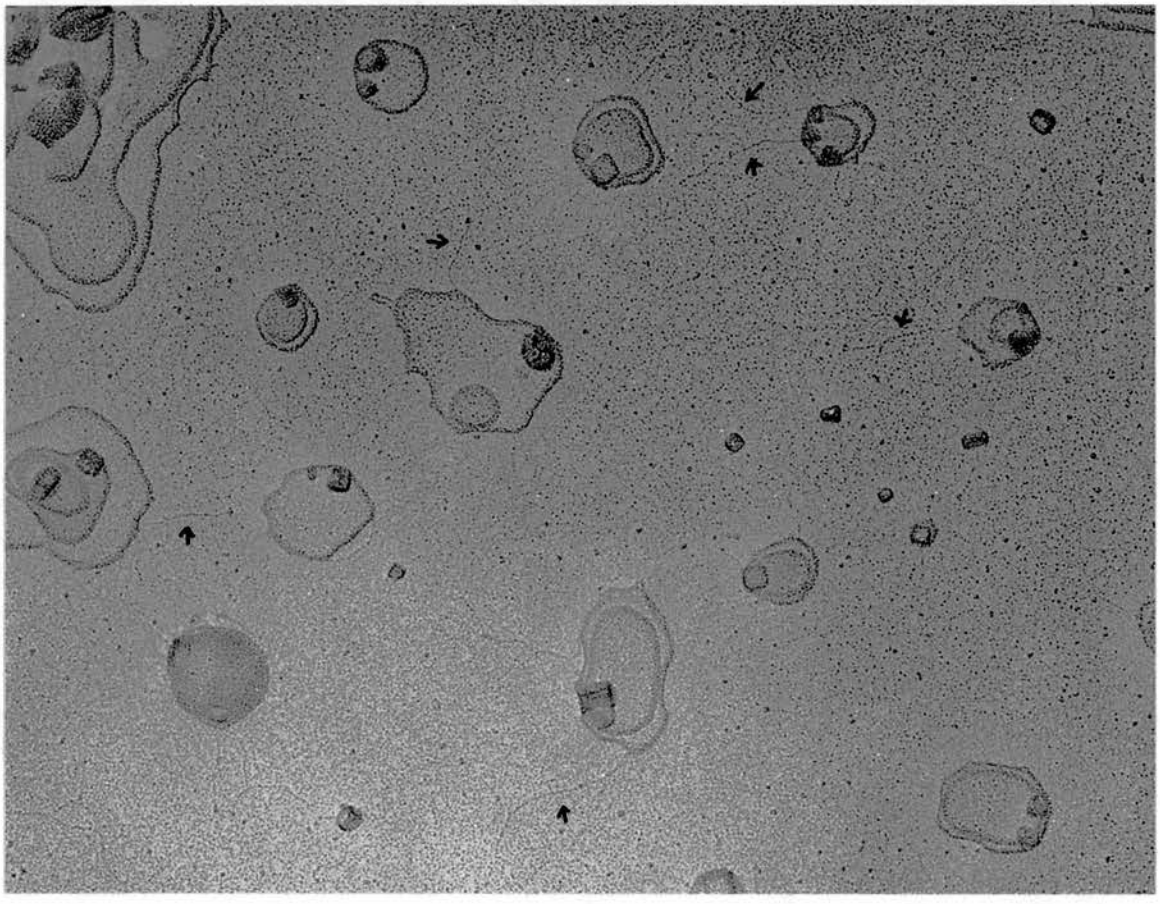


Figure 3.20 Procollagen -SPM complex prepared by freeze-drying Procollagen was incubated with SPM in 0.2 M ammonium acetate at 35 °C for 1 hour and deposited on to mica with the mica-sandwich technique. Procollagen molecules are indicated by arrows. Magnification 87,500 x

Procollagen was incubated with PC (standard concentrations) in 0.2 M ammonium acetate, 0.15 M NaCl for 1 hour at 35 °C. Density gradient binding studies suggest procollagen did not interact with DMPC in 0.15 M NaCl. The specimen was deposited on to the mica using the mica sandwich technique. The salt was removed by washing the mica-adsorbed specimen before freeze drying. No obvious signs of an interaction between procollagen and DMPC was observed.

3.3.8 Surface induced aggregation

Procollagen that had been incubated with SPM liposomes (standard concentrations) was cross-linked with glutaraldehyde (for the method, see section 2.5) and loaded on to a gel filtration column (Sephacryl S500; see section 2.6) to determine whether the liposomes induced aggregation. 5 % Brij was added to the cross-linked procollagen-SPM mixture the disperse the liposomes.

In the presence of liposomes, most of the recovered [^{3}H]procollagen was detected in a single peak centred about fractions 40-42 (V_e=33 ml; see Figure 3.21). In the absence of SPM liposomes, most of procollagen (standard concentration) was detected in a similar position to the above (V_e=34 ml; see Figure 3.22). This peak was assumed to represent monomeric procollagen since the solubility limit of procollagen (1-1.5 mg/ml; Mould *et al.*, 1990) is far greater than the concentration used in this study. The results suggest SPM liposomes do not induce procollagen to aggregate (see section 3.4.10).

3.4 Discussion

3.4.1 Procollagen-SPM interaction

At physiological conditions of pH, ionic strength and temperature, there was no evidence of procollagen binding to PC, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol. In contrast, type I procollagen bound strongly to SPM in a

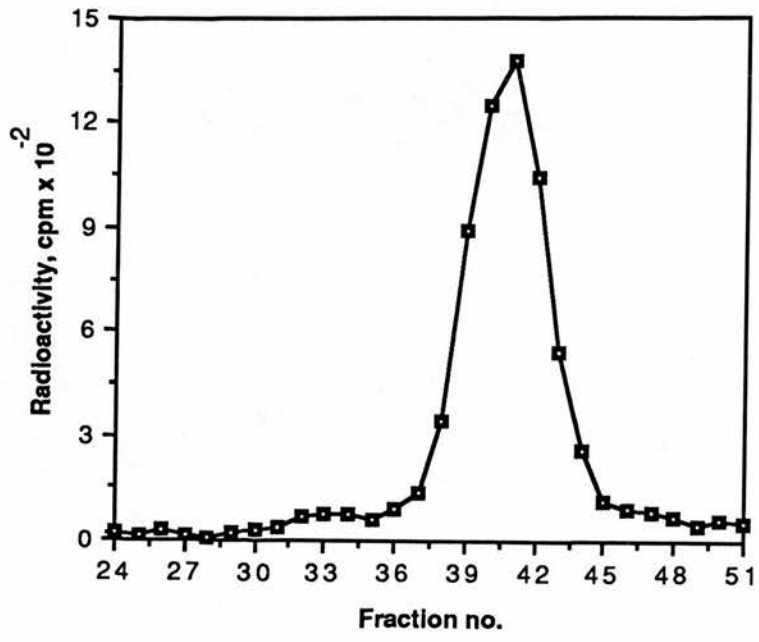


Figure 3.21 SPM liposomes do not induce procollagen to aggregate

Gel filtration chromatography of [^{3}H]procollagen that had been incubated with SPM liposomes at 35 °C, and then fixed with 1 % glutaraldehyde.

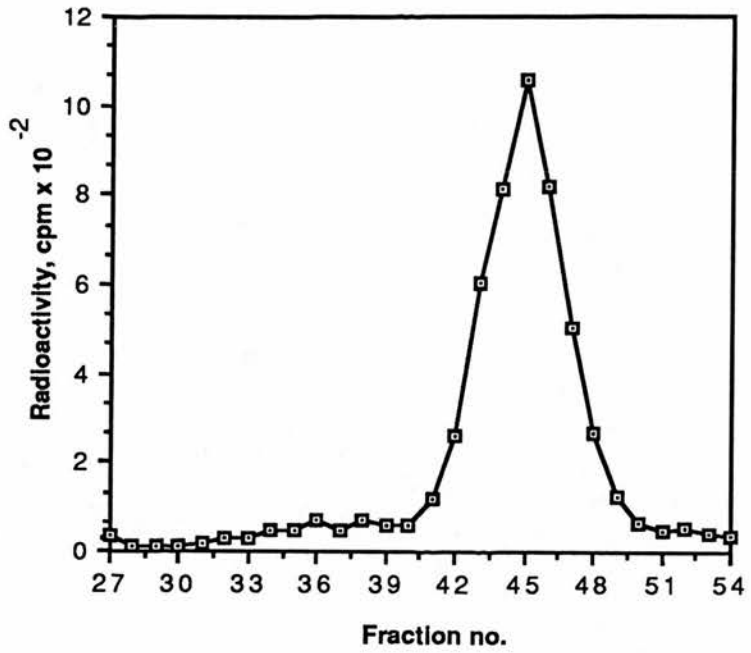


Figure 3.22 Gel filtration chromatography of procollagen on Sephacryl S500

Procollagen was fixed with 1 % glutaraldehyde before being loaded onto the column. The running buffer was 10 mM sodium phosphate buffer, pH 7.4/ 0.15 M NaCl/ 0.5 % Brij 35·

saturable manner. The procollagen was in equilibrium with the SPM-procollagen complex. Binding analysis gave an apparent dissociation constant of 2.6 nM. This procollagen concentration is probably much lower than one would expect in the pericellular space or in secretory vesicles[13] Therefore, a significant interaction between procollagen and SPM is possible *in vivo*.

All forms of acetylcholineserase having a collagen-like tail also bind to SPM liposomes with almost no binding to PC (Watkins *et al.*, 1977; Cohen and Barenholz, 1984). Non-collagenous proteins such as 5'-nucleotidase and the hemolytic toxin of sea anemone also bind specifically to SPM (for details, see section 3.4.6).

3.4.2 Nature of the interaction

A high ionic strength (1.0 M NaCl) only slightly diminished the interaction of acetylcholinesterase with SPM (Watkins *et al.*, 1977). Similarly, most of the procollagen remained bound to SPM up to a salt concentration of 1.2 M, which suggests the interaction is not electrostatic in nature. In fact, the % total procollagen bound increases, although slightly, as the salt concentration is increased. This suggests the possible involvement of a hydrophobic interaction (see below).

The change in enthalpy (ΔH) for a hydrophobic interaction is positive, and therefore, one would expect the % of total procollagen bound to decrease as the temperature is lowered. However, the amount of procollagen bound at 4 °C was similar to that at 35 °C, which suggests there is no obviuos hydrophobic interaction involved.

Hydrophobic interactions have been suggested to be responsible for the interaction of the collagen-like protein SP-A with 85 % DPPC/ 15 % DPPG (King *et al.*, 1983). It also been suggested to be the principal mode of interaction between insulin and DMPC on the sole basis that the amount of insulin bound was unaffected by increasing the ionic strength up to 1.58 M NaCl (Wiessner and Hwang, 1982). However, this suggestion appears to be incorrect, since the same workers show that the amount of bound insulin increases as the temperature is decreased (from 20 to 10 °C) within the

[13] Immunocytochemical studies suggest a very high concentration of procollagen in both secretory vesicles (Trelstad and Hayashi, 1979) and in the pericellular space (Phelps *et al.*, 1985).

gel crystalline state.

3.4.3 Involvement of hydrogen bonds

Cohen and Barenholz (1984) suggested that the interaction between acetylcholinesterase and SPM involves hydrogen bonds between the collagen domain and the interface region of SPM. The interface region in SPM contains an hydroxyl group and an amide bond which are good candidates for hydrogen bonding (see Figure 3.23). Hydrogen bonding involving these groups can be partially stabilised by the relatively low dielectric constant of the SPM interface region (Barenholz and Thompson, 1980). The hydrogen bond donor capability is not found in the interface region of PC (see Figure 3.23).

The major region of procollagen that interacts with SPM is the C-propeptide (for detailed discussion, see section 3.4.5.1). The C-propeptide contains a relatively high content of acidic amino acid residues (potential hydrogen bond acceptors). Since procollagen binds specifically to SPM, and the interaction does not appear to be electrostatic or hydrophobic in nature, it is likely that it involves hydrogen bonding. The hydrogen bonds may form between the hydrogen bond donors in the interface region and the procollagen C-propeptide (for further details, see section 3.4.4.3).

3.4.4 Properties of SPM

3.4.4.1 Lipid crystalline phases

Phospholipids can exist in two very distinct physical states. Below the phase transition temperature, the lipids exist in the gel crystalline state. The acyl chains of the lipids are fully extended with the C-C bonds in the all trans postion. In the gel state of PC and SPM, the acyl chains are tilted to the normal plane of the bilayer[14] (Barenholz and Thompson, 1980; New, 1990).The molecules do not undergo lateral diffusion but pack together in a quasi-hexagonal array. The presence in SPM of the amide linkage

[14] SPM is tilted to a far greater degree than PC, see Figure 3.24

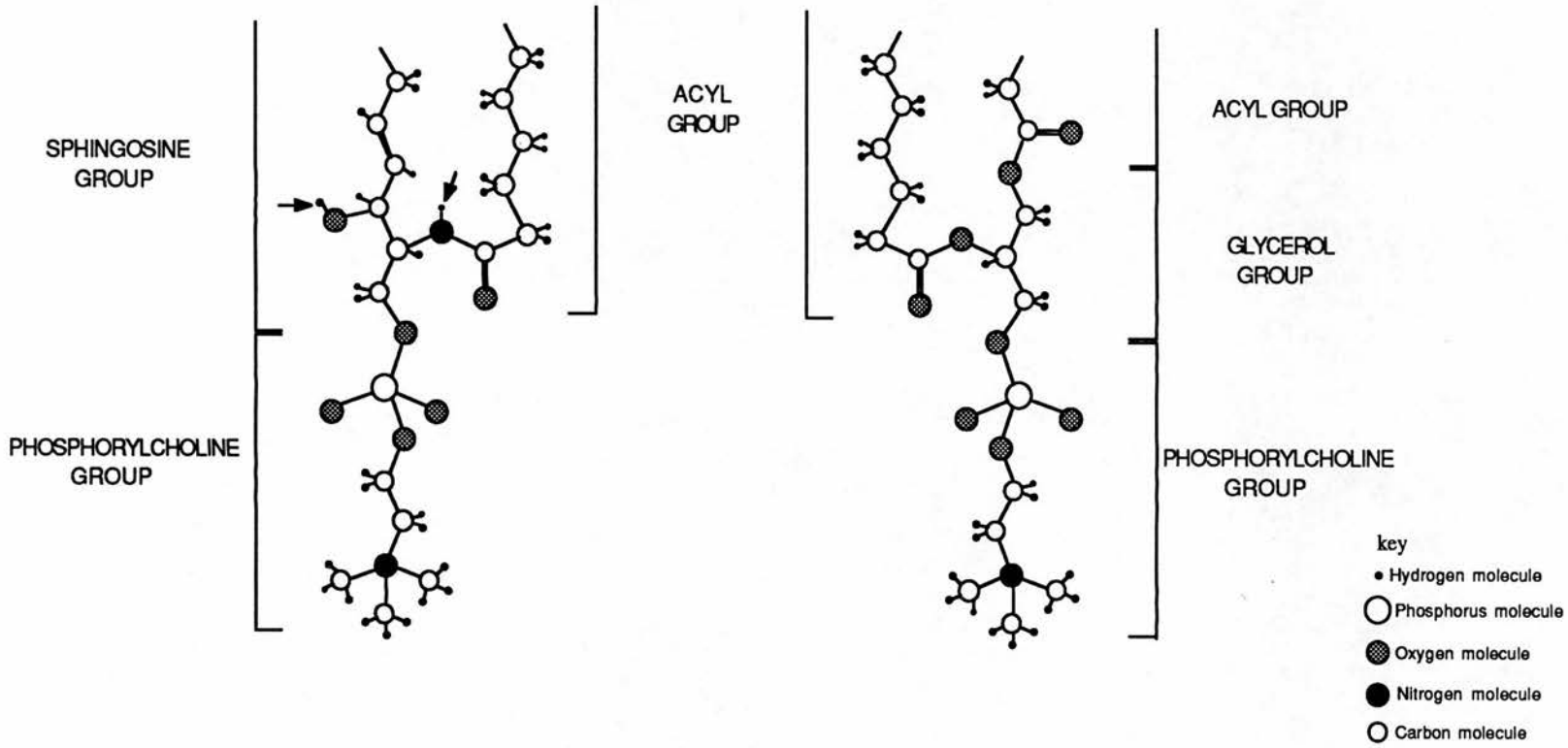


Figure 3.23 A comparison of the structure of the interfacial region of sphingomyelin (left) with that of phosphatidylcholine (right). Arrows indicate good candidates for hydrogen bonding (adapted from Barenholz and Thompson, 1980).

and hydroxyl group in the interfacial region can give rise to hydrogen bond interactions with the phosphate group. This may explain the highly ordered gel phase in SPM compared to PC (see section 3.4.4.5).

Above the phase transition temperature, the molecules are able to undergo fast lateral and rotational diffusion and also, the headgroup region is also relatively mobile. Rotational isomers about the C-C bond form leading to kink formation. The lipid phase transition is a highly cooperative event occurring over a few degrees centrigade.

3.4.4.2 Gel-crystalline phase dependent interaction

Insulin only bound to DMPC below the phase transition of the lipid (Wiessner and Hwang, 1982). These workers suggested that the packing structure of the phospholipid molecules on the surface of the liposomes may play an important role in the binding of insulin. The binding of SP-A to 85 %DPPC/ 15 % DPPG was also considerably reduced when the temperature of the interaction was above the phase transition temperature (King *et al.*, 1983).

The lack of procollagen binding with SPM at 39 °C suggested the interaction preferred SPM in the gel crystalline state[15]. SPM has a relatively high phase transition temperature (about 41 °C)[16]. Therefore, SPM/ PC mixtures were prepared which enabled the phase dependence of the interaction to be studied at a much lower temperature. This is because an increase in the mol % PC of a SPM/ PC mixture results in a decrease in its phase transition temperature (Lentz *et al.*, 1981). At 35 °C, there was little procollagen bound when the lipid mixture contained 14 mol % PC. However, binding was restored when the temperature was reduced to 4 °C. Therefore, procollagen only binds to SPM in the gel-crystalline phase.

3.4.4.3 Involvement of hydrogen bonds

The interaction has been proposed to involve hydrogen bonds (see section 3.4.3).

[15] Denatured procollagen interacts with SPM (see section 3.3.5.1).

[16] Natural PC's have a high content of unsaturated fatty acyl chains and have phase transitions well below 37 °C.

The geometric arrangement of the hydrogen bond donor and acceptor is very important, where, optimal hydrogen bonds have a linear geometry. The orientation of the SPM molecules in the closely packed structure of the gel crystalline state may favour the formation of hydrogen bonds with procollagen. Because the molecule is tilted in the bilayer (see Figure 3.24), the sphingosine group will be approximately perpendicular to the plane of the membrane. In the liquid crystalline state, the acyl chains are perpendicular to the bilayer and therefore, the sphingosine group will be in a different orientation. The different orientation of the SPM molecule, and the fast axial rotation and lateral diffusion of the lipid may partly explain the disruption of the interaction in the liquid crystalline phase.

Cohen and Barenholz (1984) showed that acetylcholinesterase binds to SPM at 37 °C and 4 °C to the same extent. The workers concluded from this observation that the interaction was unaffected by the physical state of the lipid. However, 37 °C may be below the phase transition range of SPM[17]. The temperature (higher limit) of their study was limited by the thermal inactivation of acetylcholinesterase. The interaction between the enzyme and SPM may well be phase dependent.

3.4.4.4 SPM domains

At 4 °C, the interaction of procollagen with the SPM/ PC mixture containing concentrations of DMPC greater than 40 mol % was substantially reduced. The interaction of acetylcholinesterase with 33 mol % PC (at 4 °C) was also considerably reduced (Cohen and Barenholz, 1984). At 4 °C, SPM molecules will form a tightly packed crystalline structure. The above result may be explained by a disruption of this closely packed structure by the presence of PC (see below). This would suggest the packing structure is as important as the orientation of the molecule for an interaction to occur.

SPM has a more highly ordered gel phase compared to PC (see section 3.4.4.1). In over 50 % of naturally occurring SPM[18] molecules the two hydrocarbon chains

[17] Procollagen binds to SPM at 37 °C.

[18] The acyl chain composition of the bovine brain SPM (Avanti) was not available.

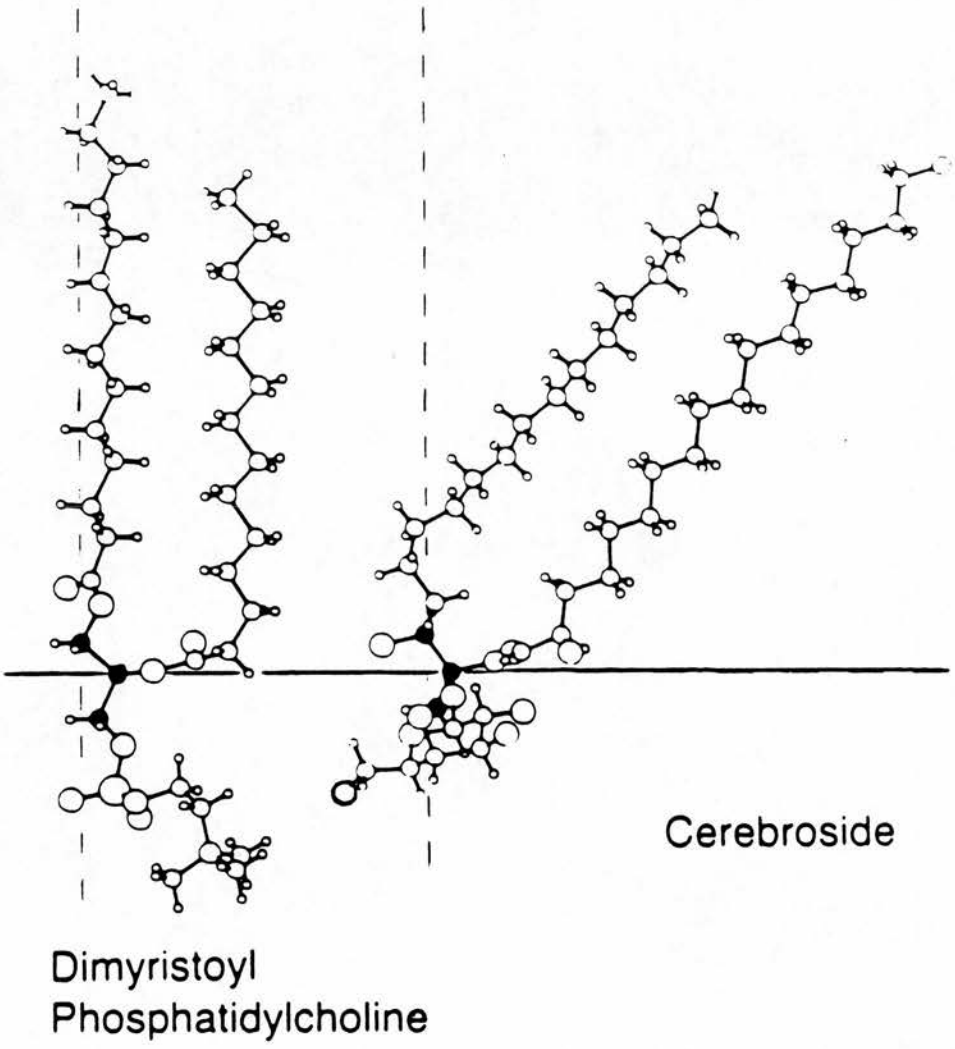


Figure 3.24 The orientation of lipids in the bilayer

Cerebrosides (including SPM) and to a lesser extent dimyristoylphosphatidylcholine are tilted in the bilayer. The glycerol and sphingosine backbone are represented by filled circles. The dashed line is perpendicular to the bilayer (adapted from Gennis, 1989).

comprising the hydrophobic region differ in length by more than 7 methylene residues (for details, see Barenholz and Thompson, 1980). Therefore, one would expect the presence of DMPC to disrupt the packing of such molecules.

The different interaction between SPM molecules to those between SPM and PC (Barenholz and Thompson, 1980) also results in an asymmetric distribution of the lipids in the mixture. For a 1:1 molar ratio of SPM and DPPC, the SPM is more concentrated in the outer leaflet and the PC in the inner leaflet (Barenholz and Thompson, 1984). Therefore, the actual concentration of PC in the outer leaflet of the SPM/ PC mixture required to abolish the interaction may be less than 40 mol %.

A large % of SPM molecules appears to be required for the interaction to procollagen. However, the % of SPM in plasma membranes varies between only 10-20 % depending on the species and cell type[19]. Watkins *et al* (1977) suggested the relatively strong interactions between SPM molecules may lead to the formation of domains in biological membranes. This provides a means by which acetylcholinesterase may specifically interact with the plasma membrane of Electrophorus electricus, where only 5 % of the total lipid is SPM. SPM mixtures isolated from mammalian tissues are unique amongst the cell's phospholipid components in undergoing a phase separation over a temperature range that includes 37 °C (Lentz *et al.*, 1981). Therefore, cell membranes may well contain domains of SPM in the gel phase (Rintoul *et al.*, 1979).

3.4.4.5 Other features of the interaction

At 30 °C, N-palmitoylsphingosine phosphorylcholine (C_{16}SPH) undergoes a transition between the gel phases analogous to the pretransition observed with PC (Lentz *et al.*, 1981). Such a pretransition in bovine brain SPM liposomes may explain the larger amount of procollagen bound at 35 °C compared to 4 °C.

From analysis of the binding, about 7 molecules of procollagen are bound per SPM liposome. In the calculation of this value, the liposomes were assumed to be unilamellar. However, electron microscopy after negative staining shows the presence of some large multilamellar vesicles, containing at least 4 bilayers (see Figure 3.25).

[19] Though about 35 % of total lipid in matrix vesicles is represented by SPM (see section 3.4.9.3).

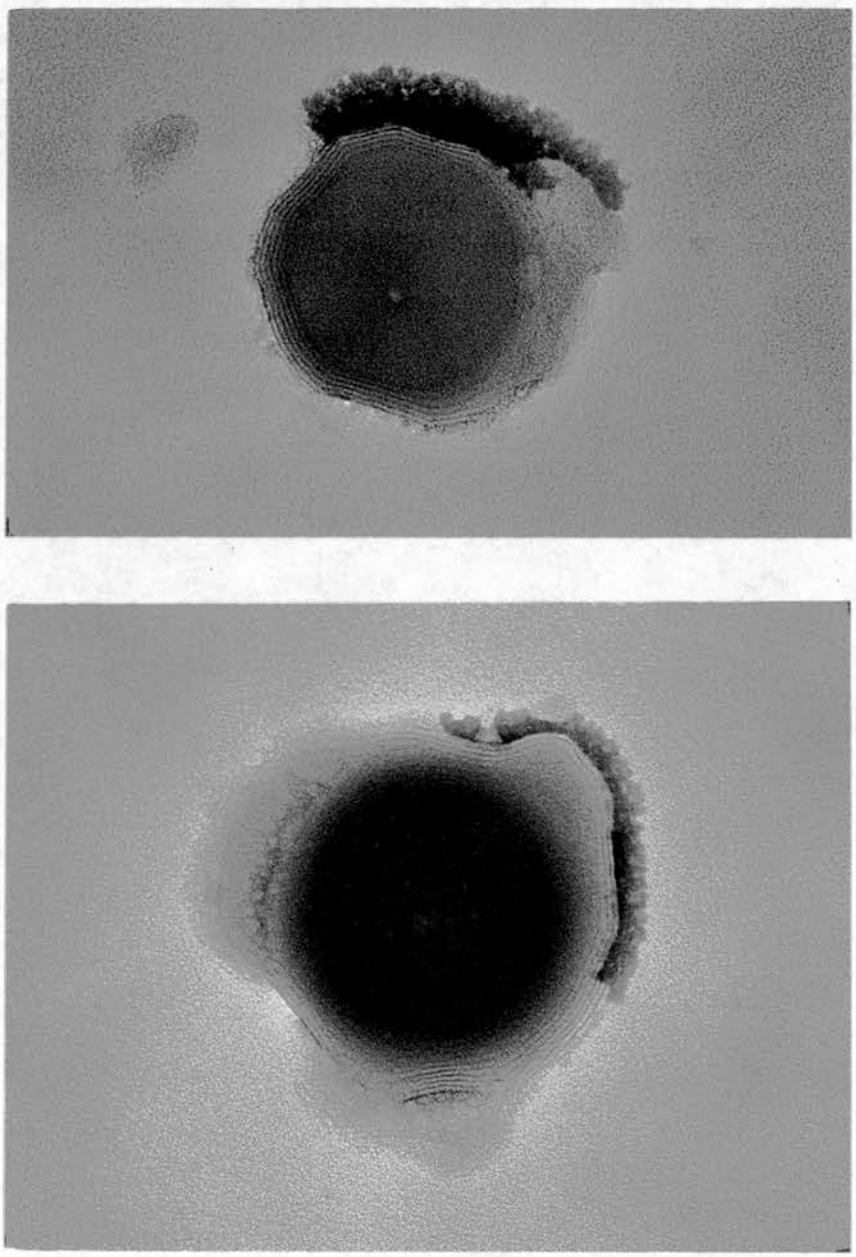


Figure 3.25 Negatively stained SPM liposomes. Magnification 150,000 x.

Therefore, more than 7 procollagen molecules may be bound per SPM liposome.

3.4.5 Region of procollagen involved with the interaction

3.4.5.1 C-propeptide

From the competitive inhibition study (section 3.3.5.1.) it appears that the C-propeptide[20] interacts with SPM. Up to a 15 fold molar excess, the inhibition observed with unlabelled C-propeptide was equivalent to the effect of added unlabelled procollagen. However, there was little further inhibition by the unlabelled C-propeptide at a molar excess greater than 15 fold. In contrast, unlabelled procollagen continued to inhibit [^{3}H]procollagen binding to SPM. This suggets that another binding site exists on the SPM molecule (see section 3.3.5.2).

[^{125}I]C-propeptide bound to SPM in a saturable manner. From Scatchard analysis the number of C-propeptide molecules bound per SPM liposome was determined to be 37. This is 5 fold grester than the number of procollagen molecules bound to SPM. Fewer procollagen molecules may be bound due to steric hinderance effects caused by its collagen domain.

From analysis of the binding, the dissociation constant was determined to be 2.3 nM, which is similar to that for procollagen. Studies where procollagen was both denatured and reduced suggest the conformation of the C-propeptide is important for the interaction (see section 3.3.5.1). A structure for the C-propeptide has not yet been determined. Another possible explanation may be that the the combination of another region on the procollagen molecule with the C-propeptide may be required for the interaction.

No interaction was observed with collagenase-treated acetylcholinesterase and SPM (Cohen and Barenholz, 1984). They concluded from this observation that the collagen domain was involved in the interaction, however, only a weak interaction between [^{14}C]collagen and SPM was found. These results can also suggest that the globular region at the end of the collagen domain may be important for the interaction

[20] For a description of the primary sequence of the C-propeptide see section 1.4.2.2

with SPM (for details of the primary structure of acetylcholinesterase, see Krejci *et al.*, 1991). Cysteine residues exist within this globular region, and therefore it may function in a similar manner to the disulphide bonded C-propeptide as the nucleation site of triple helix formation (see section 1.5.3). A globular domain also exists at the end of the collagenous domain of SP-A. No sequence similarities between the globular domain of acetylcholinesterase, SP-A and the C-propeptide have been found (preliminary study by D.J.S. Hulmes).

Type II procollagen also bound to SPM. The region of the molecule involved in the interaction was not determined. There are features of structural conservation within the fibrillar collagen genes coding for the C-propeptide (see section 1.3.1). The type II procollagen C-propeptide may well be involved in the interaction with SPM (for the implications, see section 3.4.9.3).

3.4.5.2 Collagen domain

Only 30 % of [^{3}H]collagen was bound to SPM at 4 °C. In similar conditions, Cohen and Barenholz (1984) also found only a slight interaction between [^{14}C]collagen and SPM. The lack of inhibition of [^{3}H]procollagen by unlabelled collagen can be partly explained by this weak interaction. Since procollagen binds mainly with the C-propeptide, the presence of a different binding site for the collagen domain would also explain this lack of inhibition.

To determine whether a separate site exists for the collagen domain, the binding of [^{3}H]collagen to SPM should be investigated in the presence of unlabelled C-propeptide. A lack of an inhibition by the unlabelled C-propeptide would confirm the presence of a separate binding site.

The binding may involve an electrostatic interaction between the dipoles surrounding the collagen domain and the phosphorylcholine headgroup of SPM. Such a mechanism has been proposed for the interaction of collagen with PC (for more discussion, see section 3.4.7).

3.4.6 Protein-SPM interactions

Proteins other than acetylcholinesterase and procollagen have been shown to bind specifically to SPM. The 5'-nucleotidase from rat liver plasma membrane preferentially binds to SPM (Widnell and Unkeles, 1968). Rabbit kidney [Na^+-K^+]ATPase and the hemolytic toxin of sea anemone bind strongly to SPM but not to dispersions of PC or other lipids (Sood *et al.*, 1972; Linder *et al.*, 1977). Sequence similarities between these proteins have not been identified.

3.4.7 Interaction of collagenous proteins with PC

Procollagen was not found to bind to PC in physiological conditions of pH, ionic strength and temperature. Other studies have also failed to show an interaction between collagenous proteins and PC (see Table 3.2). Watkins *et al* (1977) and Cohen and Barenholz (1984) observed no interaction between acetylcholinesterase and egg PC at 0 °C and 4 °C, respectively. There was also no association between [^{14}C]labelled-collagen and egg PC at neutral pH and 4 °C (Cohen and Barenholz, 1984).

However, there have been a few observations of interaction between collagenous proteins and PC at neutral pH (see Table 3.2). The collagen domain of SP-A (see section 3.1.3) has been shown to be important for the interaction with lipid mixtures consisting predominantly of DPPC at 37 °C (King *et al.*, 1983; Ross *et al.*, 1986). Martinez del pozo *et al* (1989) have demonstrated the interaction of collagen fibrils with DMPC at neutral pH (for details, see section 3.1.4).

In the absence of salt, procollagen bound to PC at neutral pH. The interaction was weaker (on the basis of % bound in similar conditions) than that between procollagen and SPM. The binding in only an absence of salt suggests an electrostatic nature to the interaction. Dipoles exist around the collagen molecule which help to maintain a stabilising water shell (Silver, 1982; 1983). Such dipoles may interact with the phosphorylcholine headgroup of PC[21].

[21] The binding of spectrin to PS (Maksymiw *et al.*, 1987) and actin to positively charged liposomes (Rioux and Gicquad, 1985) also involve electrostatic interactions.

Protein	Type of PC	Interaction	Conditions	Time min.	Temp. ℃	Lipid : Protien ratio	Reference
collagen	DMPC DPPC	+	100 mM acetic acid	30 25	15	700 : 1	Martinez del pozo *et al.*, 1988
collagen fibrils	DMPC	+	30 mM Tris-HCl pH 7.0, 30 mM Na_2HPO_4, 2.5 mM acetic acid, 200 mM NaCl	—	30	250 : 1	Martinez del pozo *et al.*, 1989
SP-A	mainly DMPC[1]	+	5 mM sodium borate pH 7.4, 0.1 M NaCl, 3 mM $CaCl_2$	30	37	1800 : 1	King *et al.*, 1983
SP-A	mainly[2] DPPC	+	20 mM Tris-HCl pH 7.4, 0.1 M NaCl, 5 mM$CaCl_2$	120	37	330 : 1 to 8000 : 1	Ross *et al.*, 1986
acetyl-cholinesterase	egg PC	-	10 mM phosphate buffer pH 7.0, 0.1 M NaCl	30	0	—	Watkins *et al.*, 1977
acetyl-cholinesterase	egg PC	-	10 mM phosphate buffer pH 7.0, 0.1 M NaCl	30	4	27000 : 1	Cohen and Barenholz, 1988
collagen	egg PC	-	10 mM phosphate buffer pH 7.0, 0.1 M NaCl	30	4	12000 : 1	Cohen and Barenholz, 1988

Table 3.2 A comparison of studies that have either shown an interaction (+) or not (-) between collagenous proteins and PC.

[1] 85 % DMPC, 15 % DPPG

[2] 65 % DPPC, 20 % egg PC, 7.5 % egg PG, 7.5 % soya PI

No interaction of procollagen with PC was detected in 0.1 M acetic acid. In contrast, Martinez del pozo *et al* (1988) demonstrated the interaction of collagen with both DMPC and DPPC in 0.1 M acetic acid using a sedimentation-type binding assay. With this assay, collagen remained at the top of a 8.5/ 17/ 40 % gradient after centrifugation at 200,000 g for 5 hours. In the absence of collagen, [^{14}C]labelled-PC was shown to migrate to the 8.5/ 17 % interface, whereas in the presence of collagen, the PC remained at the top of the gradient. Martinez del pozo *et al.* (1988) also used differential scanning calorimetry to show collagen modifies the enthalpy change of the PC phase transition. This was interpreted as a peripheral interaction between collagen and PC in acetic acid. Any interaction between PC and procollagen in acetic acid is probably too weak to be detected in the conditions of the flotation-type binding assay.

3.4.8 Protein-PC interactions

A wide range of proteins have been shown to interact with PC. These include insulin (Wiessner and Hwang, 1982; Oomen and Kaplan, 1987), plasma fibronectin (Rossi and Wallace, 1982) and serum amyloid A (Bausserman *et al.*, 1983). However, sequences or structure responsible for the interaction have not been identified.

3.4.9 Cartilage mineralisation

The calcification of cartilage in the hypertrophic zone of the growth plate or in freeze fracture healing is an essential component of endochondral bone formation. An area in which procollagen-SPM interactions may well play a role *in vivo* is in biomineralisation as mediated by matrix vesicles (see below).

3.4.9.1 Chondrocalcin

Sequence analysis of chondrocalcin has revealed that it is the C-propeptide of type II procollagen (van der Rest *et al.*, 1986). This protein is most concentrated in calcified cartilages, although it has also been detected in developing epiphyseal cartilage (Poole

and Rosenberg, 1986). *In vitro,* the intracellular chondrocalcin content is much greater in growth plate cells compared to epiphyseal chondrocytes (Hinek *et al.*, 1987). Immunoelectron microscopic studies have localised deposits of chondrocalcin in high concentrations, where and when cartilage calcifies (Poole *et al.*, 1984; Poole and Rosenberg, 1986). The fact that chondrocalcin binds strongly to hydroxyapatite (Poole *et al.*, 1984) and is localised at the site where the initial major focal calcification of cartilage is observed suggests it has a role as a nucleation agent.

3.4.9.2 Matrix vesicles

Matrix vesicles are cell derived structures abundant in the extracellular matrix of mineralising tissues that initiate deposition of mineral in developing bones and teeth (Anderson, 1969; Ali *et al.*, 1970; Ali, 1987). The vesicles have been shown to derive from chondrocyte microvilli, where retraction of the supporting microfilament network is essential for the release of these structures (Hale and Wuthier, 1987). Changes in membrane lipid composition characteristic of matrix vesicles occur during microvillus formation (Wuthier, 1989). There is a marked enrichment of PS, SPM and lysophospholipids (Hale and Wuthier, 1987).

The matrix vesicles contain a variety of enzymes such as alkaline phosphatase, lactate dehydrogenase and phospholipase A2 (Genge *et al.*, 1990). The vesicles also contain a family of phospholipid (PS)-dependent Ca^{2+}-binding proteins which were found to be immunologically related to the annexin family of proteins (Genge *et al.*, 1989; Genge *et al.*, 1990; for further information on the annexins, see section 4.3.1.1). The annexin-like proteins in conjunction with PS may provide the vesicles with sink conditions necessary for the accumulation of large amounts of Ca^{2+} and Pi[22] (see below). Alternatively, Genge *et al.* (1990) suggest that the annexin-like proteins may play a role in matrix vesicle formation.

Both type X and type II collagen were shown to bind to matrix vesicles(Wu *et al.*, 1989). Graded salt extraction selectively released type II collagen from the matrix vesicle which suggests the interaction involves a collagen-binding protein. A

[22] Inorganic phosphate is represented by Pi.

chondrocyte protein known to bind to type II collagen, anchorin CII (for details, see section 4.3.1.1) has been shown to have strong homology to the annexin-like proteins in matrix vesicles (Genge *et al.*, 1992). One of the matrix vesicle annexin-like proteins may play a role in linking the matrix vesicle membrane to collagen fibrils.

Matrix vesicles isolated from avian growth plate cartilage contain large amounts of Ca^{2+} and Pi (Wuthier, 1977). Matrix vesicles produced by cultures of growth plate chondrocytes rapidly accumulate large amounts of Ca^{2+} and Pi (Wu *et al.*, 1989; Wuthier, 1989). These form the first crystalline phase octacalcium phosphate which is then converted to apatite.

The mechanism by which Ca^{2+} and Pi accumulate into the matrix vesicle remains largely unknown. A Na^{+}-dependent Pi transport system in matrix vesicles isolated from avian growth plate cartilage has been characterised (for details, see Montessuit *et al.*, 1991). The annexin-like proteins may provide the necessary sink conditions to maintain a proper ion gradient for rapid Ca^{2+} influx into matrix vesicles. A protease-sensitive cation porter probably exists to control the rate of influx (Wuthier, 1989).

An increase in intraluminal Ca^{2+} concentration appears to activate membrane breakdown (Wuthier, 1989). The high levels of phospholipase A_2 may play a major role in this process. Wu *et al* (1989) suggest that the interaction between collagen and matrix vesicles would facilitate the spread of mineral into the matrix.

3.4.9.3 Implications of a C-propeptide-SPM interaction

Chondrocalcin has been observed in the immediate vicinity of matrix vesicles (Poole *et al.*, 1984; Oliver *et al.*, 1987). However, an association as of yet has not been reported. The matrix vesicles contain a high SPM content, i.e. 35 % of total lipid as apposed to 8 % in chondrocytes (Hale and Wuthier, 1987). An explanation for this enrichment in SPM has not been forwarded. Since most of the SPM wiil be in the outer leaflet of the matrix vesicle membrane (Barenholz and Thompson, 1980), this region will contain about 70 % of the lipid. Studies with SPM/ PC mixtures in the gel-crystalline phase show binding of procollagen at this concentration. However, a slight reduction in the SPM content (about 5-10 %) led to a substantial reduction in binding.

This could provide a mechanism for controlling any possible interaction between chondrocalcin and the matrix vesicles.

The following hypothetical scenario can be envisaged for the role of SPM in cartilage calcification. Annexin mediated Ca^{2+} influx and binding to matrix vesicle acidic phospholipids leads to an ordering and lateral phase separation of membrane lipid microdomains (Haverstick and Glaser, 1987; Prigent-Dachary *et al.*, 1986), and hence SPM binding to extra-vesicular chondrocalcin. The increase in intraluminal Ca^{2+} activates phospholipase A_2 mediated breakdown of matrix vesicles, and then Ca^{2+} and Pi released into the matrix interact with chondrocalcin to initiate mineralisation. Further work is required to determine whether such a scenario operates *in vivo*.

3.4.10 Surface induced aggregation

Whether procollagen is secreted in monomeric or an aggregated form into the extracellular milieu is unclear (for details, see section 1.6). Procollagen aggregation is strongly favoured by adsorption to a mica surface (see section 1.8.3.1). Mould *et al* proposed that such an aggregation may be favoured at the cell surface, and that this would assist both propeptide cleavage and the subsequent assembly of the processed procollagen into fibrils.

The electrostatic interaction between an actin filament with the surface of a positively charged liposome favours the lateral attraction of other filaments in register (Rioux and Gicquaud, 1985). A similar mechanism was proposed for the formation of in-register aggregates of procollagen. However, procollagen does not aggregate in the presence of SPM liposomes in conditions where binding is expected.

The solubility limit of purified chick type I procollagen in phosphate buffered saline is in the range 1-1.5 mg/ ml (Mould *et al.*, 1990). Above this concentration, bundles of molecules (SLS-like aggregates) and D-periodic assemblies of procollagen have been observed. The surface induced aggregation observed by Mould and Hulmes (1987) probably took place as a result of high, local concentrations of procollagen molecules, at, or close to the mica surface.

CHAPTER 4

CELL SURFACE COLLAGEN BINDING PROTEINS AND PROTEOGLYCANS

4.1 General introduction

The exact site of procollagen processing in relation to the cell surface is unknown. Several studies have suggested that procollagen processing may take place at or near the cell surface (for details, see section 1.7). Retention of procollagen and/ or the procollagen proteinases at the cell surface may involve membrane bound proteoglycans (PG) or collagen binding proteins. This chapter contains preliminary observations of the effect of heparin and dextran sulphate (Section 4.2) and a collagen binding protein anchorin CII (section 4.3) on procollagen processing.

4.2 Heparin/ heparan sulphate and procollagen processing

There are 4 main types of glycosaminoglycans (GAG), hyaluronic acid, chondroiton/ dermatan sulphate, keratan sulphate and heparin/ heparan sulphate. Heparan sulphate and heparin have the same backbone structure, one which is notably different from other glycosaminoglycans in its α1,4 linkage between the N-acetylglucosamine and the D-glucuronic acid[1] .The degree of N-sulphation is the major characteristic that distinguishes heparan sulphate from heparin. Less than 50 % of the N-acetyl groups are normally converted to N-sulphation in heparan sulphate, whereas usually 70 % or more are converted in heparin.

Only heparan sulphate and chondroitin sulphate have been identified associated with the cell surface (Toole, 1991). Several distinct forms of cell-surface associated heparan sulphate proteoglycans (PG) have been characterised (David, 1991). These PGs are intercalated into plasma membranes by transmembrane hydrophobic domians in the core protein (Marynen *et al.*, 1989) or by phosphatidylinositol constituents of the PGs (David *et al.*, 1990). Fibroglycan is an integral membrane protein with a transmembrane domain of 25 amino acids (Marynen *et al.*, 1989). It has been shown to bind avidly to collagen and fibronectin through the intermediate of its heparan sulphate

[1] L-iduronic acid is produced by the epimerisation of D-glucuronic acid at the position where the carboxyl group is located.

chains. The cell surface associated heparan sulphate PGs have been proposed to act as cell surface 'catalysts' that can influence cell growth, modulate matrix assembly and remodelling, and control the activity of extracellular proteinases (Gallagher *et al.*, 1990; Giudo, 1990; David, 1991). In related work, it has been shown that procollagen processing in fibroblast cultures is enhanced by the presence of either 0.01 % dextran sulphate or 5 % polyethylene glycol in the culture medium (Bateman and Golub, 1990; see section 1.7). The effect of polyethylene glycol is probably via a volume exclusion mechanism, though the mechanism of dextran sulphate enhancement of processing is unknown. The aim of this study was to determine the effect of heparin (as an analogue for heparan sulphate) and dextran sulphate on the activity of purified procollagen N- and C-proteinases.

4.2.1 Materials and methods

4.2.1.1 Electrophoretic assay

An electrophoretic assay was employed to determine the extent of cleavage of the N- or C-propeptide of procollagen. The proα (I) chains and their processed forms were separated by SDS-PAGE and quantitated by densitometry (Goldberg *et al.*, 1975; Hojima *et al.*, 1985).

Chick embryo tendon procollagen in storage buffer[2] was diluted with an equal volume of 10 mM $CaCl_2$/ 0.01 % NaN_3, and then further diluted with 0.05 M Tris-HCl pH 7.5/ 0.15 M NaCl/ 5 mM $CaCl_2$/ 0.01 % NaN_3 (assay buffer) to a final concentration of 12 µg/ ml. Partially purified C-proteinase (see section 2.3) and N-proteinase (a generous gift from D.J.S.Hulmes) were diluted 10-fold with 0.05 M Tris-HCl pH 7.5/ 5 mM $CaCl_2$, and then further diluted with assay buffer. The final dilution of the C-proteinase and the N-proteinase were 200 and 150-fold, respectively. These dilutions gave a basal activity of about 10 % so that any enhancement of proteinase activity would be clearly observed. The assay consisted of 10 µl [^{3}H]procollagen, 20 µl of partially purified N- or C-proteinase and 20 µl heparin or dextran sulphate or

[2] 0.1 M Tris-HCl pH 7.4/ 0.4 M NaCl

assay buffer to determine the basal activity. The concentration of heparin or dextran sulphate (in assay buffer) added to the assay mixture ranged from 0.1-5 mg/ ml. The effect of the neutral polymer polyethylene glycol (PEG) 4000 (1.5 or 5 mg/ ml) on procollagen processing was also determined. The assay mixture was incubated for 4 hours at 35 °C. One quarter the volume of 'stop' buffer (100 mM Tris-HCl, pH 7.5/ 125 mM EDTA) was added to each tube to terminate the assay. The samples were then prepared for SDS-PAGE (see Figure 2.2). After electrophoresis, the gels were processed for fluorography as described in section 2.2.3.

4.2.1.2 Rotary shadowing

Electron microscopy after rotary shadowing was used to investigate the interaction of procollagen with dextran sulphate. The specimens were glycerol dried and prepared by the mica-sandwich technique (see section 2.8.3). 10 µl procollagen (105 µg/ ml) in 0.05 M Tris-HCl pH 7.5/ 0.15 M NaCl/ 5 mM $CaCl_2$/ 0.01 % NaN_3 was added to 20 µl dextran sulphate and 56 µl glycerol (65 %). The final dextran sulphate concentration was 0.5 mg/ ml. The mixture was incubated for 1 hour at 25 °C and then prepared for rotary shadowing (for the protocol, see Figure 2.6). Glycerol dried specimens containing only procollagen were also prepared.

4.2.2 Results

4.2.2.1 Determination of the enzyme activity

Procollagen was processed by either the N- or C-proteinase in the presence of heparin or dextran sulphate (for details of procollagen processing, see section 1.7). Fluorograms were analysed with a Joyce-Loebl Chromoscan 3 scanning densitometer. Each band on the fluorogram can be assigned to a species containing type I collagenous sequences on the basis of comparison with known locations (Prockop and Tuderman, 1982; see figure 4.1). The amounts of radioactivity in each band were determined from the integrated peak areas in the densitometric scans. Care was taken when exposing the

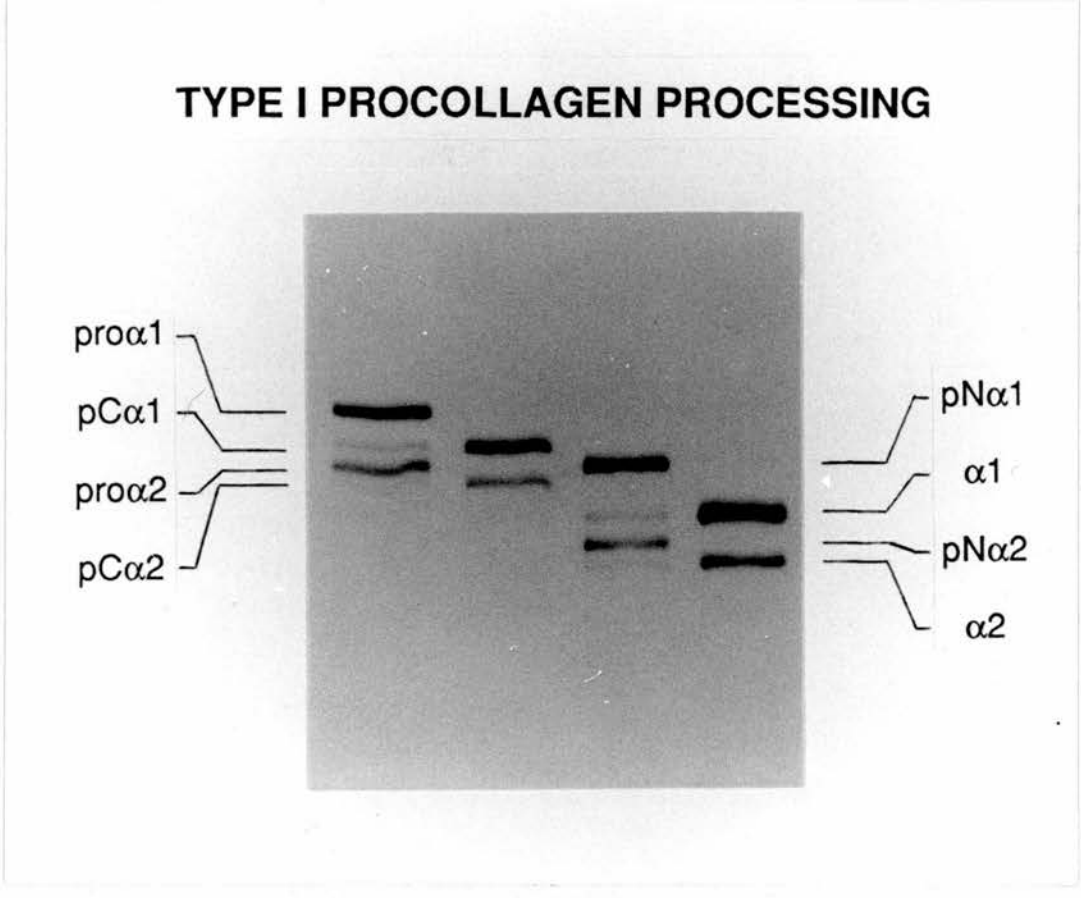


Figure 4.1 Fluorogram showing the positions of migration of proα1, proα2, pCα1, pCα2, pNα1, pNα2, α1 and α2 chains on SDS-PAGE.

(Courtesy of Dr D.J.S. Hulmes)

fluorogram to remain within the linear response of the film. Since the proα2 (I) band migrates only slightly slower than the pNα1 (I) band, these bands could not be resolved for quantitation. A value of the integral for pNα1 (I) was calculated from the following equations (Mellor *et al.*, 1991),

$$[\text{pro}\alpha 1] + [\text{pC}\alpha 1] + [\text{pN}\alpha 1] + [\alpha 1] = 66.7\ \%$$

$$[\text{pro}\alpha 2] + [\text{pC}\alpha 2] + [\text{pN}\alpha 2] + [\alpha 2] = 33.3\ \%$$

The proteinase activity was determined from the % of the proα1 intermediate relative to all the proα1 chains, i.e. for the N-proteinase activity, [pCα1]/ [pCα1] + [proα1][3] . The enzyme activity (U/ ml) was calculated as the amount of enzyme which cleaves 1 μg procollagen in 1 hour at 35 °C.

4.2.2.2 Effect of heparin and dextran sulphate on proteinase activity

Heparin was found to substantially decrease C-proteinase activity (see figures 4.2 and 4.3). The difference in C-proteinase activity in the presence of 5 mg/ ml heparin was about -150 U/ ml. In contrast, heparin (0.1-5 mg/ ml) had no distinct effect on N-proteinase activity (see Figure 4.3).

Dextran sulphate was found to enhance both N- and C-proteinase activity[4] (see figure 4.4). Only 50 μg/ml was required to significantly enhance both N- and C-proteinase activity. A considerably greater concentration of PEG (20-50 mg/ ml) was needed to show an enhancement of proteinase activity (see figure 4.4).

4.2.2.3 Rotary shadowing

When procollagen (12 μg/ ml) was observed by electron microscopy after rotary shadowing, about 70 % of the molecules appeared as monomers (Figure 3.19). In contrast, procollagen (12 μg/ ml) that had been incubated with 0.5 mg/ ml dextran

[3] For the N-proteinase activity, the % of pCα1 present in the absence of enzyme was subtracted.

[4] The proteinase assay with dextran sulphate was performed by D.J.S.Hulmes.

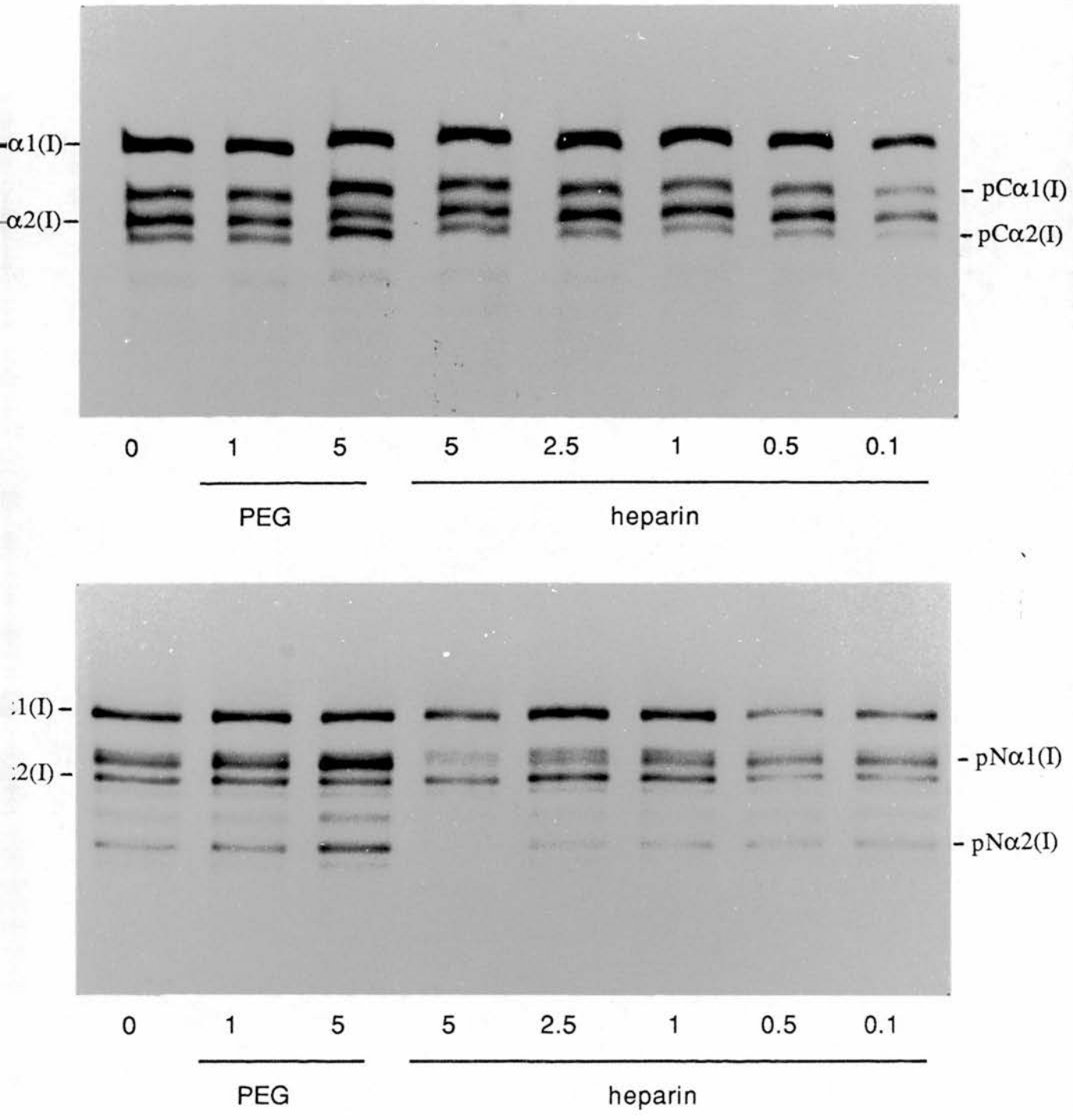


Figure 4.2 Fluorogram showing the effect of heparin on the processing of type I procollagen. (A) N-proteinase (B) C-proteinase. Enzymes were assayed in assay buffer alone (0) or different concentrations (mg/ml) of polyethylene glycol (PEG) or heparin.

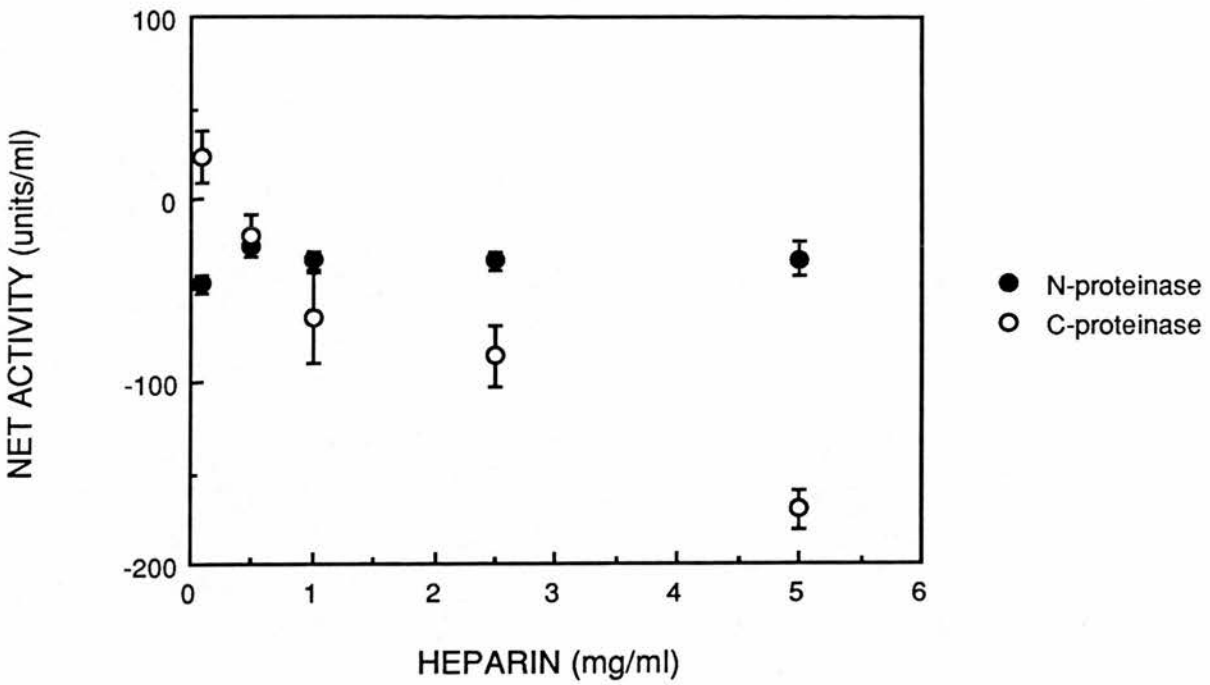


Figure 4.3 The effect of heparin on the activity of the N- and C-proteinases. Proteinase activity (units/ml) in assay buffer alone was subtracted from activity in the presence of heparin.

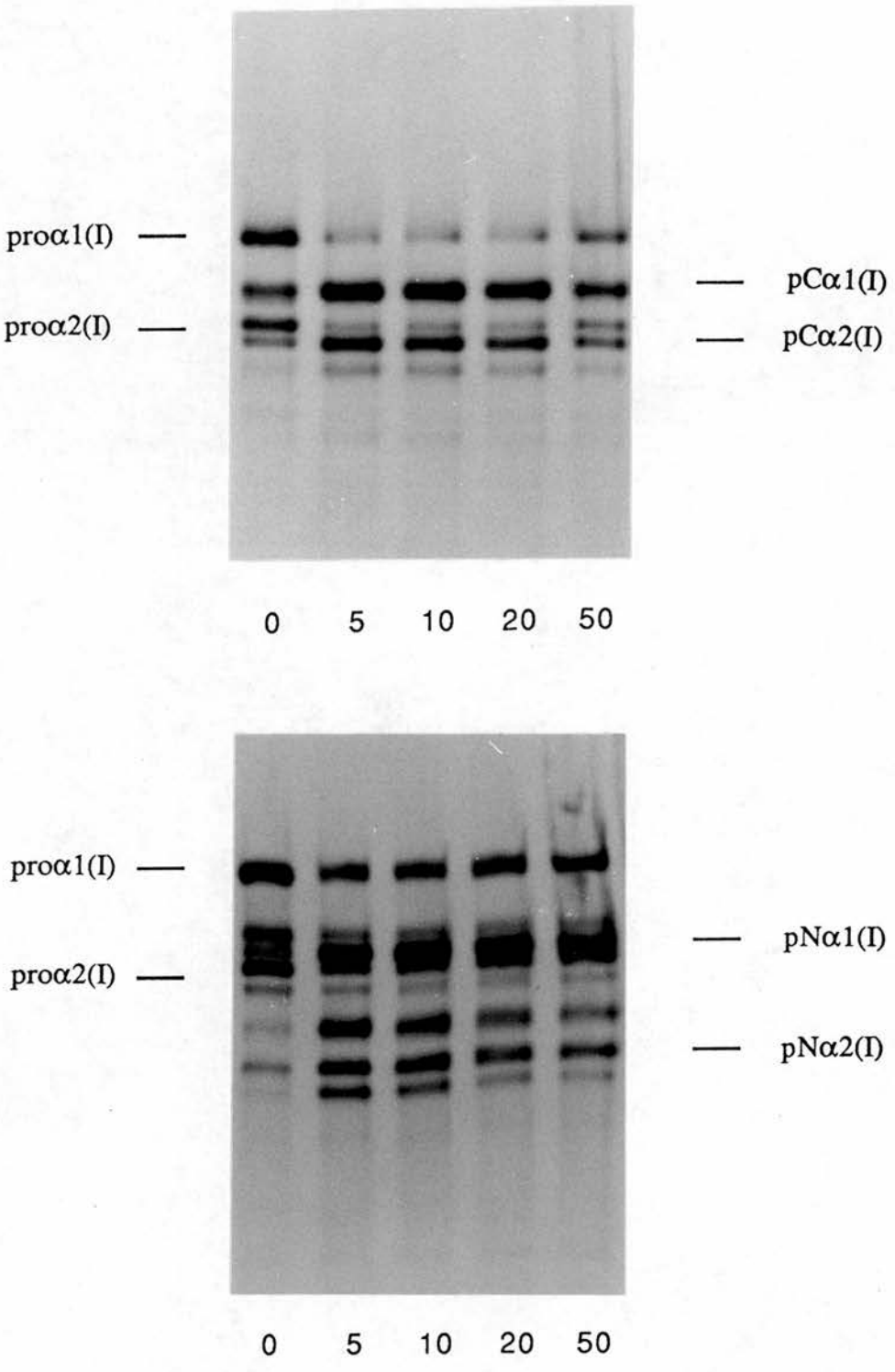


Figure 4.4 Fluorogram showing the effect of dextran sulphate on the processing of type I procollagen. (A) N-proteinase (B) C-proteinase. The pro α chains, their processing intermediates and the concentrations of dextran sulphate (μg/ml) are indicated.

sulphate appeared only in poorly defined aggregates (see figure 4.5). Clearly defined procollagen monomers or aggregates were not observed (see discussion).

4.3 Anchorin CII

4.3.1 Introduction

4.3.1.1 Collagen-binding proteins

The existence of collagen-binding proteins on the cell surface was first suggested from the binding studies of radiolabelled type I collagen and their peptides to fibroblasts (Goldberg, 1979; Goldberg and Burgeson, 1982). The binding was shown to be reversible, saturable and specific for sequences contained within the helical portions of the α1 (I) and α2 (I) chains (Goldberg, 1979). The N-propeptide of type I procollagen was also shown to inhibit binding to a lesser extent. The binding was competitvely inhibited by native types II and III collagen, but not by other collagenous molecules, e.g. acetylcholinesterase and type IV and V collagen (Goldberg and Burgeson, 1982).

Many types of collagen binding proteins have now been described. The collagen binding integrins are α1β1, α2β1 and α3β1 (Wayner and Carter, 1987; Staatz *et al.*, 1989; Ignatius *et al.*, 1990). The involvement of the collagen arg-gly-asp containing sequences in the interaction with the integrins is unclear (see Ruoslahti, 1991). A 46 kDa collagen binding glycoprotein from rat skeletal myoblasts has been characterised (Nandan *et al.*, 1988; Clark *et al.*, 1991). The collagen-glycoprotein interaction was inhibited by arg-gly-asp containing peptides. Several other glycoproteins similar in size and properties have been described from various cell types (see Clark *et al.*, 1991).

4.3.1.2 Anchorin CII

Anchorin CII is a collagen-binding protein (Mr 34 kDa) first isolated from chondrocyte plasma membranes (Mollenhauer and Von der Mark, 1983). Subsequent

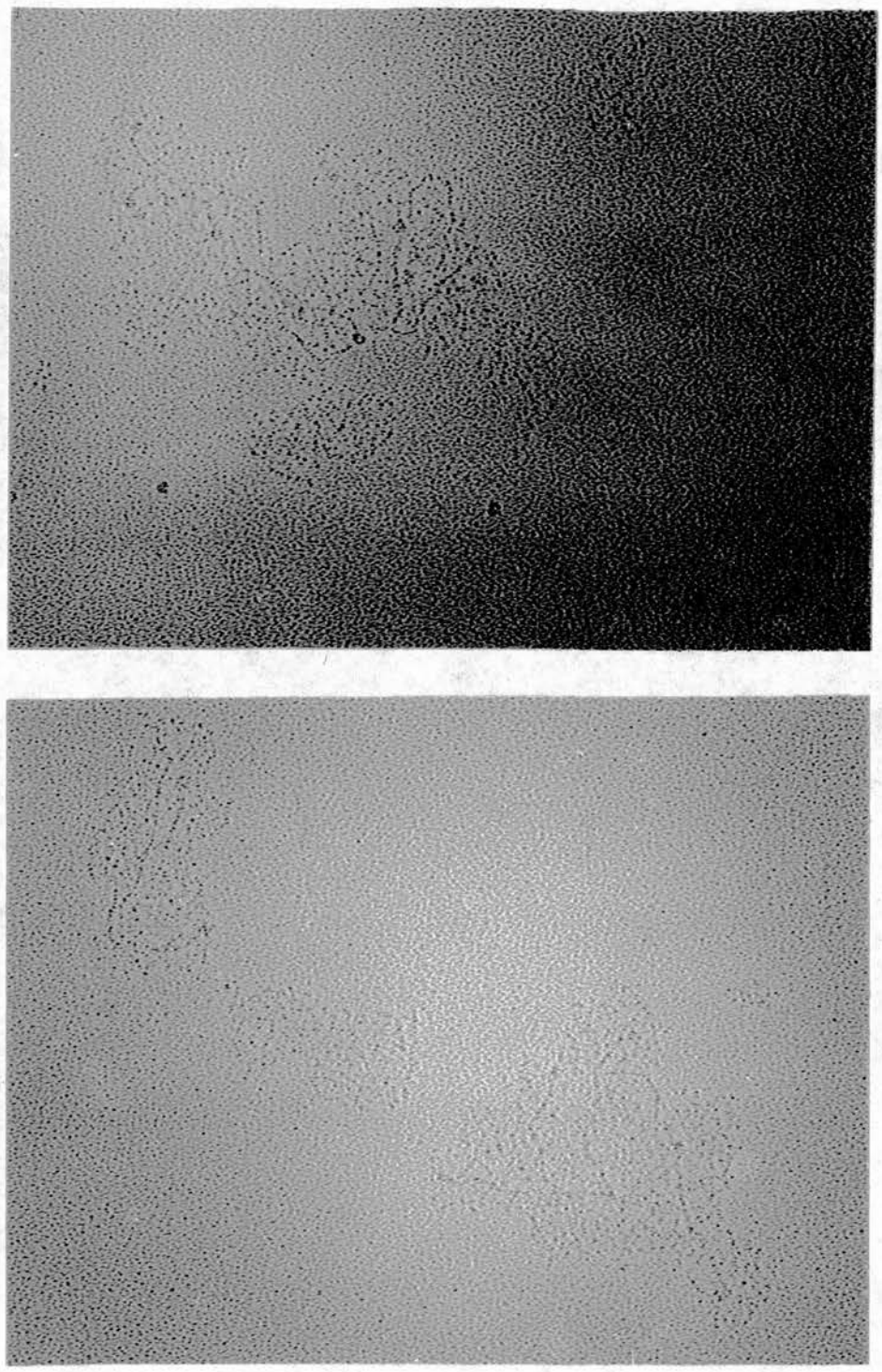


Figure 4.5 Micrographs of rotary shadowed procollagen incubated with dextran sulphate. Magnification 150,000 x.

studies have suggested that fibroblasts may also contain an anchorin CII-like protein (Von der Mark *et al.*, 1985). The cDNA sequence of anchorin CII (Fernandez *et al.*, 1988; Pfäffle *et al.*, 1988) shows considerable similarity with those in human and bovine calpactin, lipocortin and endonexin which are members of a family of Ca^{2+} and phospholipid binding proteins usually described as the annexins (for details of the annexins, see Crompton *et al.*, 1988; Haigler *et al.*, 1989). Whilst the annexins have been located to the inner-side of the plasma membranes, Pfäffle *et al* (1988) provide evidence for the localisation of anchorin CII to the outer cell surface. This is consistent with the notion that the protein is involved in the interaction of chondrocytes and fibroblasts with extracellular collagen.

4.3.1.3 Dermatosparaxis

Persistence of large amounts of pN-collagen is the basis of a heritable disorder of connective tissues called dematosparaxis (see section 1.12), originally seen in cattle and sheep, where deficient N-propetide processing leads to highly abnormal collagen fibrils (Fjølstad and Helle, 1974; Hulmes *et al.*, 1989).

The deficient processing may be due to a defective enzyme or ancilliary protein. A defective activity of the N-proteinase has never been directly demonstrated. Fibroblasts from dermatosparactic animals differ from healthy controls in their interaction with collagen (Delvoye *et al.*, 1985, 1986; Mauch *et al.*, 1986). In contrast with normal fibroblasts, the dermatosparactic fibroblasts failed to contract collagen gels. Mauch *et al* (1988) correlated the inability of the dermatosparactic fibroblasts to attach to collagen with the defficiency of a 34 kDa protein. This protein was recognised by an antiserum to chicken anchorin CII.

4.3.1.4 Purpose of the study

The purpose of the study was to purify anchorin CII from chicken sternal cartilage, and then to determine the effect of anchorin CII (incorporated into PS liposomes) on N-proteinase activity using the electrophoretic assay described in section

4.2.1.1.However, the purification of anchorin CII was not achieved because of problems with the protocol described below.

4.3.2 The purification of anchorin CII

The protocol for the purification of anchorin CII from adult chicken sternal cartilage was provided by K.von der Mark. The following procedures were undertaken at 4 °C.

4.3.2.1 Extraction

All adherent non-cartilage tissue (bone, muscle and perichondrium) were removed from about 30 xyphoid processes from 4, 6, and 8 week old chicken broilers (obtained from the AFRC Institute of Animal Physiology and Genetics, Edinburgh Research Station). The sterna were cut into pieces, and then ground with crushed ice in a meat grinder. The cartilage pellet was homogenised in 500 ml 50 mM Tris-HCl pH 7.4/ 2.5 mM EDTA/ 0.5 M NaCl/ 8.5 % sucrose with a Waring blender, and then centrifuged for 1 hour at 11,000 g at 4 °C. The supernatant was dialysed against 50 mM Tris-HCl pH 7.4/ 2.5 mM EDTA and then clarified by centrifugation at 54,000 g for 1 hour.

4.3.2.2 DEAE-Cellulose chromatography

A DEAE-Cellulose column (2.6 x 11 cm) was packed in the same way described in section 2.1.3. The column was equilibrated with 50 mM Tris-HCl pH 7.4/ 2.5 mM EDTA. The supernatant was applied at a flow rate of 36 ml/ hour. The column was then washed with 2 column volumes of equilibration buffer. A linear salt gradient (300 ml total; 0-430 mM) produced with a gradient former (Bethesda Research Laboratories, Inc) was applied to the column. Fractions of 6 ml were collected and A_{280} determined. The fractions containing anchorin CII were determined by SDS-PAGE (see figure) followed by immunoblotting[5] (see figure 4.6).

[5] Anti-anchorin CII antibody was a generous gift of K.von der Mark.

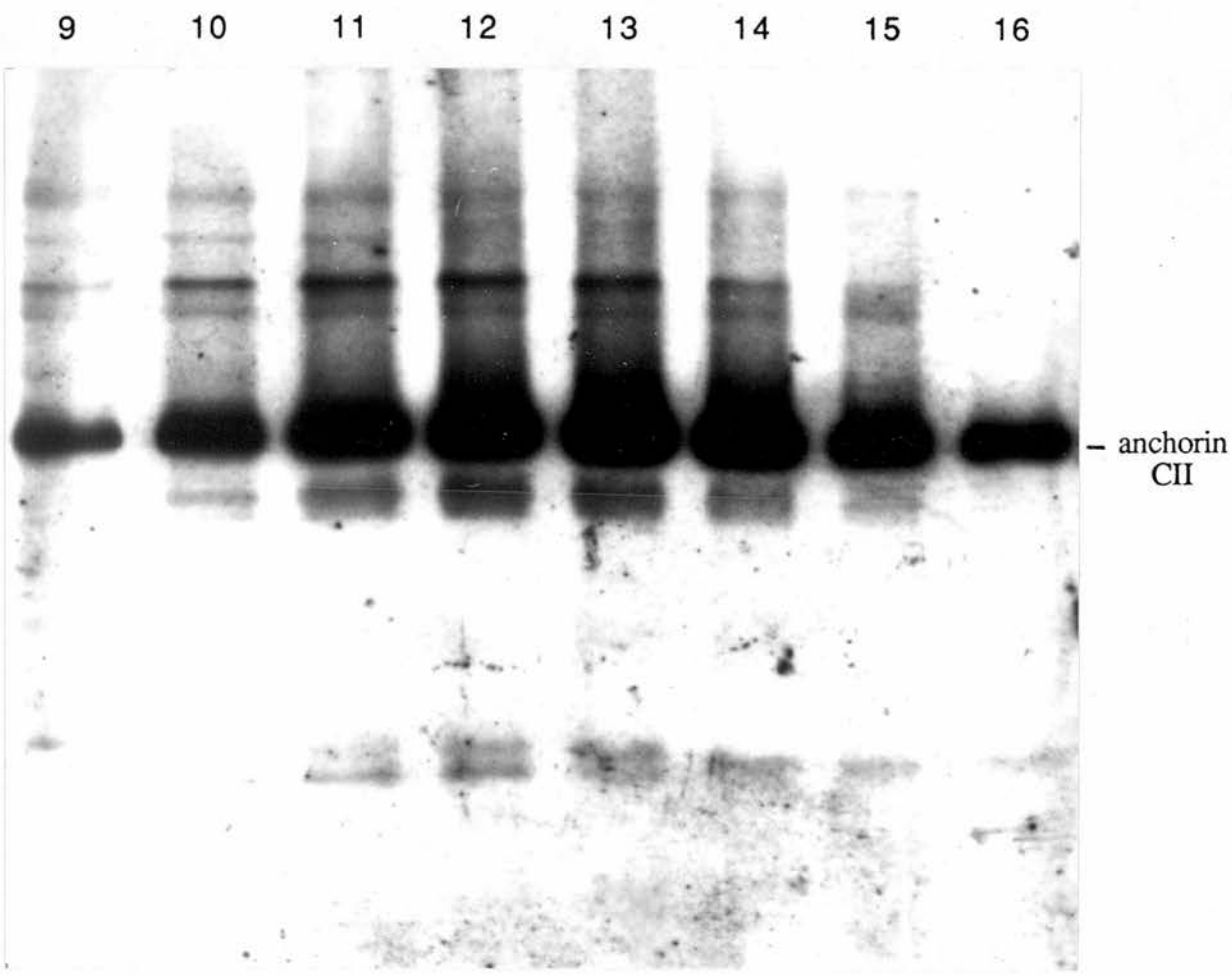


Figure 4.6 Western blotting detection of anchorin CII in the fractions eluted from the DEAE-Cellulose column. Fractions 9 to 16 were pooled for the subsequent purification step.

4.3.2.3 Preparation for Mono S chromatography

The anchorin CII containing fractions were pooled and concentrated to 1 ml using an ultrafiltration cell (10 ml capacity Amicon cell) with a YM 10 membrane (molecular weight exclusion limit 10,000). The concentrate was then diluted gradually by 10-fold with Mono S buffer (20 mM sodium acetate pH 5.6). However, this resulted in the formation of a precipitate. This should not have occurred according to the protocol. The mixture was centrifuged at 50,000 g for 1 hour at 4 °C. Immunoblotting of the supernatant with anti-anchorin CII revealed anchorin CII was not present. Therefore, anchorin CII must have been present in the precipitate. However, since the precipitate would not redissolve in 20 mM sodium acetate pH 5.6/ 0.2 M NaCl the purification was abandoned. Further work is required to determine a salt concentration which will allow anchorin CII to remain in solution (Mono S buffer) and yet still bind to the Mono S column.

4.4 Discussion

The interaction of collagenous molecules with cells has been demonstrated (Goldberg, 1982; Mollenhauer and von der Mark, 1983; Phelps *et al.*, 1985). Such an interaction may aid the segregation of the collagenous molecules and help to determine the spatial specificity of their assembly. The interaction may also assist both propeptide cleavage (see below) and subsequent assembly of processed procollagen into fibrils. The mechanism involved in the interaction of the cell membrane with collagenous molecules are poorly understood.

4.4.1 Heparin

Since the C-proteinase requires 5-10 mM Ca^{2+} for optimal activity (see section 1.7.2), the heparin inhibition of C-proteinase activity may be due to a reduction in the

free Ca^{2+} concentration[6] caused by heparin chelation. This acidic polymer has been shown to bind to Ca^{2}. However, rather curiously, heparin had no obviuos effect on N-proteinase activity which also requires about 5-10 mM Ca^{2+} for optimal activity (see section 1.7.1).

The inhibition of the C-proteinase may result from an interaction between heparin and procollagen. Heparan sulphate has been shown to bind to collagen (Koda *et al.*, 1985; Marynen *et al.*, 1989). Further work is required to determine whether cell surface heparan sulphate PGs inhibit C-proteinase activity. This can provide a mechanism for ensuring fibrillogenesis does not take place at the cell surface.

4.4.2 Dextran sulphate

Procollagen was completely processed to collagen by the addition of 0.1 mg/ ml dextran sulphate to human fibroblast culture medium (Bateman and Golub et al., 1990; see section 1.7). The enhancement of both N- and C-proteinase activity by dextran sulphate was confirmed with the use of the proteinase assay. The neutral polymer PEG also enhanced proteinase activity but only at a considerably greater concentration (20-50 mg/ ml). Therefore, the effect of dextran sulphate is not due to an exclusion effect.

The fact that dextran sulphate enhanced both proteinase activities suggests it interacts with the procollagen rather than the proteinases. When procollagen was incubated with dextran sulphate only large poorly defined aggregates were observed with rotary shadowing. The interaction of dextran sulphate with procollagen would explain the poorly defined structures. The results of the proteinase assays suggest that the dextran sulphate-induced procollagen aggregate is a better substrate for both the N- and C-proteinase. Further work is required to characterise these aggregates.

4.4.3 Interaction of type XI collagen with a polyanion at the cell surface

Cartilage fibrils are heterotypically assembled from type II, IX and XI collagen

[6] The assay contains 5 mM Ca^{2+}.

(see section 1.8.3.3). Type XI collagen has been immunolocalised on to the surface of chondrocytes in culture (Smith *et al.*, 1989). The association was suggested to be ionic in nature which is consistent with a previous suggestion that type II collagen is retained at the cell surface by interactions with a cell surface polyanion (Smith et al., 1985). Smith *et al* (1989) proposed the association of type XI collagen with the cell surface may be a step in the assembly of the fibrils.

4.4.4 Anchorin CII

Anchorin CII may be an ancillary protein for the procollagen N-proteinase. It may function in a similar way to that proposed for the enhancer[7] of the C-proteinase (see section 1.7.2). The enhancer has been suggested to act by presenting the procollagen molecule to the C-proteinase in a favourable conformation, increasing thereby the catalytic rate. Further work is required to determine the role of anchorin CII in the cleavage of the N-propeptide, and to characterise its interaction with procollagen.

[7] Enhancer has been suggested to be a cell-surface protein (Kessler and Adar, 1986).

REFERENCES

Abermann, R., Salpeter, M.M., and Bachmann, L. (1972). High resolution and shadowing. In: Principles and Techniques of Electron Microscopy. Biological Applications, Hayat, M.A. editor. pp. 197-217.Van Norstrand Reinhold, New York

Adachi, E. and Hayashi, T. (1986). *In vitro* formation of hybrid fibrils of type V collagen and type I collagen. Limited growth of type I collagen into thick fibrils by type V collagen. Connect.Tiss.Res. 14:257-266

Adachi, E. and Hayashi, T. (1987). Comparison of axial banding patterns in fibrils of type V collagen and type I collagen. Collagen.Rel.Res. 7:27-38

Adar, R., Kessler, E. and Goldberg, B. (1986). Evidence for a protein that enhances the activity of type I procollagen C-proteinase. Collagen Rel.Res. 6:267-277

Alexander, C.M., and Werb, Z. (1989). Proteinases and extracellular matrix remodeling. Curr.Opin.Cell Biol. 1:974-982

Ali, S.Y. (1987). Analysis of matrix vesicles and their role in the calcification of epiphyseal cartilage. Fed.Proc. 35:135-142

Ali, S.Y., Sajdera, S.W., and Anderson, H.C. (1970). Isolation and characterisation of calcifying matrix vesicles from epiphyseal cartilage. Proc.Natl.Acad.Sci.USA 67:1513-1523

Anderson, H.C. (1969). Vesicles associated with calcification in the matrix of epiphyseal cartilage. J.Cell.Biol. 41:59-72

Aycock, R.S., Raghow, R., Stricklin, G.P., Seyer, J.M., and Kang, A.H. (1986). Post-transcriptional inhibition of collagen and fibronectin synthesis by a synthetic homologue of a portion of the carboxyl-terminal propeptide of human type I collagen. J.Biol.Chem. 261:14355-14360

Bachinger, H.P. and Davis, J.M. (1991). Sequence specific thermal stability of the collagen triple helix. Int.J.Biol.Macromol. 13:152-156

Barenholz, Y. and Thompson, T.E. (1980). Sphingomyelins in bilayers and biological membranes. Biochim.Biophys.Acta 604:129-158

Barnes, M. (1985). Collagen in atherosclerosis. Collagen Rel.Res. 5:65-97

Bateman, J.F. and Golub, S.B. (1990). Assessment of procollagen processing defects by fibroblasts cultured in the presence of dextran sulphate. Biochem.J. 267:573-577

Bateman, J.F., Pillow, J.J., Mascara, T., Medvedec, S., Ramshaw, J.A.M. and Cole, W.G. (1987). Cell-layer associated proteolytic cleavage of the telopeptides of type I collagen in fibroblast culture. Biochem.J. 245:677-682

Bausserman, L.L., Herbert, P.N., Forte, T., *et al.* (1983). Interaction of the serum amyloid A protein with phospholipid . J.Biol.Chem. 258:10681-10688

Bennet, V.D., and Adams, S.L. (1990). Identification of a cartilage-specific promoter within intron 2 of the chick alpha2 (I) collagen gene . J.Biol.Chem. 265:2223-2230

Benson-Chanda, V., Su, M.W., Weil, D., Chu, M.L. and Ramirez, F. (1989). Cloning and analysis of the 5' portion of the human type-III procollagen gene (COL3A1). Gene 78:255-265

Berg, R.A. and Prockop, D.J. (1973). The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilising the triple helix of collagen. Biochem.Biophys.Res.Comm. 52:115-120

Berger, J., Tanzawa, K. and Prockop, D.J. (1985). Sequential cleavage of type I procollagen by procollagen N-proteinase. An intermediate containing an uncleaved proα1 (I) chain. Biochemistry 24:600-605

Berne, B.J. and Pecora, R. Dynamic light scattering, New York:Wiley (1976)

Bernengo, J.C., Ronziere, M.C., Bezot, P., Bezot, C., Herbage, D. and Veis, A. (1983). A hydrodynamic study of collagen fibrillogenesis by electric birefringence and quasielastic light scattering. J.Biol.Chem. 258:1001-1006

Birk, D.E., Fitch, J.M., Babiarz, J.P. and Linsenmayer, T.F. (1988). Collagen type I and type V are present in the same fibril in the avian corneal stroma. J.Cell Biol. 106:999-1008

Birk, D.E., Silver, F.H. and Trelstad, R.L. (1991). Matrix Assembly. In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 221-254. Plenum press, New York

Birk, D.E. and Trelstad, R.L. (1984). Extracellular compartments in matrix morphogenesis: Collagen fibril, bundle, and lamellar formation by corneal fibroblasts. J.Cell.Biol. 99:2024-2033

Birk, D.E. and Trelstad, R.L. (1986). Extracellular compartments in tendon morphogenesis: collagen fibril, bundle, and macroaggregate formation. J.Cell Biol. 103:231-240

Birk, D.E. and Trelstad, R.L. (1986). Extracellular compartments in tendon morphogenesis: Collagen fibril, bundle, and macroaggregate formation. J.Cell.Biol. 103:231-240

Birk, D.E., Zycband, E.I., Winkelmann, D.A. and Trelstad, R.L. (1989). Collagen fibrillogenesis in situ: Fibril segments are intermediates in matrix assembly. Proc.Natl.Acad.Sci.USA. 86:4549-4553

Birk, D.E., Zycband, E.I., Winkelmann, D.A. and Trelstad, R.L. (1990). Collagen fibrillogenesis in situ. Discontinuous segmental assembly in extracellular compartments. Ann.N.Y.Acad.Sci. 580:176-194,

Bonaldo, P., Russo, V., Bucciotti, F., Doliana, R. and Colombatti, A. (1990). Structural and functional features of the α3 chain indicate a bridging role for chicken collagen VI in connective tissues. Biochemistry 29:1245-1254

Bonner, W.M., and Laskey, R.A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels . Eur.J.Biochem. 46:83-88

Bornstein, P., Horlein, D., McPherson, J., Sandmeyer, S., and Gallis, B. (1982). Regulation of collagen biosynthesis. In: New Trends in Basement Membrane Research, Kühn, K., Schoene, H., and Timpl, R. editors. pp. 127-137. Raven press, New York

Bornstein, P., and McKay, J. (1988). The first intron of the α1 (I) collagen gene contains several transcriptional regulatory elements. J.Biol.Chem. 263:1603-1606

Bornstein, P., McKay, J., Liska, D.J., Apone, S., and Devarayalu, S. (1988). Interactions between the first promoter and first intron are involved in transcriptional control of α1 (I) collagen gene expression. Mol.Cell.Biol. 8:4851-4857

Bornstein, P., McKay, J., Morishima, J.K., Devarayalu, S., and Gelinas, R.E. (1987). Regulatory elements of the first intron contribute to transcriptional control of the human α1 (I) collagen gene. Proc.Natl.Acad.Sci.USA 84:8869-8873

Brewton, R.G., Wright, D.W. and Mayne, R. (1991). Structural and functional comparison of type IX collagen-proteoglycan from chicken cartilage and vitreous humour. J.Biol.Chem. 266:4752-4757

Brown, D.M., Charonis, A.S., Furcht, L.T., *et al.* (1991). An overview of role of matrix components. Diabetes Care 14:157-159

Bruckner, P. and Eikenberry, E.F. (1984). Formation of the triple helix of type I procollagen in cellulo. Temperature-dependence kinetics support a model based on cis-trans isomerisation of peptide bonds. Eur.J.Biochem. 140:391-395

Bruckner-Tuderman, L., Mitsuhashi, Y., Schnyder, U.W. and Bruckner, P. (1989). Anchoring fibrils and type VII collagen are absent from skin in severe recessive dystrophic epidermolysis bullosa. J.Invest.Dermatol. 93:3-9

Bruns, R.R., Hulmes, D.J.S., Therrien, S.F. and Gross, J. (1979). Procollagen segment-long-spacing crystallites: Their role in collagen fibrillogenesis. Proc.Natl.Acad.Sci.USA 76:313-317

Burgeson, R.E. (1987). Type VII Collagen. In: Structure and Function of Collagen Types, Mayne, R. and Burgeson, R.E. editors. pp. 145-172. Academic press, Orlando

Burgeson, R.E., Lunstrum, G.P., Rokosova, B., Rimberg, C.S., Rosenbaum, L.M. and Keene, D.R. (1990). The structure and function of type VII collagen. Ann.N.Y.Acad.Sci 580:32-43

Butkowski, R.J., Noelken, M.E. and Hudson, B.G. (1982). Estimation of the size of collagenous proteins by electrophoresis and gel chromatography. Meth.Enzymol. 82:410-423

Byers, P.H. (1990). Brittle bones-fragile molecules: disorders of collagen gene structure and expression. Trends Genet. 6:293-300

Byers, P.H., Click, E.M., Harper, E. and Bornstein, P. (1975). Interchain disulphide bonds in procollagen are located in a large non-triple helical COOH-terminal domain. Proc.Natl.Acad.Sci.USA 72:3009-3013

Bächinger, H.P., Doege, K.J., Petschek, J.P., Fessler, L.I. and Fessler, J.H. (1982). Structural implications from an electron microscopic comparison of procollagen V with procollagen I, pC-collagen I, procollagen IV and drosophila procollagen. J.Biol.Chem. 257:14590-14592

Capaldi, M.J. and Chapman, J.A. (1982). The C-terminal extra-helical peptide of type I collagen and its role in fibrillogenesis *in vitro*. Biopolymers 21:2291-2313

Chan, D., Lamande, S.R., Cole, W.G. and Bateman, J.F. (1990). Regulation of procollagen synthesis and processing during ascorbate-induced extracellular matrix accumulation *in vitro*. Biochem.J. 269:175-181

Chapman, J.A. (1989). The regulation of size and form in the assembly of collagen fibrils *in vivo*. Biopolymers 28:1367-1382

Chapman, J.A. and Hulmes, D.J.S. (1984). Electron microscopy of the collagen fibril. In: Ultrastructure of the Connective Tissue Matrix, Ruggeri, A. and Motta, P.M. editors. pp. 1-33. Martinus Nijhoff, Boston

Cho, M-I. and Garant, P.R. (1981). Sequential events in the formation of collagen secretion granules with special reference to the development of segment-long-spacing aggregates. Anat.Rec. 199:309-320

Chu, M-L., de Wet, W., Bernard, M., *et al.* (1984).Human proα (I) collagen gene structure reveals evolutionary conservation of a pattern of introns and exons. Nature 310:337-340

Chu, M.L., Zhang, R.Z., Pan, T.C., *et al.* (1990). Mosaic structure of globular domains in the human type VI collagen α3 chain: similarity to von Willebrand factor; fibronectin; actin; salivary proteins and aprotinin type protease inhibitors. EMBO J. 9:385

Chu, M.L., de Wet, W., Bernard, M.P. and Ramirez, F. (1985). Fine structural analysis of the human proα1 (I) collagen gene: promroter structure, Alu1 repeats and polymorphic transcripts. J.Biol.Chem. 260:2315-2320

Clarke, E.P., Cates, G.A., Ball, E.H. and Sanwal, B.D. (1991). A collagen-binding protein in the endoplasmic reticulum of myoblasts exhibits relationship with serine protease inhibitors. J.Biol.Chem. 266:17230-17235

Cockayne, D., and Cutroneo, K.R. (1988). Glucocorticoid coordinate regulation of type I procollagen gene expression and procollagen DNA-binding proteins in chick skin fibroblasts. Biochemistry 27:2736-2745

Cohen, R. and Barenholz, Y. (1984). Characterisation of the association of Electrophorus electricus acetylcholinesterase with sphingomyelin liposomes. Biochim.Biophys.Acta 778:94-104

Colombatti, A. and Bonaldo, P. (1991). The superfamily of proteins with von Willebrand factor type A-like domains: one theme common to components of extracellular matrix, hemostasis, cellular adhesion, and defence mechanisms. Blood 77:2305-2315

Comper, W.D. and Veis, A. (1977). The mechanism of nucleation for *in vitro* collagen fibril formation. Biopolymers 16:2113-2131

Cooper, A. (1970). Thermodynamic studies of the assembly *in vitro* of native collagen fibrils. Biochem.J. 118:355-365

Crompton, M.R., Moss, S.E. and Crumpton, M.J. (1988). Diversity in the lipocortin/ calpactin family. Cell 55:1-3

Cutroneo, K.R., Sterling, K.M. and Shull, S. (1986). Steroid Hormone Regulation of Extracellular Matrix Proteins. In: Regulation of Matrix Accumulation, Mecham, R.P. editor. pp. 120-176. Academic press, New York

David, G. (1991). Biology and pathology of the pericellular heparan sulphate proteoglycans. Biochem.Soc.Trans. 19:816-820

David, G., Lories, V., Decock, B., Marynen, P., Cassimen, J-J. and van der Berghe, H. (1990). Molecular cloning of a phosphatidylinositol-anchored membrane heparan sulphate proteoglycan from human lung fibroblasts. J.Biol.Chem. 111:3165-3176

Davidson, J.M., McEneany, L.S.G. and Bornstein, P. (1977). Intermediates in the conversion of procollagen to collagen. Eur.J.Biochem. 81:349-355

Davidson, J.M., McEneany, L.S.G. and Bornstein, P. (1979). Procollagen processing: Limited proteolysis of COOH-terminal extension peptides by a cathepsin-like protease secreted by tendon fibroblasts. Eur.J.Biochem. 100:551

Dean, P. and Watson, D. (1978). Affinity chromatography of enzymes. In: Affinity Chromatography, Hoffmann-Ostenhof, O. editor. pp. 25-38. Pergamon press, New York

de Crombrugghe, B., Vuorio, T. and Karsenty, G. (1990). Control of type I collagen genes in scleroderma and normal fibroblasts. Rheum.Dis.Clin.N.Amer. 16:109-123

Delvoye, P., Mauch, C., Krieg, T. and Lapiere, C.M. (1986). Contraction of collagen lattices by fibroblasts from patients and animals with heritable disorders of connective tissue. Br.J.Derm. 115:139-146

Delvoye, P., Nusgens, B. and Lapiere, C.M. (1985). The capacity of retracting collagen matrix is lost by dermatosparactic skin fibroblasts. J.Invest.Derm. 81:267-270

de Wet, W., Bernard, M., Chanda, V., *et al.* (1987). Organisation of the human proα2 (I) collagen gene. J.Biol.Chem. 262:16032-16036

Dickson, L.A., Ninomiya, Y., Bernard, M.P., *et al.* (1981). The exon/ intron structure of the 3' region of the proα2 (I) collagen gene. J.Biol.Chem. 256:8407-8415

Dombrowski, K.E. and Prockop, D.J. (1988). Cleavage of type I and type II procollagens by type I/II procollagen N-proteinase. Correlation of kinetic constants with the predicted conformations of procollagen substrates. J.Biol.Chem. 263:16545-16552

Dombrowski, K.E., Vogel, B.E. and Prockop, D.J. (1989). Mutations that alter the primary structure of type I procollagen have long-range effects on its cleavage by procollagen N-proteinase. Biochemistry 28:7107-7112

Dublet, B., Oh, S., Sugrue, S.P., *et al.* (1989). The structure of avian type XII collagen. Alpha1(XII) chains contain 190-kDa non-triple helical amino-terminal domains and form homotrimeric molecules. J.Biol.Chem. 264:13150-13156

Dublet, B. and van der Rest, M. (1991). Type XIV collagen, a new homotrimeric molecule extracted from fetal bovine skin and tendon, with a triple helical disulfide-bonded domain homologous to type IX and type XII collagens. J.Biol.Chem. 266:6853-6858

Edwards, R. and Woody, R. (1977). Circular dichroism of dehydrogenase complexes with cibacron blue and congo red . Fed.Proc. 36:839-839

Eikenberry, E.F. and Brodsky, B. (1980). X-ray diffraction of reconstituted collagen fibers. J.Mol.Biol. 144:397-404

Elliott, A. and Offer, G. (1978). Shape and flexibility of the myosin molecule. J.Mol.Biol. 123:505-519

Engel, J. (1991). Domains in proteins and proteoglycans of the extracellular matrix with functions in assembly and cellular activities. Int.J.Biol.Macromol. 13:147-151

Engel, J. and Prockop, D.J. (1991). The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper (Review). Annu.Rev Biophys.Biophys.Chem. 20:137-152

Erdei, A. and Reid, K.B.M. (1988). The C1q receptor. Mol.Immunol. 25:1067-1073

Eskanasy, M., Mora, R. and Simionescu, N. (1984). *In vitro* study of low density lipoprotein-collagen interaction. Morphol.Emryol. 2:147-152

Eyre, D. and Wu, J-J. (1987). Type XI or 1α2α3α collagen. In: Structure and Function of Collagen Types, Mayne, R. and Burgeson, R.E. editors. pp. 261-281. Academic press, Orlando

Eyre, D.R., Wu, J-J. and Woods, P.E. (1991). The cartilage collagens: Structural and metabolic studies. J.Rheumatol. 18:49-51

Eyre, D.R., Paz, M.A. and Gallop, P.M. (1984). Cross-linking in collagen and elastin. Ann.Rev.Biochem. 53:717-748

Farquhar, M.G. (1991). The glomerular basement membrane: A selective macromolecular filter. In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 365-418. Plenum press, New York

Fernandez, M.P., Selmin, O., Martin, G.R., *et al.* (1988). The structure of anchorin CII, a collagen binding protein isolated from chondrocyte membrane. J.Biol.Chem. 263:5921-5925

Fernandez, M.P., Young, M.F. and Sobel, M.E. (1985). Methylation of type II and type I collagen genes in differentiated and dedifferentiated chondrocytes. J.Biol.Chem. 260:2374-2378

Fessler, J.H. and Fessler, L.I. (1978). Biosynthesis of procollagen. Ann.Rev.Biochem. 47:129-162

Fessler, J.H. and Fessler, L.I. (1987). Type V Collagen. In: Structure and Function of Collagen Types, Mayne, R. and Burgeson, R.E. editors.pp. 81-103. Academic press, Orlando

Fessler, L.I., Morris, N.P. and Fessler, J.H. (1975). Procollagen: biological scission of amino and carboxyl extension peptides. Proc.Natl.Acad.Sci.USA 72:4905-4909,

Fielder-Nagy, C., Bruckner, P., Hayashi, T. and Prockop, D.J. (1981). Isolation of unhydroxylated type I procollagen. Folding of the protein *in vitro*. Arch.Biochem.Biophys. 212:668-677

Fitch, J.M., Birk, D.E., Hasty, K.A., Mainardi, C. and Linsenmayer, T.F. (1988). Corneal collagen fibrils: dissection with specific collagenases and monoclonal antibodies. Invest.Opthal.Vis.Sci. 29:1125-1136

Fjotalstad, M., and Helle, O. (1974). A hereditary dysplasia of collagen tissue in sheep. J.Pathol. 112:183-188

Fleischmajer, R., Fisher, L.W., Macdonald, E.D., Jacobs, L., Perlish, J.S. and Termine, J.D. (1991). Decorin interacts with fibrillar collagen of embryonic and adult human skin. J.Struct.Biol. 106:82-90

Fleischmajer, R., Macdonald, E.D., Perlish, J.S., Burgeson, R.E. and Fisher, L.W.

Fleischmajer, R., Perlish, J.S., Burgeson, R.E., Shaikh-Bahal, F. and Timpl, R. (1990b). Type I and type III collagen interactions during fibrillogenesis. Ann.N.Y.Acad.Sci. 580:161-175

Fleischmajer, R., Perlish, J.S., Timpl, R. and Olsen, B.R. (1988). Procollagen intermediates during tendon fibrillogenesis. J.Histochem.Cytochem. 36:1425-1432

Fleischmajer, R., Timpl, R., Tuderman, L., *et al.* (1981). Ultrastructural identification of extension amino propeptides of type I and type III collagens in human skin. Proc.Natl.Acad.Sci.USA 78:7360-7364

Fleischmajer, R., Olsen, B.R., Timpl, R., Perlish, J.S. and Lovelace, O. (1983). Collagen fibril formation during embryogenesis. Proc.Natl.Acad.Sci.USA 80:3354-3358

Fleischmajer, R., Perlish, J.S. and Olsen, B.R. (1987). Amino and carboxyl propeptides in bone collagen fibrils during embryogenesis. Cell Tissue Res. 247:105-109

Fleischmajer, R., Perlish, J.S. and Timpl, R. (1985). Collagen fibrillogenesis in human skin. Ann.N.Y.Acad.Sci. 460:246-257

Fowler, W.E., and Aebi, U. (1983). Preparation of single molecules and supramolecular complexes for high resolution metal shadowing. J.Ultrastruct.Res. 83:319-334

Fraser, R.D.B. and MacRae, T.P. (1981). Unit cell and molecular connectivity in tendon collagen. Int.J.Biol.Macromol. 3:193-200

Fraser, R.D.B., MacRae, T.P. and Miller, A. (1987). Molecular packing in type I collagen fibrils. J.Mol.Biol. 193:115-125

Freedman, R.B., Bullied, N.J., Hawkins, H.C. and Paver, J.L. (1989). Role of protein disulphide isomerase in the expression of native proteins. Biochem.Soc.Symp. 55:167-192

Fruton, C. (1974). Protease and biological control. In: , Reich, E.,, Rifkin, D.B., and Show, E. editors. pp. 33-50.

Fuller, F. and Boedtker, H. (1981). Sequence determination and analysis of the 3' region of the chicken proα1 (I) and proα2 (I) collagen messenger ribonucleic acids including the carboxyl sequences. Biochemistry 20:996-1006

Furthmayr, H. and Madri, J.A. (1982). Rotary shadowing of connective tissue macromolecules. Collagen.Rel.Res. 2:349-363

Furthmayr, H. and Timpl, R. (1971). Characterisation of collagen peptides by sodium dodecyl sulphate-polyacrylamide electrophoresis. Anal.Biochem. 41:510-516

Gallagher, J.T., Turnbull, J.E. and Lyon, M. (1990). Heparan sulphate proteoglycans. Biochem.Soc.Trans. 18:207-209

Glanville, R.W. (1987). Type IV Collagen. In: Structure and Function of Collagen Types, Mayne, R. and Burgeson, R.E. editors. pp. 43-79. Academic press, Orlando

Geetha-Habid, M., Noiva, R., Kaplan, H.A. and Lennarz, W.J. (1988). Glycosylation site binding protein. A component of oligosaccharide transferase is highly similar to three other 57 kDa luminal proteins of the ER. Cell 54:1053-1060

Gelman, R.A. and Piez, K.A. (1980). Collagen fibril formation *in vitro*. A quasielastic light scattering study of early stages. J.Biol.Chem. 255:8098-8102

Genge, R.B., Cao, X., Wu, L.N.Y., Buzzi, W.R., Showman, R.W., Arsenault, A.L., Ishikawa, Y. and Wuthier, R.E. (1992). Establishment of the primary structure of the major lipid-dependent Ca^{2+} binding proteins of chicken growth plate cartilage matrix vesicles. Identity with anchorin CII (annexin V) and annexin II. J.Bone Min.Res. 7:807-819

Genge, B.R., Wu, L.N.Y. and Wuthier, R.E. (1989). Identification of phospholipid-dependent calcium-binding proteins as constituents of matrix vesicles. J.Biol.Chem. 264:10917-10921

Genge, B.R., Wu, L.N.Y. and Wuthier, R.E. (1990). Differential fractionation of matrix vesicle proteins: Further characterisation of the acidic phospholipid-dependent Ca^{2+}-binding proteins. J.Biol.Chem. 265:4703-4710

Gennis, R.B. Biomembranes, New York:Springer-Verlag, (1989). pp. 1-533.

Gerstenfeld, L., Beldekas, J.C., Sonenshein, G.E. and Franzblau, C. (1984). Processing of procollagen types III and I in cultured bovine smooth muscle cells. J.Biol.Chem. 259:9158-9162

Glenney, J.R., Glenney, P. and Weber, K. (1981). Mapping the fodrin molecule with monoclonal antibodies: A general approach for rod-like multidomain proteins. J.Mol.Biol. 167:275-293

Glimcher, M.J. (1989). Mechanism of calcification: Role of collagen fibrils and collagen-phosphoprotein complexes *in vitro* and *in vivo*. Anat.Rec. 224:139-153

Goldberg, B., Taubman, M.B. and Radin, A. (1975). Procollagen peptidase: Its mode of action on the native substrate. Cell 4:45-50

Goldberg, B.D. (1979). Binding of soluble type I collagen molecules to the fibroblast plasma membrane. Cell 16:265-275

Goldberg, B.D. and Burgeson, R.E. (1982). Binding of soluble type I collagen to fibroblasts: specificities for native collagen types, triple helical structure, telopeptides, propeptides and cyanogen bromide derived peptides. J.Cell.Biol. 95:752-756

Goldstein, I.J., Hollerman, C.E. and Smith, E.E. (1965). Protein-carbohydrate interaction. Inhibition studies on the interaction of concanavalin A with polysaccharides. Biochemistry 4:876-883

Gong, Q.H., Fukuda, T., Parkinson, C. and Cheng, S.Y. (1988). Nucleotide sequence of a full length cDNA clone encoding a mouse cellular thyroid hormone binding protein (p55) that is homologous to protein disulphide isomerase and the β-subunit of prolyl 4-hydroxylase. Nucleic Acids Res. 16:1203-1200

Gordon, M.K., Castagnola, P., Dublet, B., *et al.* (1991). Cloning of a cDNA for a new member of the class of fibril-associated collagens with interrupted triple helices. Eur J Biochem. 201:333-338

Gordon, M.K., Gerecke, D.R., Dublet, B., van der Rest, M. and Olsen, B.R. (1989). Type XII collagen. A large multidomain molecule with partial homology to type IX collagen. J.Biol.Chem. 264:19772-19778

Gordon, M.K., Gerecke, D.R., Dublet, B., van der Rest, M., Sugrue, S.P. and Olsen, B.R. (1990). The structure of type XII collagen. Ann.N.Y.Acad.Sci. 580:8-16

Gordon, M.K., Gerecke, D.R. and Olsen, B.R. (1987). Type XII collagen: Distinct extracellular matrix component discovered by cDNA cloning. Proc.Natl.Acad.Sci.USA 84:6040-6044

Gordon, M.K., and Olsen, B.R. (1990). The contribution of collagenous proteins to tissue-specific matrix assemblies. Curr.Opin.Cell Biol. 2:833-838

Gross, J. and Bruns, R.R. (1984). Another look at fibrillogenesis. In: The Role of Extracellular Matrix in Development, Trelstad, R.L. editor. pp. 479-512. Alan R. Liss, New York

Gross, J. and Nagai, Y. (1965). Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. Proc.Natl.Acad.Sci.USA 54:1197-1204

Haas, C., Voss, T. and Engel, J. (1991). Assembly and disulphide rearrangement of recombinant surfactant protein A *in vitro*. Eur.J.Biochem. 197:799-803

Haigler, H.T., Fitch, J.M., Jones, J.M. and Schlaepfer, D.D. (1989). Two lipocortin-like proteins, endonexin II and anchorin CII, may be alternative splices of the same gene. TIBS 14:48-50

Hale, J.E., and Wuthier, R.E. (1987). The mechanism of matrix vesicle formation: Studies on the composition of chondrocyte microvilli and on the effects of microfilament-perturbing agents on cellular vesiculation. J.Biol.Chem. 262:1916-1925

Halila, R. and Peltonen, L. (1986). Purification of human procollagen type III N-proteinase from placenta and preparation of antiserum. Biochem.J. 239:47-52

Hall, C.E. (1955). Electron densitometry of stained virus particles. J.Biophys.Biochem.Cytol. 1:1-11

Hall, C.E. (1956). Visualisation of individual molecules with the electron microscope. Proc.Natl.Acad.Sci.USA 42:801-806

Hamalainen, K., Oikarinen, J. and Kivirikko, K.I. (1985). Synthesis and degradation of type I procollagen mRNAs in cultured human skin fibroblasts and the effect of cortisol. J.Biol.Chem. 260:720-725

Harris, A.K., Stopak, D. and Wild, P. (1981). Fibroblast traction as a mechanism for collagen morphogenesis. Nature 290:249-251

Harris, E.D., Welgus, H.G. and Krane, S.M. (1984). Regulation of the mammalian collagenases. Collagen Rel.Res. 4:493-512

Hartung, S., Jaenisch, R. and Breindl, M. (1986). Retrovirus insertion inactivates mouse α1(I) collagen gene by blocking initiation of transcription. Nature 320:365-367

Haverstick, D.M. and Glaser, M. (1987). Visualisation of Ca^{2+}-induced phospholipid domains. Proc.Natl.Acad.Sci.USA 84:4475-4479

Hay, E.D. (1991). Collagen and Other Matrix Glycoproteins in Embryogenesis. In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 419-462. Plenum press, New York

Heinegard, D., Björne-Perrson, A., Cöster, L., *et al.* (1985). The core protein of large and small interstitial proteoglycans from various connective tissues from distinct subgroups. Biochem.J. 230:181-194

Helseth, D.L. and Veis, A. (1981). Collagen self Assembly *in vitro*: Differentiating specific telopeptide-dependent interactions using selective enzyme modifications and the addition of free amino telopeptide. J.Biol.Chem. 256:7118-7128

Helseth, D.L. and Veis, A. (1984). Cathepsin D-mediated processing of procollagen: lysosomal enzyme involvement in secretory processing of procollagen. Proc.Natl.Acad.Sci.USA 81:3302-3306

Henkel, W. and Glanville, R.W. (1982). Covalent cross-linking between molecules of type I and type III collagen. The involvement of the N-terminal non-helical regions of the α1 (I) and α1 (III) chains in the formation of intermolecular cross-links. Eur.J.Biochem. 122:205-213

Hinek, A., Reiner, A. and Poole, R. (1987).The calcification of cartilage matrix in chondrocyte culture: Studies of the C-propeptide of type II collagen (chondrocalcin). J.Cell.Biol. 104:1435-1441

Hodge, A.J. and Petruska, J.A. (1963). Recent studies with the electron microscope on ordered aggregates of the tropocollagen macromolecule. In: Aspects of protein structure, Ramachandran, G.N. editor. pp. 289-300. Academic press, London and New York

Hodge, A.J. and Schmitt, F.O. (1960). The charge profile of the tropocollagen macromolecule and the packing arrangement in native type collagen fibrils. Proc.Natl.Acad.Sci.USA 46:186-197

Hojima, Y., McKenzie, J., van der Rest, M. and Prockop, D.J. (1989). Type I procollagen N-proteinase from chick embryo tendons. Purification of a new 500-kDa form of the enzyme and identification of the catalytically active polypeptides. J.Biol.Chem. 264:11336-11345

Hojima, Y., van der Rest, M. and Prockop, D.J. (1985). Type I procollagen carboxyl-terminal proteinase from chick embryo tendons. J.Biol.Chem. 260:15596-16003

Horlein, D., McPherson, J., Goh, S.H. and Bornstein, P. (1981). Regulation of protein synthesis: translational control by procollagen derived fragments. Proc.Natl.Acad.Sci.USA 78:6163-6167

Horton, W., Miyashita, T., Kohno, K., Hassel, J.R. and Yamada, Y. (1987). Identification of a phenotype-specific enhancer in the first intron of the rat collagen II gene. Proc.Natl.Acad.Sci.USA 84:8864-8868

Hostikka, S.L., Eddy, R.L., Byers, M.G., Hoyhtya, M., Shows, T.B. and Tryggvason, K. (1990). Identification of a distinct type IV collagen alpha chain with restricted kidney distribution and assignment of its gene to the locus of X chromosome-linked Alport Syndrome. Proc.Nat.Acad.Sci.Usa. 87:1606-1610

Houslay, M.D. and Stanley, K.K. Dynamics of Biological Membranes, Chichester: Wiley, (1982)

Hukins, D.W.L. and Woodhead-Galloway, J. (1977). Collagen fibrils as examples of smectic A biological fibres. Mol.Cryst.Liq.Cryst. 41:33-39

Hulmes, D.J.S. (1983). A possible mechanism for the regulation of collagen fibril diameter in vivo. Collagen Rel.Res. 3:317-321

Hulmes, D.J.S. (1992). The collagen superfamily-diverse structures and assembly. Essays in Biochemistry 27:49-67

Hulmes, D.J.S., Holmes, D.F. and Cummings, C. (1985). Crystalline regions in collagen fibrils. J.Mol.Biol. 184:473-477

Hulmes, D.J.S., Jesior, J-C., Miller, A., Berthet-Colominas, C. and Wolff, C. (1981). Electron microscopy shows periodic fibril structure in collagen fibril cross-sections. Proc.Natl.Acad.Sci.USA 78:3567-3571

Hulmes, D.J.S., Kadler, K.E., Mould, A.P., *et al.* (1989a). Pleomorphism in type I collagen fibrils produced by persistence of the procollagen N-propeptide. J.Mol.Biol. 210:337

Hulmes, D.J.S. and Miller, A. (1979). Quasi-hexagonal packing in collagen fibrils. Nature 282:878-880

Hulmes, D.J.S., Miller, A., Parry, D.A.D., Piez, K.A. and Woodhead-Galloway, J. (1973). Analysis of the primary structure of collagen for the origins of molecular packing. J.Mol.Biol. 79:137-148

Hulmes, D.J.S., Miller, A., White, S.W., Timmins, P.A. and Berthet-Colominas, C. (1980). Interpretation of the low-angle meridional neutron diffraction patterns from collagen fibres in terms of the amino acid sequence. Int.J.Biol.Macromol. 2:338-346

Hulmes, D.J.S., Mould, A.P., Kadler, K.E., Chapman, J.A. and Prockop, D.J. (1989b). Procollagen processing control of type I collagen fibril assembly. In: Cytoskeletal and Extracellular Proteins, Aebi, U. and Engel, J. editors. pp. 292-301.Springer, Berlin

Humphries, M.J. (1990). The molecular basis and specificity of integrin-ligand interactions. J.Cell Sci. 97:585-592

Huxley, H.E., and Zubay, G. (1960). Electron microscope observations on the structure of microsomal particles from *Escerichia coli*. J.Mol.Biol. 2:10-18

Ignatius, M.J., Large, T.H., Houde, M., *et al.* (1990). Molecular cloning of the rat integrin α1-subunit: A receptor for laminin and collagen. J.Cell.Biol. 111:709-720

Jeffrey, J.J. (1986). The Biological Regulation of Collagenase Activity. In: Regulation of Matrix Accumulation, Mecham, R.P. editor. pp. 53-98. Academic press

Jones, E.Y. and Miller, A. (1991). Analysis of structural design features in collagen. J.Mol.Biol. 218:209-219

Kadler, K.E., Hojima, Y. and Prockop, D.J. (1987). Assembly of collagen fibrils *de Novo* by cleavage of the type I pC-collagen with procollagen C-proteinase. Assay of critical concentration demonstrates that collagen self-assembly is a classical example of an entropy-driven process. J.Biol.Chem. 262:15696-15701

Kadler, K.E., Hojima, Y. and Prockop, D.J. (1988). Assembly of Type I Collagen Fibrils *de Novo*. Between 37 and 41 °C the process is limited by micro-unfolding of monomers. J.Biol.Chem. 263:10517-10523

Kadler, K.E., Hojima, Y. and Prockop, D.J. (1990). Collagen fibrils in vitro grow from pointed tips in the C- to N-terminal direction. Biochem.J. 268:339-343

Kadler, K.E., Torreblanco, A., Adachi, E., Vogel, B.E., Hojima, Y. and Prockop, D.J. (1991). A type-I collagen with substitution of a cysteine for glycine-748 in the α1(I) chain copolymerizes with normal type-I collagen and can generate fractallike structures. Biochemistry 30:5081-5088

Kagan, H.M. and Trackman, P.C. (1991). Properties and function of lysyl oxidase. Amer.J Respir.Cell Molec.Biol. 5:206-210

Kato, Y.P., Christiansen, D.L., Hahn, R.A., Shieh, S.J., Goldstein, J.D. and Silver, F.H. (1989). Mechanical properties of collagen fibres: a comparison of reconstituted and rat tail tendon fibres. Biomaterials. 10:38-42

Keene, D.R., Sakai, L.Y., Bachinger, H.P. and Burgeson, R.E. (1987). Type III collagen can be present on banded collagen fibrils regardless of fibril diameter. J.Cell.Biol. 105:2393-2402

Kessler, E. and Adar, R. (1989). Type I procollagen C-proteinase from mouse fibroblasts-purification and demonstration of a 55-kDa enhancer glycoprotein. Eur.J.Biochem. 186:115-121

Kessler, E., Adar, R., Goldberg, B. and Niece, R. (1986). Partial purification and characterization of a procollagen C-proteinase from the culture medium of mouse fibroblasts. Collagen Rel.Res. 6:249-266

Kessler, E. and Goldberg, B. (1978). A method for assaying the activity of the endopeptidase which excises the nonhelical carboxylterminal extensions from type I procollagen. Anal.Biochem. 86:463-469

Khillan, J.S., Schmidt, A., Overbeek, P.A., de Crombrugghe, B. and Westphal, H. (1986). Developmental and tissue-specific expression directed by the α2 type I collagen promoter in transgenic mice. Proc.Natl.Acad.Sci.USA 83:725-729

King, R.J., Carmichael, M.C. and Horowitz, P.M. (1983). Reassembly of lipid-protein complexes of pulmonary surfactant. J.Biol.Chem. 258:10672-10680

Kiovu, J., Myllylä, R., Helaaskoski, T., Pihlajaniemi, T., Tasanen, K. and Kivirikko, K.I. (1987). A single polypeptide acts both as the β-subunit of prolyl 4-hydroxylase and as a protein disulphide isomerase. J.Biol.Chem. 262:6447-6449

Kivirikko, K. and Myllylä, R. (1982). Post-Translational Modifications. In: Collagen in Health and Disease, Weiss, J.B. and Jayson, M.V. editors. pp. 101-120.Churchill Livingstone, Edinburgh

Kivirikko, K.I., Helaakoski, T., Tasanen, K., *et al.* (1990). Molecular biology of prolyl 4-hydroxylase. Ann.N.Y.Acad.Sci. 580:132-142

Kivirikko, K.I. and Myllylä, L. (1979). Collagen glycosyltransferases. Int.Rev.Connect.Tissue Res. 8:23-72

Kivirikko, K.I. and Myllylä, R. (1980). The hydroxylation of prolyl and lysyl residues. In: The Enzymology of Posttranslational Modification of Proteins, Friedman, R.B. and Hawkins, H.C. editors. pp. 53-104. Academic press, New York

Kivirikko, K.I. and Myllylä, R. (1984). Biosynthesis of collagen. In: Extracellular Matrix Biochemistry, Piez, K.A. and Reddi, A.H. editors. pp. 83-118.Elsevier, New York

Kivirikko, K.I. and Myllylä, R. (1985). Post-translational processing of procollagens. Ann.N.Y.Acad.Sci. 460:188-201

Koa, W.W-Y., Mai, S.H., Chou K-L.L. and Ebert, J. (1983). Mechanism for the regulation of post-translational modifications of procollagen synthesised by matrix free cells from chick embryos. J.Biol.Chem. 258:7779-7787

Koda, J.E., Repraeger, A. and Bernfield, M. (1985). Heparan sulphate proteoglycans from mouse epithelial cells: Cell surface proteoglycan as a receptor for interstitial collagens. J.Biol.Chem. 260:8157-8162

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. and Kreiger, M. (1990). Type I macrophage scavenger receptor contains α-helical and collagen- like coiled coils. Nature 343:531-535

Kohno, K., Sullivan, M. and Yamada, Y. (1985). Structure of the promoter of the rat type II procollagen gene. J.Biol.Chem. 260:4441-4447

Koller, T., Kübler, O., Portmann, R. and Sogo, J.M. (1978). High resolution physical mapping of specific binding sites of Escherichia Coli RNA polymerase on the DNA of bacteriophage T7. J.Mol.Biol. 120:121-131

Krane, S.M., Conca, W., Stephenson, M.L., Amento, E.P. and Goldring, M.B. (1990). Mechanisms of Matrix Degradation in Rheumatoid Arthritis. Ann.N.Y.Acad.Sci. 580:340-354

Krejci, E., Coussen, F., Duval, N., *et al.* (1991). Primary structure of a collagenic tail peptide of Torpedo acetylcholinesterase: co-expression with catalytic subunit induces the production of collagen-tailed forms in transfected cells. EMBO J. 10:1285-1293

Kubo, K., and Takagi, T. (1984). The α1 (I) and α2 (I) chains of collagen separate in sodium dodecyl sulphate poltacrylamide gel electrophoresis due to differences in sodium dodecyl sulphate binding capacities. Collagen Rel.Res. 4:201-208

Kuhn, K. (1987).The Classical Collagens: Types I, II, AND III. In: Structure and Function of Collagen Types, Mayne, R. and Burgeson, R.E editors. pp. 1-42. Academic press, Orlando

Kuivaniemi, H., Tromp, G. and Prockop, D.J. (1991). Mutations in collagen genes - causes of rare and some common diseases in humans (Review). FASEB J. 5:2052-2060

Kuroki, K. and Akino, T. (1991). Roles of collagenous domain and oligosaccharide moiety of pulmonary surfactant protein A in interactions with phospholipids. Biochemistry International 24:225-234

Kwan, A.P.L., Cummings, C.E., Chapman, J.A. and Grant, M.E. (1991). Macromolecular organization of chicken type-X collagen *in vitro*. J Cell Biol. 114:597-604

Labat-Robert, J., Bihari-Varga, M. and Robert, L. (1990). Extracellular matrix. FEBS Lett. 268:386-393

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680-685

Laskey, R.A., and Mills, A.D. (1975). Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur.J.Biochem. 56:335-341

Last, J.A., Armstrong, L.G. and Reiser, K.M. (1990). Biosynthesis of collagen crosslinks (Minireview). Int.J.Biochem. 22:559-564

Lawler, J., and Hynes, R.O. (1986). The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. J.Cell.Biol. 103:1635-1648

Layman, D.L. (1981). Detection of cell-associated aminoterminal procollagen peptidase activity in cultured fibroblasts. Proc.Soc.Exp.Biol.Med. 166:325-329

Leblond, C.P., and Wright, G.M. (1981). Steps in elaboration of collagen by odontoblasts and osteoblasts. Basic mechanisms of cellular secretion. Methods Cell Biol. 23:167-189

Lee, Y-M., Leiby, K.R., Allar, J., Paris, K., Lerch, B. and Okarma, T.B. (1991). Primary structure of bovine conglutinin, a member of the C-type animal lectin family. J.Biol.Chem. 266:2715-2723

le Lous, M., Boudin, D., Salmon, S. and polonovski, J. (1982). The affinity of type I collagen for lipid *in vitro*. Biochim.Biophys.Acta 708:26-32

Lentz, B.R., Hoechi, M. and Barenholz, Y. (1981). Acyl chain order and lateral domain formation in mixed phosphatidylcholine-sphingomyelin multilamellar and unilamellar vesicles. Biochemistry 20:6803-6809

Leung, M.K.K., Fessler, L.I., Greenberg, D.B. and Fessler, J.H. (1979). Separate amino and carboxyl procollagen peptidases in chick embryo tendon. J.Biol.Chem. 254:224-232

Lichtenstein, J.R., Martin, G.R., Kohn, L.D., Byers, P.H. and McKusick, V.A. (1973). Defect in conversion of procollagen to collagen in a form of Ehlers-Danlos Syndrome. Science 182:298-300

Limeback, H.F. and Sodek, J. (1979). Procollagen synthesis and processing in periodontal ligament in vivo and in vitro. Eur.J.Biochem. 100:541-550

Linder, R., Bernheimer, A.W. and Kim, K-S. (1977). Interaction between SPM and a cytosolin from the sea anemone *Stoichactis helianthus*. Biochim.Biophys.Acta 467:290-300

Linsenmayer, T.F. (1991). What is extracellular matrix ? In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 7-44. Plenum press, New York

Linsenmayer, T.F., Fitch, J.M. and Birk, D.E. (1990a). Heterotypic collagen fibrils and stabilising collagens: Controlling elements in corneal morphogenesis. Ann.N.Y.Acad.Sci. 580:143-160

Linsenmayer, T.F., Fitch, J.M., Gross, J. and Mayne, R. (1985). Are collagen fibrils in the developing avian cornea composed of two different collagen types? Evidence from monoclonal antibody studies. Ann.N.Y.Acad.Sci. 460:231-245,

Livne, E., Oliver, C., Leapman, R.D., Rosenberg, L.C., Poole, A.R. and Silbermann, M. (1987). Age-related changes in the role of matrix vesicles in the mandibular condylar cartilage. J.Anat. 150:61-74,

Lu, J., Thiel, S., Wiedemann, H., Timpl, R. and Reid, K.B.M. (1990). Binding of the pentamer/ hexamer forms of mannan-binding protein to zymosan activates the proenzyme $C1r_2C1s_2$ complex of the classical pathway of complement without involvement of C1q. J.Immunol. 144:2287-2295

Maity, S.N., Golumbek, P.T., Karsenty, G. and de Crombrugghe, B. (1988). Selective activation of transcription by a novel CCAAT binding factor. Science 241:582-585

Maksymiw, R., Sui, S.F., Gaub, H. and Sackmann, E. (1987). Electrostatic coupling of spectrin dimers to phosphatidylserine containing lipid lamellae. Biochemistry 26:2983-2990

Malhotra, R., Thiel, S., Reid, K.B.M. and Sim, R.B. (1990). Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J.Exp.Med. 172:955-959

Marchi, F. and Leblond, C.P. (1983). Collagen biogenesis and assembly into fibrils as shown by ultrastructural and [^{3}H]proline radioautographic studies on the fibroblasts of the rat foot pad. Am.J.Anat. 168:167-197

Marchi, F. and Leblond, C.P. (1984). Radioautographic charaterisation of successive compartments along the rough endoplasmic reticulum-golgi pathway of collagen precursors in foot pad fibroblasts of [^{3}H]proline injected rats. J.Cell Biol. 98:1705-1709

Marchini, M., Morocutti, M., Ruggeri, A., Koch, M.H.J., Biji, A. and Roveri, N. (1986). Differences in the fibril structure of corneal and tendon collagen. An electron microscopy and X-ray diffraction investigation. Conn.Tiss.Res. 15:269-281

Marchini, M. and Ruggeri, A. (1984). Ultrastructural aspects of freeze-etched collagen fibrils. In: Ultrastructure of the Connective Tissue Matrix, Ruggeri, A. and Motta, P.M. editors. pp. 89-94. Martinus Nijhoff, Boston

Marti, T., Kosen, P.A., Honory, K., *et al.* (1984). The structure of the carbohydrate units of the carboxyl-terminal peptide of procollagen as elucidated by 500 MHz 1H-NMR spectroscopy. Biochim.Biophys.Acta 779:305-312

Martinez del Pozo, A., Onaderra, M., Laynez, J. and Gavilanes, J. (1988).Interaction of type I collagen with phosphatidylcholine vesicles. Collagen Rel.Res. 8:133-144

Martinez del Pozo, A., Onaderra, M., Laynez, J. and Gavilanes, J. (1989). Interaction of type I collagen fibrils with phospholipid vesicles. Matrix 9:405-410

Marynen, P., Zhang, J., Cassiman, J-J., van der Berghe, H. and David, G. (1989). Partial primary structure of the 48- and 90-kDa core proteins of cell surface associated heparan sulphate proteoglycans of lung fibroblasts. Prediction of an integral membrane domain and evidence for multiple distinct core proteins at the cell surface of human lung fibroblasts. J.Biol.Chem. 262:7017-7024

Massoulie, J. (1980). The polymorphism of cholinesterases and its physiological significance. TIBS 5:160-164

Mauch, C., von der Mark, K., Helle, O., Mollenhauer, J., Pfäffle, M. and Krieg, T. (1988). A defective cell surface collagen-binding protein in dermatosparactic sheep fibroblasts. J.Cell.Biol. 106:205-211

Mayne, R. and Burgeson, R.E. Structure and Function of Collagen Types, Orlando:Academic press, (1987). pp. 1-317.

McKeon, C., Ohkubo, H., Pastan, I. and de Crombrugghe, B. (1982). Unusual methylation pattern of the α2 (I) collagen gene. Cell 29:203-210

McPherson, J.M., Hörlein, D., Abbot-Brown, D. and Bornstein, P. (1982). Inhibition of protein synthesis *in vitro* by procollagen derived fragments is associated with changes in protein phosphorylation. J.Biol.Chem. 257:8557-8560

Mecham, R.P. and Heuser, J.E. (1991). The Elastic Fibre. In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 79-106. Plenum press, New York

Meek, K.M., Chapman, J.M. and Hardcastle, R.A. (1979). The staining pattern of collagen fibrils. Improved correlation with sequence data. J.Biol.Chem. 254:10710-10714

Mellor, S.J., Atkins, G.L. and Hulmes, D.J.S. (1991). Developmental changes in the type-I procollagen processing pathway in chick-embryo cornea. Biochem.J 276:P3, 777-784

Mendler, M., Eich-Bender, S.G., Vaughan, L., Winterhalter, K.H. and Bruckner, P. (1989). Cartilage contains mixed fibrils of collagen types II, IX, and XI. J.Cell Biol. 108:191-197

Miller, E.J. (1985). The structure of the fibril forming collagens. Ann.N.Y.Acad.Sci 460:1-13

Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981). Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. Biochemistry 20:833-840

Miyahara, M., Hayashi, K., Berger, J., *et al.* (1984). Formation of collagen fibrils by enzymic cleavage of precursors of type I collagen *in vitro*. J.Biol.Chem. 259:9891-9898

Miyahara, M., Njieha, F.K. and Prockop, D.J. (1982). Formation of collagen fibrils *in vitro* by cleavage of procollagen with procollagen proteinases. J.Biol.Chem. 257:8442-8448

Mollenhauer, J. and von der Mark, K. (1983). Isolation and characterisation of a collagen-binding glycoprotein from chondrocyte membranes. EMBO Journal 2:45-50

Montessuit, C., Caverzasio, J. and Bonjour, J-P. (1991). Characterisation of a Pi transport system in cartilage matrix vesicles: Potential role in the calcification process. J.Biol.Chem. 266:17791-17797

Morikawa, T., Tuderman, L. and Prockop, D.J. (1980). Inhibitors of procollagen N-protease. Synthetic peptides with sequences similar to the cleavage site in the proα1 (I) chain. Biochemistry 19:2646-2650

Morris, N.P., Fessler, L.I., Weinstock, A. and Fessler, J.H. (1975). Procollagen secretion and assembly in embryonic chick bone. J.Biol.Chem. 250:5719-5726,

Mould, A.P. and Hulmes, D.J.S. (1987). Surface induced aggregation of type I procollagen. J.Mol.Biol. 195:543-553

Mould, A.P., Hulmes, D.J.S., Holmes, D.F., Cummings, C., Sear, C.H.J. and Chapman, J.A. (1990). D-periodic assemblies of type I procollagen. J.Mol.Biol. 211:581-594

Mould, P.A., Holmes, D.F., Kadler, K.E. and Chapman, J.A. (1985). Mica sandwich technique for preparing macromolecules for rotary shadowing. J.Ultrastruct.Res. 91:66-76

Muragaki, Y., Jacenko, O., Apte, S., Mattei, M.G., Ninomiya, Y. and Olsen, B.R. (1991). The α2(VIII) collagen gene. A novel member of the short chain collagen family located on the human chromosome 1. J.Biol.Chem. 266:7721

Murphy, G., Hembry, R.M., Hughes, C.E., Fosang, A.J. and Hardingham, T.E. (1990). Role of regulation of metalloproteinases in connective tissue turnover. Biochem.Soc.Trans. 18:812-815

Murphy, G., Nagase, H. and Brinckerhoff, C.E. (1988). Relationship of procollagenase activator, stromelysin and matrix metalloproteinase. Collagen Rel.Res. 8:389-391

Myers, J.C., and Emmanuel, B.S. (1987). Chromosomal localisation of human collagen genes. Collagen Rel.Res. 7:149-159

Na, G.C. (1989a). Monomer and oligomer of type I collagen: molecular properties and fibril assembly. Biochemistry 28:7161-7167

Na, G.C., Butz, L.J. and Carroll, R.J. (1986). Mechanism of *in vitro* collagen fibril assembly. Kinetic and morphological studies. J.Biol.Chem. 261:12290-12299

Na, G.C., Phillips, L.J. and Freire, E.I. (1989b). *In vitro* Collagen Fibril Assembly: Thermodynamic studies. Biochemistry 28:7153-7161

Nandan, D., Cates, G.A., Ball, E.H. and Sanwal, B.D. (1988). A collagen-binding protein involved in the differentiation of myoblasts recognizes the Arg-Gly-Asp sequence. Exp.Cell Res. 179:289-297

New, R.R.C. Liposomes, Oxford:IRL press, (1990). pp. 1-301.

Nikkari, T. and Heikkinen, E. (1968). Lipids of collagen preparations. Acta Chem.Scand. 22:3047-3069

Ninomiya, Y., Showalter, A.M., van der Rest, M., Seidah, N.G., Chretien, M. and Olsen, B.R. (1984). Structure of the carboxyl propeptide of chicken type II procollagen determined by DNA and protein sequence analysis. Biochemistry 23:617-624

Ninomiya, Y.P., Castagnola, D., Gercke, D., *et al.* (1990). The molecular biology of collagens with short triple helical domains . In: Extracellular Matrix Genes, Sandell, L., and Boyd, C.D. editors. pp. 79-125. Academic press, New York

Nist, C., von der Mark, K., Hay, E.D., *et al.* (1975). Localisation of procollagen in chick corneal and tendon fibroblasts with ferritin-conjugated antibodies. J.Cell.Biol. 65:75-87

Nittmann, J. and Stanley, H.E. (1986). Tip splitting without interfacial tension and dendritic growth patterns arising from molecular anisotropy. Nature 321:663-668

Njieha, F.K., Morikawa, T., Tuderman, L. and Prockop, D.J. (1982). Partial purification of a procollagen C-proteinase. Inhibition by synthetic peptides and sequential cleavage of type I procollagen. Biochemistry 21:757-764

Nusgens, B.V., Goebels, Y., Shinkai, H. and Lapiere, C.M. (1980). Procollagen type III N-terminal endopeptidase in fibroblast culture. Biochem.J. 191:699-706

Öbrink, B. (1973). A study of the interactions between monomeric tropocollagen and glycosaminoglycans. Eur.J.Biochem. 33:387-400

O'Hara, P.J., Read, W.K., Romane, W.M. and Bridges, C.H. (1970). A collagenous tissue dysplasia of calves. Lab.Invest. 23:307-314

Olsen, B.R. (1991). Collagen Biosynthesis . In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 177-220.Plenum press, New York

Oomen, R.P., and Kaplan, H. (1987). Competitive labeling as an approach to defining the binding surfaces of proteins: Binding of monomeric insulin to lipid bilayers. Biochemistry 26:303-308

Oosawa, F. and Asakura, S. Thermodynamics of the Polymerisation of Protein, London:Academic press, (1975). pp. 1-204.

Özgunes, N. and Artvinli, S. (1988). Important data in understanding the interaction between the cell and collagen. Biochem.Med.Met.Biol. 40:269-275

Paglia, L.M., Wiestner, M., Duchene, M., *et al.* (1981). Effects of procollagen peptides on the translation of type II collagen messenger ribonucleic acid and on collagen biosynthesis in chondrocytes. Biochemistry 20:3523-3527

Pajean, M., Huc, A. and Herbage, D. (1991). Stabilisation of liposomes with collagen. Int.J.Pharm. 77:31-40

Parry, D.A.D and Craig, A.S (1979). Electron microscope evidence for an 80 Å unit in collagen fibrils. Nature 282:213-215

Payne, K.J., King, A. and Holmes, D.F. (1986). Collagen fibrillogenesis *in vitro*: an investigation of the thermal memory effect and of the early events occurring during fibril assembly using dynamic light scattering. Biopolymers 25:1185-1207

Peltonen, L., Halila, R. and Ryhanen, L. (1985). Enzymes converting procollagens to collagens. J.Cell.Biochem. 28:15-21

Penttinen, R.P., Kobayashi, S. and Bornstein, P. (1988).Transforming growth factor-β increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. Proc.Natl.Acad.Sci.USA 85:1105-1108

Perez-Castiñeira, J.R. and Apps, D.K. (1990). Vacuolar H^+-ATPase of adrenal secretory granules. Rapid partial purification and reconstitution into proteoliposomes. Biochem.J. 271:127-131

Perlish, J.S., Timpl, R. and Fleischmajer, R. (1985). Collagen synthesis regulation by the aminopropeptide of procollagen I in normal and scleroderma fibroblasts. Arthritis Rheum. 28:647-651

Pesciotta, D.M., Dickson, L.A., Showalter, A.M., *et al.* (1981). Primary structure of the carbohydrate-containing regions of the carboxyl-propeptides of type I procollagen. FEBS lett. 125:170-174

Pfäffle, M., Ruggiero, F., Hofmann, H., *et al.* (1988). Biosynthesis, secretion and extracellular localisation of anchorin CII, a collagen-binding protein of the calpactin family. EMBO 7:2335-2342

Phelps, R.G., Martinez-Hernandez, A. and Goldberg, B.D. (1985). Ultrastructural immunocytochemical localisation of type I procollagen in cultured human fibroblasts. Collagen Rel.Res. 5:405-414

Piérard, G.E., Lê, T., Herman, J-F., Nusgens, B.V. and Lapiére, C.M. (1986). Morphometric study of cauliflower collagen fibrils in dermatosparaxis of the calves. Collagen Rel.Res. 6:481-492

Piez, K.A. (1984). Molecular and Aggregate Structures of the Collagens. In: Extracellular Matrix Biochemistry, Piez, K.A. and Reddi, A.H. editor. pp. 1-39. Elsevier, New York

Pihlajaniemi, T. and Tamminen, M. (1990). The α1 chain of type XIII collagen consists of three collagenous and four noncollagenous domains and its primary transcript undergoes complex alternative splicing. J.Biol.Chem. 265:16922-16928

Ploetz, C., Zycband, E.I. and Birk, D.E. (1991). Collagen fibril assembly and deposition in the developing dermis-segmental deposition in extracellular compartments. J.Struct.Biol. 106:73

Poole, A.R., Pidoux, I., Reiner, A., Choi, H. and Rosenberg, L.C. (1984). Association of an extracellular protein (chondrocalcin) with the calcification of cartilage in endochondral bone formation. J.Cell.Biol. 98:54-65

Poole, A.R., and Rosenberg, L.C. (1986). Chondrocalcin and the calcification of cartilage. Clin.Orthop.Rel.Res. 208:114-118

Prigent-Dachary, J., Faucon, J-F., Boisseau, M.R. and Dufourcq, J. (1986). Topology of the binding site of blood-clotting factors in model membranes. A fluorescent study. Eur.J.Biochem. 155:133-140

Pringle, G.A. and Dodd, C.M. (1990). Immunoelectron microscopic localization of the core protein of decorin near the d and e bands of tendon collagen fibrils by use of monoclonal antibodies. J.Histochem.Cytochem. 38:1405-1413

Prockop, D.J. (1990). Mutations that alter the primary structure of type I collagen. The perils of a system for generating large structures by the principle of nucleated growth (Minireview). J.Biol.Chem. 265:15349-15352

Prockop, D.J. and Kivirikko, K.I. (1984). Heritable diseases of collagen. New Eng.J.Med. 311:376-386

Prockop, D.J., Kivirikko, K.I., Tuderman, L. and Guzman, N.A. (1979). The biosynthesis of collagen and its disorders. New Engl.J.Med. 301:13-23 (part 1); 77-85 (part 2)

Prockop, D.J., Olsen, A., Kontusaari, S., *et al.* (1990). Mutations in Human Procollagen Genes. Consequences of the Mutations in Man and in Transgenic Mice. Ann.N.Y.Acad.Sci. 580:330-339

Prockop, D.J., and Tuderman, L. (1982). Post-translational enzymes in the biosynthesis of collagen: Extracellular enzymes. Meth.Enzymol. 82:305-319

Pusey, P.N. (1989). Dynamic light scattering by partially-fluctuating media. In: Dynamic properties of biomolecular assemblies , Harding, S.E. and Rowe, A.J. editors. pp. 90-102.Royal Society of Chemistry, Cambridge

Rabinovitz, J.L. and Shapiro, I.M. (1972). Lipids of bovine skin collagen. Arch.Oral Biol. 17:547-553

Raghow, R. and Thompson, J.P. (1989). Molecular mechanisms of collagen gene expression (Review). Mol.Cell Biochem. 86:5-18

Raghow, R., Postlewaite, A.E., Keski-Oja, J., Moses, H.L. and Kang, A.H. (1987). Transforming growth factor-β increases steady state levels of type I procollagen and fibronectin messanger RNAs posttranscriptionally in cultured human dermal fibroblasts. J.Clin.Invest. 79:1285-1288

Ramachandran, G.N. and Ramakrishnan, C. (1976). Molecular structure. In: Biochemistry of collagen, Ramachandran, G.N. and Reddi, A.H. editors. pp. 45-84. Plenum press, New York

Ramirez, F. and Di Liberto, M. (1990). Complex and diversified regulatory programs control the expression of vertebrate collagen genes. FASEB.J. 4:1616-1623

Ramshaw, J.M., Bateman, J.F. and Cole, W.G. (1984). Precipitation of collagens by polyethylene glycols. Anal.Biochem. 141:361-365

Raspanti, M., Ottani, V. and Ruggeri, A. (1989). Different architectures of the collagen fibril: morphological aspects and functional implications. Int.J.Biol.Macromol. 11:367-371

Razin, A., and Riggs, A. (1980). DNA methylation and gene function. Science 210:604-610

Reynolds, J.A., and Tanford, C. (1970). Binding of dodecyl sulphate to protein at high binding ratios. Proc.Natl.Acad.Sci.USA 66:1002-1007

Rintoul, D.A., Chou, S.M. and Silbert, D.F. (1979). Physical characterisation of sterol-depleted LM-cell plasma membranes. J.Biol.Chem. 254:10070-10077

Rioux, L. and Gicquaud, C. (1985). Actin paracrystalline sheets formed at the surface of positively charged liposomes. J.Ultrastruct.Res. 93:42-49

Robins, S.P. (1982). Turnover and Cross Linking of Collagen . In: Collagen in Health and Disease, Weiss, J.B., and Jayson, M.V. editors. pp. 84-95.Churchill Livingstone

Ross, G.F., Notter, R.H., Meuth, J. and Whitsett, J.A. (1986). Phospholipid binding and biophysical activity of pulmonary surfactant-associated protein (SAP)-35 and its non-collagenous carboxyl-terminal domains. J.Biol.Chem. 261:14283-14291

Rossi, J.D. and Wallace, B.A. (1983). Binding of fibronectin to phospholipid vesicles. J.Biol.Chem. 258:3327-3331

Rossi, P. and de Crombugghe, B. (1987). Identification of a cell-specific transcriptional enhancer in the first intron of the mouse α2 (type I) collagen gene. Proc.Nat.Acad.Sci.USA 84:5590-5594

Ruggeri, A., Benazzo, F. and Reale, E. (1979). Collagen fibrils with straight and helicoidal microfibrils: A freeze fracture and thin section study. J.Ultrastruct.Res. 68:101-108

Ruggiero, F., Pfäffle, M., von der Mark, K. and Garrone, R. (1988). Retention of carboxypropeptides in type II collagen fibrils in chick embryo chindrocyte cultures. Cell Tissue Res. 252:619-624

Ruoslahti, E. (1988). Fibronectin and its Receptors. Ann.Rev.Biochem. 57:375-413

Ruoslahti, E. (1991). Integrins as Receptors for Extracellular Matrix . In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 343-363. Plenum press, New York

Ryan, M.C. and Sandell, L.J. (1990). Differential expression of a cysteine-rich domian in the amino-terminal propeptide of type II (cartilage) procollagen by alternative splicing of mRNA. J.Biol.Chem. 265:10334-10339

Sage, E.H., and Bornstein, P. (1991). Extracellular proteins that modulate cell-matrix interactions: SPARC, tenascin, and thrombospondin. J.Biol.Chem. 266:14831-14834

Sage, H. and Bornstein, P. (1987). Type VIII collagen. In: Structure and Function of Collagen Types, Mayne, R. and Burgeson, R.E. editors. pp. 173-194. Academic press, Orlando

Sakai, L.Y., Keene, D.R., Morris, N.P. and Burgeson, R.E. (1986). Type VII collagen ia a major structural component of anchoring fibrils. J.Cell.Biol. 103:1577-1586

Sandell, L.J., and Boyd, C.D. (1990). Conserved and divergent sequence and functional elements within collagen genes. In: Extracellular Matrix Genes, Sandell, L.J., and Boyd, C.D. editors. pp. 1-56. Academic press, New York

Santoro, C., Mermod, N., Andrews, P.C. and Tjian, R. (1988). A family of human CCAAT-box-binding proteins active in transcription and DNA replication: cloning and expression of multiple cDNAs. Nature 334:218-224

Saus, J., Quinones, S., MacKrell, A., *et al.* (1989). The complete primary structure of mouse α2(IV) collagen. Alignment with mouse α1(IV) collagen. J.Biol.Chem. 264:6318-6324

Saus, J., Wieslander, J., Langeveld, J.P.M., Quinones, S. and Hudson, B.G. (1988). Identification of the Goodpasture antigen as the α3 (IV) chain of collagen IV. J.Biol.Chem. 263:13374-13380

Sawada, H., Konomi, H. and Hirosawa, K. (1990). Characterization of the collagen in the hexagonal lattice of Descemet's membrane: its relation to type VIII collagen. J.Cell Biol. 110:219-227

Schmid, T.M. and Linsenmayer, T.F. (1990). Immunoelectron microscopy of type X collagen: supramolecular forms within embryonic chick cartilage. Develop.Biol. 138:53-62

Schofield, J.D., Uitto, J. and Prockop, D.J. (1974). Formation of interchain disulphide bonds and helical structure during biosynthesis of procollagen by embryonic tendon cells. Biochemistry 13:1801-1806

Scott, J.E. (1988). Proteoglycan-fibrillar collagen interactions. Biochem.J. 252:313-323

Scott, J.E. (1990). Proteoglycan-collagen interactions and subfibrillar structure in collagen fibrils. Implications in the development and ageing of connective tissue. J.Anat. 169:23-35

Scott, J.E. and Bosworth, T.R. (1990). A comparative biochemical and ultrastructural study of proteoglycan- collagen interactions in corneal stroma. Functional and metabolic implications. Biochem.J. 270:491-497

Scott, J.E. and Orford, C.R. (1981). Dermatan sulphate-rich proteoglycans associates with rat tail-tendon collagen at the d-band in the gap region. Biochem.J. 197:213-216

Scott, P.G., Winterbottom, N., Dodd, C.M., Edwards, E. and Pearson, C.H. (1986). A role for disulphide bridges in the protein core in the interaction of proteodermatan sulphate and collagen. Biochem.Biophys.Res.Commun. 138:1348-1354,

Sellar, G.C., Blake, D.J. and Reid, K.B.M. (1991). Characterisation and organisation of the genes encoding the A-, B- and C- chains of human complement subcomponent C1q. Biochem.J. 274:481-490

Seyedin, S.M. and Rosen, D.M. (1990). Matrix proteins of the skeleton. Curr.Opin.Cell Biol. 2:914-919

Shaw, L.M. and Olsen, B.R. (1991). FACIT collagens: diverse molecular bridges in extracellular matrices. Trends Biochem.Sci. 16:191-194

Shotton, D.M., Burke, D.E. and Branton, D. (1979). The molecular structure of human erythrocyte spectrin. J.Mol.Biol. 131:303-329

Siegel, R.C. (1974). Biosynthesis of collagen cross-links: Increased activity of purified lysyl oxidase with reconstituted collagen fibrils. PNAS 71:4826-4830

Siegel, R.C. and Schumaker, V.N. (1983). Measurement of the association constants of the complexes formed between intact C1q or pepsin treated C1q stalks and the unactivated or activated $C1r_2C1s_2$ tetramers. Mol.Immunol. 20:53-66

Silver, F.H. (1981). Type I collagen fibrillogenesis *in vitro*. Additional evidence for the assembly mechanism. J.Biol.Chem. 256:4973-4977

Silver, F.H. (1982). A molecular model for linear and lateral growth of type I collagen fibrils. Collagen Rel.Res. 2:219-229

Silver, F.H. (1983). A 2 step model for lateral growth of collagen fibrils. Collagen Rel.Res. 3:167-180,

Silver, F.H. and Trelstad, R.L. (1980). Type I collagen in solution. Structure and properties of fibril fragments. J.Biol.Chem. 255:9427-9433,

Skinner, M.K. and Griswold, M.D. (1983). Fluorographic detection of radioactivity in polyacrylamide gels with 2, 5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem.J. 209:281-284

Smith, G.N., Hasty, K.A. and Brandt, K.D. (1989). Type XI collagen is associated with the chondrocyte surface in suspension culture. Matrix. 9:186-192

Smith, J.W. (1968). Molecular pattern in native collagen. Biopolymers 219:157-158

Sonohara, S., Brentani, R.R., Andrade, H.F. and Machado-Santelli, G.M. (1981). Procollagen biosynthesis and processing in guinea pig fibroblast culture medium. Braz.J.Med.Biol.Res. 14:267-276

Sood, C.K., Sweet, C. and Zull, J.E. (1972). Interaction of kidney (Na^+-K^+)ATPase with phospholipid model membrane systems. Biochim.Biophys.Acta 282:429-434

Sporn, M.B., and Roberts, A.B. (1989). Transforming growth factor-β: multiple actions and potential clinical applications. JAMA 262:938-941

Staatz, W.D., Rajpara, S.M., Wayner, E.A., Carter, W.G. and Santoro, S.A. (1989). The membrane glycoprotein Ia-IIIa (VLA-2) complex mediates the Mg^{2+}-dependent adhesion of platelets to collagen. J.Cell.Biol. 108:1917-1924

Steinmann, B., Tuderman, L., Peltonen, L., Martin, G.R., McKusick, V.A. and Prockop, D.J. (1980). Evidence for a structural mutation of procollagen type I in a patient with the Ehlers-Danlos Syndrome type VII. J.Biol.Chem. 255:8887-8893

Stopak, D., Wessels, N.K. and Harris, A.K. (1985). Morphogenetic rearrangement of injected collagen in developing chicken limb buds. Proc.Natl.Acad.Sci.USA 82:2804-2808

Su, M.W., Benson-Chanda, V., Vissing, H. and Ramirez, F. (1989). Organization of the exons coding for pro α1(II) collagen N-propeptide confirms a distinct evolutionary history of this domain of the fibrillar collagen genes. Genomics. 4:438-441

Suarez, G., Oronsky, A.L., Bordas, J. and Koch, M.H. (1985). Synchrotron radiation X-ray scattering in the early stages of *in vitro* collagen fibril formation. Proc.Natl.Acad.Sci.USA 82:4693-4696

Sugrue, S.P., Gordon, M.K., Seyer, J., Dublet, B., van der Rest, M. and Olsen, B.R. (1989). Immunoidentification of type XII collagen in embryonic tissues. J.Cell Biol. 109:939

Svoboda, K.K., Nishimura, I., Sugrue, S.P., Ninomiya, Y. and Olsen, B.R. (1988). Embryonic chicken cornea and cartilage synthesize type XI collagen molecules with different amino terminal domains. Proc.Natl.Acad.Sci.USA 85:7469-7500

Sykes, B. (1990). Bone disease cracks genetics. Nature 348:18-20

Tall, A.R., Small, D.M. and Lees, R (1978). Interaction of collagen with the lipids of tendon xanthomata. J.Clin.Invest. 62:836-846

Tanzawa, K., Berger, J. and Prockop, D.J. (1985). Type I procollagen N-proteinase from whole chick embryos. Cleavage of a homotrimer of proα1(I) chains and the requirement for procollagen with a triple-helical conformation. J.Biol.Chem. 260:1120-1126

Tanzer, M.L., Church, R.L., Yaeger, J.A., Wampler, D.E. and Park, E.D. (1974). Procollagen intermediate forms containing several types of peptide chains and non-collagen peptide extensions at NH_2 and COOH ends. Proc.Natl.Acad.Sci.USA 71:3009-3019

Taubman, M.B. and Goldberg, B. (1976). The processing of procollagen in cultures of human and mouse fibroblasts. Arch.Biochem.Biophys. 173:490-494

Theil, S. and Reid, K.B.M. (1989). Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. FEBS lett. 250:78-84

Tikka, L., Elomaa, O., Pihlajaniemi, T. and Tryggvason, K. (1991). Human α1 (XIII) gene. J.Biol.Chem. 266:17713-17719

Timpl, R. and Engel, J. (1987). Type VI Collagen. In: Structure and Function of Collagen Types, Mayne, R. and Burgeson, R.E. editors. pp. 105-143. Academic press, Orlando

Titani, K., Kumar, S., Takio, K., Ericson, L.H. and Wade, R.D. (1986). Amino acid sequence of human von Willebrand factor. Biochemistry 25:3171-3184

Tolstochev, P., Berg, R.A., Rennard, S.I., Bradley, K.H., Trapnell, PC. and Crystal, R.G. (1981). Procollagen production and procollagen mRNA levels and activity in human lung fibroblasts during periods of rapid and stationary growth. J.Biol.Chem. 256:3135-3140

Toole, B.P. (1991). Proteoglycans and Hyaluronan in Morphogenesis and Differentiation. In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 305-341. Plenum press, New York

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc.Natl.Acad.Sci.USA 76:4350-4354

Trelstad, R.L. (1971). Vacuoles in the embryonic chick corneal epithelium, an epithelium which produces collagen. J.Cell Biol. 48:689-694

Trelstad, R.L. (1977). Mesenchymal cell polarity and morphogenesis of chick cartilage. Dev.Biol. 59:153-163

Trelstad, R.L. and Birk, D.E. (1984). Collagen fibril assembly at the surface of polarised cells. In: Extracellular Matrix in Development, Trelstad, R.L. editor. pp. 513-543. Liss, New York

Trelstad, R.L. and Hayashi, K. (1979). Tendon collagen fibrillogenesis: Intracellular subassemblies and cell surface changes associated with fibril growth. Dev.Biol. 71:228-242

Trus, B.L. and Piez, K.A. (1980). Compressed microfibril models of the native collagen fibril. Nature 286:300-301

Tuderman, L., Kivirikko, K.I. and Prockp, D.J. (1978). Partial purification and characterisation of a neutral protease which cleaves the N-terminal propeptides from procollagen. Biochemistry 17:2948-2954

Tuderman, L. and Prockop, D.J. (1982). Procollagen N-proteinase. Properties of the enzyme purified from chick embryo tendons. Eur.J.Biochem. 125:545-549

Tyler, J.M., and Branton, D. (1980). Rotary shadowing of extended molecules dried from glycerol. J.Ultrastruct.Res. 71:95-102

Uitto, J., Allan, R.E. and Polak, K.L. (1979). Conversion of type II procollagen to collagen. Extracellular removal of the amino- and carboxyl-terminal extensions without preferential sequence. Eur.J.Biochem. 99:97-103

Uitto, J. and Prockop, D.J. (1974). Biosynthesis of cartilage procollage. Influence of chain association and hydroxylation of prolyl residues of the folding of the polypeptides into the triple helical conformation. Biochemistry 13:4586-4591

Uldbjerg, N. and Danielsen, C.C. (1988). A study of the interaction *in vitro* between type I collagen and a small dermatan sulphate proteoglycan. Biochem.J. 251:643-648

Untracht, S.H. and Shipley, G.G. (1977). Molecular interactions between lecithin and sphingomyelin: Temperature and composition dependent phase separation. J.Biol.Chem. 252:4449-4457

Upholt, W.B., and Sandell, L.J. (1986). Exon/ intron organisationof the chicken type II procollagen gene: intron size distribution suggests a minimal intron size. Proc.Natl.Acad.Sci.USA 83:2325-2329

van der Rest, M., Aubert Foucher, E., Dublet, B., Eichenberger, D., Font, B. and Goldschmidt, D. (1991). Structure and function of the fibril-associated collagens. Biochem.Soc Trans. 19:820-824

van der Rest, M. and Garrone, R. (1991). Collagen family of proteins. Faseb J 5:2814-2823

van der Rest, M. and Mayne, R. (1987). Type IX Collagen. In: Structure and Function of Collagen Types , Mayne, R. and Burgeson, R.E. editors. pp. 195-222. Academic press, Orlando

van der Rest, M. and Mayne, R. (1988). Type IX collagen proteoglycan from cartilage is covalently cross-linked to type II collagen. J.Biol.Chem. 263:1615-1618

van der Rest, M., Rosenburg, L.C., Olsen, B.R. and Poole, A.R. (1986). Chondrocalcin is identical to the C-propeptide of type II procollagen. Biochem.J. 297:923-925

Vaughan, L., Mendler, M., Huber, S., *et al.* (1988). D-perodic distribution of collagen type IX along cartilage fibrils. J.Cell.Biol. 106:991-997

Veis, A., Anesey, J., Yuan, L. and Levy, S.J. (1973). Evidence for an amino-terminal extension in high molecular weight collagens from mature bovine skin. Proc.Natl.Acad.Sci.USA 70:1464-1467

Veis, A., Miller, A., Leibovich, S.J. and Traub, W. (1979). The limiting collagen microfibril. The minimum structure demonstrating native axial periodicity. Biochim.Biophys.Acta 576:88-98

Vogel, B.E., Dolez, R., Kadler, K.E., Hojima, Y., Engel, J. and Prockop, D.J. (1988). A substitution of cysteine for glycine 748 of the alpha1 chain produces a kink at this site in the procollagen I molecule and an altered N-proteinase cleavage site over 225 nm away. J.Biol.Chem. 263:19249-19255

Vogel, K.G., Paulsson, M. and Heingard, D. (1984). Specific inhibition of type I and type II collagen fibrillogenesis by the low molecular mass proteoglycan of tendon. Biochem.J. 223:587-597

Vogel, K.G. and Trotter, J.A. (1987). The effect of proteoglycans on the morphology of collagen fibrils fromed *in vitro*. Collagen Rel.Res. 7:105-114

von der Mark, K., Mollenhauer, J., Müller, P.K. and Pfäffle, M. (1985). Anchorin CII, a type II collagen-binding glycoprotein from chondrocyte membranes. Ann.N.Y.Acad.Sci. 460:214-223

Voss, T., Eistetter, H., Schäfer, K.P. and Engel, J. (1988). Macromolecular organisation of natural and recombinant lung surfactant protein SP 28-36: Structual homology with the complement factor C1q. J.Mol.Biol. 201:219-227

Walker, M., Knight, P. and Trinick, J. (1985). Negative staining of myosin molecules. J.Mol.Biol. 184:535-542

Wallace, D.G. and Thompson, A. (1983). Description of collagen fibril formation by a theory of polymer crystallisation. Biopolymers 22:1793-1811

Ward, N., Hulmes, D.J.S. and Chapman, J.A. (1986). Collagen self assembly *in vitro*: Electron microscopy of initial aggregates formed during the lag phase. J.Mol.Biol. 190:107-112

Watkins, M.S., Hitt, A.S. and Bulger, J.E. (1977). The binding of 18S acetylcholinesterase to sphingomyelin and the role of the collagen-like tail. Biochem.Biophys.Res.Commun. 79:640-647

Wayner, E.A., and Carter, W.G. (1987). Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosarcoma cells possessing unique α and β subunits. J.Cell.Biol. 105:1873-1884

Weiner, A.L., Carpenter-Green, S.S., Soehngen, E.C., Lenk, R.P. and Popescu, M.C. (1985). Liposome-collagen gel matrix: a novel sustained drug delivery system. J.Pharm.Sci. 74:922-925

Weiner, F.R., Czaja, M.J., Jefferson, D.M., *et al.* (1987). The effect of dexamethasone on in vitro collagen gene expression. J.Biol.Chem. 262:6955-6958

Weinstock, M. and Leblond, C.P. (1974). Synthesis, migration, and release of precursor collagen by odontoblasts as visualised by radioautography after [^{3}H]proline administration. J.Cell Biol. 60:92-127

Widnell, C. and Unkeless, J.C. (1968). Partial purification of a lipoprotein with 5'-nucleotidase activity from membranes of rat liver cells. Proc.Natl.Acad.Sci.USA 61:1050-1057

Wiessner, J.H. and Hwang, K.J. (1982). Binding of insulin to the external surface of liposomes. Effect of surface curvature, temperature, and lipid composition. Biochim.Biophys.Acta 689:490-498

Wiestner, M.T., Kreig, D., Horlein, R.W., Glanville, R., Fietsek, P. and Müller, P.K. (1979). Inhibiting effect of procollagen peptides on collagen biosynthesis in fibroblast cultures. J.Biol.Chem. 254:7016-7023

Williams, B.R., Gelman, R.A., Poppke, D.C. and Piez, K.A. (1978). Collagen fibril formation. Optimal *in vitro* conditions and preliminary kinetic results. J.Biol.Chem. 253:6578-6585

Williams, R.C., and Wyckoff, R.W.G. (1945). Electron shadow micrography of the tobacco mosaic virus. Science 101:594-596

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

Woodhead-Galloway, J. and Machin, P.A. (1976). Modern theories of liquids and the diffuse equatorial X-ray scattering from collagen. Acta Cryst. A32:368-372

Wright, G.M., and Leblond, C.M. (1981). Immunohistochemical localisation of procollagen. Type I procollagen antigenicity in osteoblasts and prebone (osteoid). J.Histochem.Cytochem. 29:791-804

Wright, J.R., Borchelt, J.D. and Hagwood, S. (1989). Lung surfactant apoprotein SP-A (26-36 kDa) binds with high affinity to isolated alveolar type II cells. Proc.Natl.Acad.Sci.USA 86:5410-5414

Wu, C.H., Donovan, C.B.and Wu, G.Y. (1986). Evidence for pretranslational regulation of collagen synthesis by procollagen propeptides. J.Biol.Chem. 261:10482-10484

Wu, L.N.Y., Sauer, G.R., Genge, B.R. and Wuthier, R.E. (1989). Induction of mineral deposition by primary cultures of chicken growth plate chondrocytes in ascorbate-containing media. Evidence of an association between matrix vesicles and collagen. J.Biol.Chem. 264:21346-21355

Wuthier, R.E. (1977). Electrolytes of isolated epiphyseal chondrocytes, matrix vesicles, and extracellular fluids.Calcif.Tiss.Res. 23:125-133

Wuthier, R.E. (1989). Mechanism of *de Novo* Mineral Formation by Matrix Vesicles. Connective Tiss.Res. 22:27-33

Yada, T., Suzuki, S., Kobayashi, K., *et al.* (1990). Occurrence in chick embyro vitreous humor of a type-IX collagen proteoglycan with an extraordinarily large chondroitin sulfate chain and short alpha1 polypeptide. J.Biol.Chem. 265:6992-6999

Yamada, K.M. (1991). Fibronectin and Other Cell Interactive Glycoproteins. In: Cell Biology of Extracellular Matrix, Hay, E.D. editor. pp. 111-146. Plenum press, New York

Yamada, Y., Kühn, K. and de Crombrugghe, B. (1983). A conserved nucleotide sequence, coding for a segment of the C-propeptide, is found at the same location in different collagen genes. Nucleic Acids Res. 11:2733-2744

Yamada, Y., Lian, G., Mudryj, M., Obsci, S. and de Crombrugghe, B. (1984). Conservation of the sizes for one but not another class of exons in two chick collagen genes. Nature 310:333-337

Yamada, Y., Miyashita, T., Savagner, P., *et al.* (1990). Regulation of the collagen II gene in vitro and in transgenic mice. Ann.N.Y.Acad.Sci. 580:81-87

Yamada, Y., Mudryj, M. and de Crombrugghe, B. (1983). A uniquely conserved regulatory signal is found around the translation initiation site in three different collagen genes. J.Biol.Chem. 258:14914-14919

Yamagata, M., Yamada, K.M., Yamada, S.S., *et al.* (1991). The complete primary structure of type-XII collagen shows a chimeric molecule with reiterated fibronectin type-III motifs, von Willebrand factor-A motifs, a domain homologous to a noncollagenous region of type-XI collagen, and short collagenous domains with an Arg-Gly-Asp site. J Cell Biol. 115:209-221

Yamaguchi, N., Benya, P.D., van der Rest, M. and Ninomiya, Y. (1989). The cloning and sequencing of α1(VIII) collagen cDNAs demonstrate that type VIII collagen is a short chain collagen and contains triple-helical and carboxyl-terminal non-triple helical domains similar to those of type X collagen. J.Biol.Chem. 264:16022-16029

Yurchenco, P.D. and Schittny, J.C. (1990). Molecular architecture of basement membranes (Review). FASEB.J. 4:1577-1590

APPENDIX

Presentations and Publications

Poster presentations:

"Lipid interactions with type I procollagen"
A.A. Choglay and D.J.S. Hulmes
British Connective Tissue Society/ British Society for Cell Biology, Oxford, 1990.

"Procollagen-sphingomyelin interactions"
A.A. Choglay and D.J.S. Hulmes
Third International Symposium on Extracellular Matrix Macromolecules, Jerusalem, 1991.

"Procollagen-sphingomyelin interactions"
A.A. Choglay and D.J.S. Hulmes
British Connective Tissue Society, Lancaster, 1991

Publication:

"Procollagen binding to sphingomyelin"
A.A. Choglay, I.F. Purdom and D.J.S. Hulmes
Journal of Biological Chemistry, in press (pre-print attached).

6/10/92

J. Biol. Chem.
Submitted
10/10/92
in press

rocollagen binding to sphingomyelin[1]

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Summary

The interactions of [^{3}H]procollagen I with various phospholipids were studied by density gradient centrifugation. At physiological conditions of pH, ionic strength and temperature, there was no evidence for procollagen binding to phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol or phosphatidylserine liposomes. In contrast, procollagen I bound strongly to sphingomyelin liposomes, in a reversible and saturable manner, with an apparent dissociation constant (K_d) of 2.6 nM. Binding occurred over a range of temperatures (4 °C to 37 °C) and was relatively unaffected by salt concentrations up to 1.2 M NaCl. Binding was observed in phosphate buffers but not in the presence of high concentrations of Tris or Hepes. Bovine serum albumin had no effect on procollagen binding to sphingomyelin, neither did unlabelled type I collagen, with or without the non-helical telopeptides. Procollagen II and denatured procollagen I also bound to sphingomyelin. Procollagen binding to sphingomyelin at 35 °C was considerably reduced when small amounts of phosphatidylcholine were present, though binding was partially restored when the temperature was reduced below the corresponding phase transition temperature. Purified, unlabelled procollagen C-propeptides successfully competed for binding, and ^{125}I-labelled C-propeptides bound to sphingomyelin in the absence of procollagen. Weaker binding to sphingomyelin, mediated by the collagen triple helical region, was also observed, but this was dominated by the sphingomyelin C-propeptide interaction. The data suggest a novel mechanism for matrix vesicle mediated biomineralisation.

Several studies have pointed to a role for plasma membranes in the assembly and function f the extracellular matrix (Hay, 1991). Cell surface receptors for collagens, fibronectins and ther matrix components have been identified (Albelda and Buck, 1990; Ruoslahti, 1991; lynes, 1990, 1992). Cell-matrix interactions are intimately involved in extracellular fibronectin ssembly (Mosher *et al.*, 1991; Chernousov *et al.*, 1991; Limper *et al.*, 1991), and in collagen ssembly, collagen fibrils are extruded into extracellular compartments via membraneous, cell- rface invaginations (Birk *et al.*, 1991). Fibril forming collagens (Hulmes, 1992; van der Rest nd Garrone, 1991) are synthesised in precursor form, pro-collagens, with N- and C-terminal ropeptide extensions (Olsen, 1991). Kinetic studies indicate that procollagen processing can cur close to the plasma membrane (Morris *et al.*, 1975; Mellor *et al.*, 1991) and there is direct evidence for the involvement of an anchorin-like, cell surface collagen binding protein N-terminal procollagen processing (Mauch *et al.*, 1988). Furthermore, the C-propeptides of pe I procollagen have been immunolocalised to the plasma membrane (Phelps *et al.*, 1985). oth N- and C-terminal propeptides are thought to play a role in feedback inhibition of collagen osynthesis (Wu *et al.*, 1991) which suggests specific cell recognition mechanisms for these mains. Though much attention has been given to the identification of cell surface receptors for tracelluar matrix macromolecules, the role of membrane lipids in the assembly and function of ECM is largely unknown.

We have shown that surface effects can have a pronounced influence on the aggregation procollagen I (Mould and Hulmes, 1987). On a mica surface, association via the C- peptides leads to the formation of spoke-like arrays and in-register parallel bundles of lecules. It has been suggested that interactions with the cell surface could direct the form of embly of newly synthesised collagens and their precursors (Goldberg and Burgeson, 1982; uld and Hulmes, 1987; Smith *et al.*, 1989). Collagen interactions with matrix vesicles have o been implicated in the formation of bone (Wu *et al.*, 1989, 1991c; Genge et al, 1992). eractions of type I collagen with phosphatidylcholine have been described (Martinez del Pozo *al.*, 1988, 1989) and there have been several reports of lipid association with collagen

(Barnes, 1985; Tall *et al.*, 1978; Le Lous *et al.*, 1982; Ozgunes and Artvinli, 1988; Pajean *et al.*, 1991) and collagen-like proteins (Kuroki and Akino, 1991; Ross *et al.*, 1986; Watkins *et al.*, 1977; Cohen and Barenholz, 1984). Here we examine procollagen interactions with liposomes of defined composition, in physiological buffer conditions. There is strong binding to sphingomyelin, and the interaction appears to be mediated mainly by the C-terminal propeptides.

EXPERIMENTAL PROCEDURES

Reagents - Bovine brain SPM[4] and DMPC were purchased from Avanti Polar Lipids, Pelham, Alabama, USA. Bovine spinal chord phosphatidylserine, wheat germ phosphatidylinositol and egg phosphatidylethanolamine were supplied by Lipid Products, S Nutfield, Surrey, UK. [^{14}C]DOPC (specific activity 3.89 GBq/mmol) was purchased from Amersham, Aylesbury, Bucks, UK. Pepsin (from porcine gastric mucosa) was supplied by Boehringer Mannheim, Lewes, E Sussex, UK. Other reagents were from Sigma, Poole, Dorset, UK or BDH/ Merck, Poole, Dorset, UK.

Preparation of procollagens and their structural domains - [^{3}H]procollagen I was prepared from the culture medium of 17 day chick embryo tendon cells, as described (Mould *et al.*, 1990). The specific activity was typically 29,000 cpm/ µg. Pepsinised [^{3}H]collagen I was prepared from the [^{3}H]procollagen I by buffer exchange into 0.5 M acetic acid using Amicon C-10 microcentrators, followed by addition of pepsin to an enzyme:substrate weight ratio of 1:10, and digestion for 18 h at 4 °C. The [^{3}H]collagen was precipitated by the addition of NaCl to a final concentration of 2 M, followed by centrifugation at 50,000 g for 1 h at 4 °C. Finally the pellet was re-dissolved in 50 mM Tris/ HCl and the buffer was exchanged for assay buffer (150 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, pH 7.4) with Amicon C-10 microconcentrators. SDS-PAGE and fluorography (Mellor *et al.*, 1991) showed the procollagen propeptides to be absent (not shown). [^{3}H]Procollagen II was purified from the medium of freshly isolated

17 day chick embryo sternal chondrocytes, as described by Dehm and Prockop (1973) and Curran and Prockop (1982), except that polyethylene glycol and DEAE-Sephacel were used for the procollagen precipitation and ion exchange chromatography steps (Mould *et al.*, 1990), respectively.

Procollagen I C-propeptides were prepared from the medium of cultured chick embryo tendons, otherwise used for the preparation of procollagen C-proteinase (Hojima *et al.*, 1985), using a modified version of the procedure described by Olsen *et al.* (1977). Typically 3 litres of culture medium (tendons from 100 dozen embryos) were used. All purification procedures were at 4°C. Unbound material from the Green A Dye Matrix column (Hojima *et al.*, 1985) was applied directly, at a flow rate 25 ml/h, to a column of concanavilin A - Sepharose (Pharmacia; 5 cm x 2.6 cm), previously equilibrated in 50 mM Tris-HCl, 300 mM NaCl, 1mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.4 (measured at room temperature). After washing with two column volumes of equilibration buffer, bound material was eluted, at 25 ml/h, with equilibration buffer containing 1 M methyl α-D-mannopyranoside. Fractions (10 ml) were collected and the absorbance measured at 280 nm. The single elution peak (not shown) was pooled and dialysed against DEAE starting buffer (2 M urea, 50 mM Tris/HCl pH 8.6, measured at room temperature). The dialysed protein was applied at 10 ml/h to a column of DEAE-Sephacel (Pharmacia; 5 cm x 2.6 cm), previously equilibrated and then washed with two column volumes of DEAE starting buffer. Bound material was eluted at 10 ml/h with a 1000 ml linear gradient of zero to 300 mM NaCl in DEAE starting buffer. Protein was measured by absorbance at 280 nm and fractions (7 ml) were analysed, both reduced and non-reduced, by SDS-PAGE with 12 % acrylamide in the separating gel, followed by Coomassie Blue staining. Peak fractions consisted solely of procollagen C-propeptides (not shown) and the yield from 100 dozen embryos was approximately 5 mg protein. For some experiments, the C-propeptides were ^{125}I-iodinated (by Dr. D.K. Apps) using IodoGen (Pierce, Oud Beijerland, The Netherlands), according to the manufacturer's instructions. The specific activity of the [^{125}]C-propeptides was 564,700 cpm/μg.

Unlabelled collagen I (a gift from Mr J.R.E. MacBeath) was prepared from lathyritic rat kins as described (Payne *et al.*, 1986), with the inclusion of a DEAE-Sephacel ion-exchange hromatography step (to remove proteoglycans; Miller, 1971) following neutral salt recipitation, and with omission of the ethanol precipitation step. For some experiments, the on-helical telopeptide regions were removed by pepsin treatment (as above).

Preparation and characterisation of liposomes - Lipids were obtained either lyophilised SPM, DMPC) or in chloroform/ methanol (2:1 v/v; phosphatidylserine, phos-phatidylinositol, hosphatidylethanolamine). When necessary, solvent was removed by drying the lipids to a thin lm in a glass vial, in a stream of nitrogen.The dried film or lyophilised lipid was dissolved (at concentration of 10 mM) by stirring overnight in 1 ml of 50 mM n-octyl-β-D-glucoside previously purged by bubbling with a stream of nitrogen). The detergent/ lipid molar ratio was :1 to effectively solubilise the lipids and hence minimise the formation of lipid aggregates Mimms *et al.*, 1981). Liposomes were formed by removal of detergent on a mini desalting olumn (Bio-Gel P6-DG), as described by Perez-Castineira and Apps (1990). The gel (1 ml) as equilibrated in assay buffer, supported on glass wool and centrifuged for 2 min at 1200 m (110 g) in a microcentrifuge tube pierced by a needle at the tip. Detergent dissolved lipid 00 µl) was applied, the microcentrifuge tube was supported on a 10 ml centrifuge tube, and e turbid liposome solution was collected by centrifugation, as above. The liposome solution as diluted 1:1 with assay buffer, then stored at 4°C in an atmosphere of nitrogen and used ithin 8 hours. Lipid recovery after detergent solubilisation and desalting was measured in ntrol experiments by the addition of trace amounts of [^{14}C]DOPC.

Liposomes were analysed by photon correlation spectroscopy (Pusey, 1989) to termine their translational diffusion coefficient, and hence their diameter. The measurements ere made on a Malvern 4700c system with an Argon-ion (100 to 250 mW) or He-Ne (40 mW) ser. Liposome samples were filtered through 0.45 µm Nuclepore filters and analysed in 1 cm ameter cylindrical quartz cells. Measurements were made at 25 °C at a scattering angle of 90°. ata were accumulated from several runs (typically 10) each of duration 10 sec, with automatic

ejection of runs in which the integrated intensity differed significantly from the mean.

Liposome preparations were also examined by electron microscopy, after rotary shadowing with platinum of freeze-dried specimens prepared by the "mica-sandwich" technique (Mould *et al.*, 1985), or by negative staining with 1 % (w/v) phosphotungstic acid at neutral pH (New, 990). Grids were examined in a Philips CM12 transmission electron microscope.

Density gradient binding assay - Unbound proteins were separated from lipid and lipid-protein complexes by centrifugation in a stepwise sucrose gradient. Gradients consisted of 0, 30, 40 and 60 % sucrose in phosphate buffered saline (or otherwise), with the sample to be analysed initially in the 40 % sucrose region. The gradient was chosen so that liposomes floated to the 0/ 30% sucrose interface and protein sedimented to the 40/ 60 % sucrose interface. In a standard assay, [^{3}H] procollagen I (approximately 1 mg/ml) in storage buffer (0.4 M NaCl, 0.1 M Tris-HCl, pH 7.5) was diluted with an equal volume of H_2O (to lower both Tris and NaCl concentrations) and then further diluted with assay buffer, or dialysed against assay buffer, so that the final Tris concentration was below 0.5 mM. A 50 µl aliquot of this diluted procollagen (typically 9 µg/ml or 20 nM) was added to 100 µl liposomes (0.45 mM), followed by 300 µl of 60 % sucrose (in assay buffer) to give a final concentration of 40 % sucrose. For the competitive inhibition studies, an additional 50 µl of the putative inhibitor (in assay buffer) was also present and the amount of 60 % sucrose was adjusted accordingly to give the final 40 % concentration. The protein/ lipid mixture was incubated for 1 h at the selected temperature before being incorporated into the stepwise sucrose gradient. Gradients were formed in 5 ml centrifuge tubes, by upwards displacement with increasing sucrose concentrations, 0 % (1ml), 30 % (2ml), 40 % (450 µl or 600 µl incubation mixture) and 60 % (600 µl). Centrifugation was in a Beckman SW50.1 rotor at 41000 rpm (200,000 g) for 4 hours at the selected temperature, usually 35 °C. After centrifugation, liposomes were always present at the 0/ 30 % sucrose interface, as shown by the visible turbidity. A bung was inserted in the top of each tube, with tubing connected to a peristaltic pump, then a hole was made in the the base and fractions (approximately 150 µl) were collected in microcentrifuge tubes. For each fraction, ^{3}H or ^{14}C

cpm were detected by liquid scintillation counting using Cocktail T (BDH, Poole, Dorset, UK) or ^{125}I cpm were measured in a gamma counter..

RESULTS

Characterisation of liposomes - Liposomes were prepared by detergent dialysis using DMPC, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine or SPM. Photon correlation spectroscopy gave the following Z-average mean diameters (and polydispersity): DMPC liposomes 156 nm (36 % polydispersity), SPM liposomes 175 nm (28 % polydispersity). The SPM liposomes were also examined by electron microscopy, after rapid freeze drying and rotary shadowing, and their number average diameter was 116 +/- 5 nm (SE, = 70).This latter value was used in subsequent estimations of the number of binding sites per PM liposome (see below). By negative staining, the SPM liposomes appeared multi-lamellar not shown).

Procollagen-liposome interactions - Possible interactions of [^{3}H]procollagen I with hospholipids were studied by density gradient centrifugation, using a flotation-type binding ssay in which the procollagen lipid incubation mixture was initially incorporated into the 40 % egion of a discontinuous 0/ 30/ 40/ 60 % sucrose gradient (see Materials and Methods). In the bsence of lipid, essentially all the [^{3}H]procollagen sedimented to the 40/ 60 % sucrose nterface, leaving only 9.1 +/- 0.4 % (S.E., n = 6) of total recovered cpm in the upper half of he gradient (Fig. 1(a)). In the absence of protein, DMPC migrated to the 0/ 30 % sucrose nterface, as shown with DMPC liposomes containing added [^{14}C]DOPC (Fig. 1(b)). When ^{3}H]procollagen I was added to unlabelled DMPC, in physiological conditions of pH, ionic rength and temperature, there was no evidence of any interaction, with only 12.5 +/- 1.5 % SE, n = 3) of recovered cpm in the upper half of the gradient (Fig. 1(c)). Similar results were otained with liposomes of phosphatidylethanolamine, phosphatidylinositol or phosphatidyl-rine (data not shown). With SPM liposomes, however, most of the [^{3}H]procollagen co-

migrated with the lipid at the 0/ 30 % sucrose interface (Fig. 1(d)). The amount of procollagen bound to the lipid was determined from the total cpm in the upper half of the gradient. In standard assay conditions (2 nM procollagen , 0.1 mM phosholipid, 10 mM phosphate, 150 mM NaCl, pH 7.4, 35 °C), 85.6 +/- 0.8 % (S.E., n=6) of the procollagen recovered from the gradient was found in the bound fractions. SDS-PAGE and fluorography showed that the procollagen chains were intact in both the bound and unbound fractions (not shown). Total recovery of cpm throughout the gradient was 82.5 +/- 1.8 % (S.E.; n=6) of cpm applied in the original incubation mixture.

SPM binding was also observed with [^{3}H] procollagen II, where 85.1 ± 2.3 % (S.E.; =4) was bound at 35 °C in standard assay conditions (not shown). SPM interactions with procollagen I were abolished in the presence of both Tris (50 mM Tris-HCl pH 7.4, 0.15 M NaCl) and Hepes (20 mM Hepes pH7.4, 0.15 M NaCl) buffers, though low concentrations of Tris (0.5 mM) had no effect. Procollagen I binding to SPM was also observed at 4 °C in 0.1 M acetic acid, though again no binding was seen with DMPC liposomes in similar conditions (data not shown).

Concentration dependence of the procollagen-SPM interaction - At a fixed concentration of [^{3}H]procollagen I (2 nM), the proportion of total cpm in the bound fractions increased with increasing concentrations of SPM (Fig. 2). Optimal binding required freshly prepared liposomes and a concentration of 0.1 mM SPM was chosen both to maximise binding and to maintain a homogeneous dispersion of lipid.

To study the reversibility of the protein-lipid interaction, the procollagen-SPM complex was isolated from the bound fractions, and then further incubated for 1 h in 40 % sucrose followed by re-centrifugation. Only 70 % of the procollagen remained in the bound fractions (not shown), showing that the procollagen was in equilibrium with the procollagen-SPM complex.

At a fixed concentration of SPM (0.1 mM), the amount of [^{3}H]procollagen bound increased with concentration, and approached saturation (Fig. 3). Direct non-linear least squares

fitting to the binding data (courtesy of Dr G L Atkins) gave an apparent dissociation constant (K_d) of 2.55 +/- 0.14 nM with a saturation limit of 5.05 +/- 0.08 nM. From the amount of procollagen bound at saturation, and assuming a single lipid bilayer with a value of 0.58 nm^2 for the surface area of the SPM headgroup in the gel crystalline phase (Barenholz and Thompson, 1980; see below), the number of procollagen molecules bound per SPM liposome was approximately 7. As the liposomes were known to be multi-lamellar, this number must be regarded as a lower limit.

Nature of the procollagen-SPM interaction - The nature of the procollagen-SPM interaction was investigated by varying the buffer conditions, as follows. To study the effect of ionic strength, NaCl was added to the procollagen-SPM incubation mixture (prepared in 10 mM phosphate buffer in the absence of NaCl) from a stock solution in 60 % sucrose. When separated by density gradient centrifugation, with the NaCl concentration throughout the gradient adjusted accordingly, the proportion of procollagen in the bound fractions increased slightly with increasing salt concentration, from 68.8 +/- 13.1 % (SE, n=3) in the absence of NaCl to 94.0 +/- 3.0 % (SE, n=2) in 1.2 M NaCl. These observations suggest that the interaction is not electrostatic in nature. The procollagen-SPM interaction was also examined in phosphate buffered saline over a range of temperatures (Fig. 4). Between 4 and 37 °C, a small decrease was observed in the proportion of [^{3}H]procollagen bound, suggesting that hydrophobic interactions are also not important for binding.

At 39 °C, a sharp decrease in binding was found. This is close to the denaturation temperature of chick procollagen I (42 °C; Hayashi *et al.*, 1979), so to examine whether the lack of binding at 39 °C was due to protein denaturation, the procollagen was first denatured by heating to 60 °C for 5 min. When incubated and centrifuged at 35 °C with SPM liposomes in standard buffer conditions, denatured procollagen bound almost as efficiently as native procollagen (Fig. 4). Therefore the decrease in binding at 39 °C was not attributable to procollagen denaturation.

DMPC/ SPM mixtures and lipid phase dependent binding - An alternative interpretation

for the reduced binding at 39 °C was the possible requirement for SPM to be in its gel crystalline phase, since 39 °C is close to the crystalline/ fluid phase transition temperature of SPM from bovine brain (41 °C; Ultracht and Shipley, 1977). Lentz *et al.* (1981) have shown that the lipid phase transition temperature is changed when liposomes are prepared from mixtures of SPM and DMPC. Increasing amounts of DMPC lower the approximate phase transition temperature from 41°C to 24 °C (Lentz *et al.*, 1981). Therefore, liposomes were prepared from SPM/ DMPC mixtures (total phospholipid concen-tration 0.1 mM) and their interactions with [^{3}H]procollagen were measured, in standard buffer conditions, at 4 °C and at 35 °C.

At 35 °C, the presence of small amounts of DMPC greatly reduced the amount of [^{3}H]procollagen bound (Fig. 5). No more than 20 % binding was observed when the DMPC content was 10 % or greater. In contrast, at 4 °C, maximum procollagen binding was observed with mixtures containing up to 26 % DMPC, and there was a sharp transition in the percentage bound with a mid-point at approximately 35 % DMPC. A temperature of 4 °C is well below the phase transition temperature for any of the SPM/ DMPC mixtures. These data suggest that procollagen only interacts with the gel-crystalline phase of SPM, and that the interaction requires at least 65 % SPM when the procollagen concentration is 2 nM.

Procollagen domains involved in the interaction - In order to determine which region(s) of the procollagen molecule were important for the interaction with SPM, binding of [^{3}H]procollagen I was investigated by competitive inhibition with either unlabelled procollagen or separated procollagen domains. As expected, unlabelled procollagen successfully competed for binding of [^{3}H]procollagen to SPM (Fig. 6), when assayed at 35 °C in standard buffer conditions. In contrast, no inhibition was observed in the presence of highly purified lathyritic rat skin collagen I, when assayed at 4 °C (to avoid collagen aggregation) in standard buffer conditions. The interaction was also unaffected in the presence of pepsin treated collagen (i.e. with shortened non-triple helical telopeptides) up to a molar excess of 30 fold. With bovine serum albumin, only a slight inhibition was observed at a molar excess of 100 fold, with 71 % of total procollagen remaining in the bound fractions (not shown).

When unlabelled purified procollagen C-propeptides were present in the competitve inhibition binding assay, inhibition was observed that appeared to be equivalent up to a 15 fold molar excess to the effect of added unlabelled procollagen. These observations suggested that procollagen I bound to SPM via its C-propeptides. The presence of C-propeptides at a molar excess greater than 15 fold was found to have little further effect on the inhibition of procollagen binding. At a 54 fold molar excess, 40 % of the [^{3}H]procollagen remained bound to SPM. This suggested additional, weaker binding elsewhere on the procollagen molecule and remote from the C-propeptides.

To further examine the role of the procollagen C-propeptides in binding to sphingomyelin, direct binding studies were carried out with ^{125}I-labelled C-propeptides from chick procollagen I. When incubated with SPM liposomes in standard buffer conditions, [^{125}I]C-propeptides were found to bind in a saturable manner (Fig. 7). Direct non-linear least squares fitting to the binding data gave an apparent dissociation constant (K_d) of 2.32 +/- 0.53 nM with a saturation limit of 25.6 +/- 0.7 nM. The dissociation constant was similar to that for procollagen, though the saturation limit was about five-fold greater. Therefore, procollagen binds to SPM with a similar affinity to its isolated C-propeptide domain, but there appear to be more binding sites per liposome for the C-propeptides.

Though the lack of inhibition of [^{3}H]procollagen binding to SPM by unlabelled collagen suggested that the binding mechanism was independent of the mature collagen domain, others (Cohen and Barenholz, 1984) have reported binding of collagen to SPM. To investigate this, [^{3}H]collagen I was prepared by pepsinisation of [^{3}H]pro-collagen I, and binding to SPM was studied in standard assay buffer at 4 °C (to minimise collagen aggregation). At a [^{3}H]collagen I concentration of approximately 2 nM, 29.1 % ± 0.7 % (S.E., n=4) was bound to spingomyelin, compared to 93.5 +/- 0.9 % (S.E., n=10) of [^{3}H]procollagen bound in identical conditions (4 °C). Thus weak binding via the collagen domain also seems important for binding to SPM.

Binding is predominantly via the C-propeptide domain - We have shown that procollagen I binds specifically to SPM in physiological conditions of pH, ionic strength and temperature. In the same buffer conditions, there was no evidence for procollagen binding to DMPC, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol. SPM binding was also observed with procollagen II. The binding of procollagen I was saturatable, with an apparent dissociation constant (K_d) of 2.6 nM. From the competitive inhibition studies, binding appeared to occur via the C-propeptides, since at low inhibitor concentrations the effect of unlabelled C-propeptides was equal (on a molar basis) to that of unlabelled procollagen, while unlabelled collagen, with or without the non-helical telopeptides, was ineffective. Furthermore, ^{125}I-labelled C-propeptides were found to bind to SPM with a similar K_d (2.3 nM) to that of [^{3}H]procollagen I. The observation that binding was unaffected following denaturation of procollagen further points to a role for the C-propeptides, since this region of the molecule remains relatively intact (due to interchain disulphide bonds) after denaturion of the triple-helix.

At high inhibitor concentrations, unlabelled procollagen competed more efficiently for binding of [^{3}H]procollagen to SPM than did the isolated C-propeptides. This observation suggests that while binding occurs predominantly via the C-propeptide domain, additional weaker binding is mediated by at least one region elsewhere on the procollagen molecule. The observed weak binding of ^{3}H-labelled pepsinised collagen to SPM supports this interpretation and suggests that one such region is the main triple-helical domain. Binding of collagen to SPM liposomes has also been reported by Cohen and Barenholz (1984). Weak binding via the triple helical domain is consistent with the failure of unlabelled collagen to inhibit procollagen binding to SPM, when strong binding continues to be mediated by the C-propeptides.

Protein - SPM interactions - The asymmetric form of acetylcholinesterase also binds specifically to SPM, with almost no binding to phosphatidylcholine (Watkins *et al.*, 1977; Cohen and Barenholz, 1984). The asymmetric form of this enzyme has a collagen-like tail

(Hulmes, 1992) and binding to SPM is abolished following treatment with bacterial collagenase (Cohen and Barenholz, 1984). This observation was originally interpreted in terms of a direct role for the collagenous triple-helical region in binding (Cohen and Barenholz, 1984), but in the light of our results with procollagen, it is possible that binding of acetylcholinesterase to SPM may also be mediated by the globular foot of its collagenous "stalk", since collagenase treatment would sever the link between the foot region and the catalytic subunits. In accord with our observations on procollagen, the acetylcholinesterase molecule bound more strongly to SPM than collagen alone (Cohen and Barenholz, 1984), and the interaction was insensitive to ionic strength (up to 1 M NaCl; Watkins *et al.*, 1977) and temperature (4 °C and 37 °C; Cohen and Barenholz, 1984). The amino acid sequence of the collagen tail from *Torpedo* acetylcholinesterase has recently been published (Krejci *et al.*, 1991), but we have found no sequence homologies between its terminal non-collagenous sequences and the procollagen C-propeptides. Cohen and Barenholz (1984) suggested that binding of acetylcholinesterase is mediated by hydrogen binding in the interface region of SPM, which contains hydroxyl and amide groups not found in phosphatidylcholine.

Collagen-lipid interactions - We found no evidence for procollagen binding to phosphatidylcholine in physiological buffer conditions, in agreement with the observations of Watkins *et al.* (1977) and Cohen and Barenholz (1984) on acetylcholinesterase and collagen. Martinez del Pozo *et al.* (1988) reported binding of collagen to both DMPC and DPPC liposomes in 0.1 M acetic acid, as determined by density gradient sedimentation, fluorescence polarisation and differential scanning calorimetry, while in our flotation binding assay with procollagen I and DMPC liposomes in the same buffer conditions, we found no evidence for such an interaction (not shown). Furthermore, at neutral pH, Martinez del Pozo *et al.* (1989) reported an interaction between DMPC and collagen fibrils, and Ross *et al.* (1986) found the collagenous domain of the lung surfactant protein SP-A to be important for its interaction with (predominantly) DPPC, while Mollenhauer and von der Mark (1983) found no binding of ^{125}I-labelled collagen II to egg lethicin liposomes. Our flotation binding assay revealed a weak

interaction between DMPC and procollagen I at neutral pH but only in conditions of low ionic strength (not shown). We conclude that any interactions between DMPC and procollagen in physiological buffer conditions are too weak to be detected in the conditions of our flotation binding assay.

Importance of the of the gel to liquid crystalline lipid phase transition - We found that the procollagen-SPM interaction was relatively insensitive to temperature over the range 4 to 37 °C. At 39 °C, however, the extent of binding decreased considerably, an effect that could not be ascribed to procollagen denaturation since denatured procollagen bound equally well as native procollagen at 35 °C. SPM is unique among phospholipids in that its gel to liquid crystalline phase transition (41 °C at the mid-point for bovine brain SPM; Untracht and Shipley, 1977) occurs close to the physiological range of temperature (Lentz *et al.*, 1981), hence the lack of procollagen binding at 39 °C suggested that the interaction required SPM to be in its gel crystalline state. To test this hypothesis, liposomes were prepared from mixtures of SPM and DMPC, where the phase transition temperature decreases to about 24 °C as the mole percentage of DMPC increases to 100 % (Lentz *et al.*, 1981). The presence of 10 mol % DMPC in the lipid mixtrure dramatically reduced the extent of procollagen binding at 35 °C. With 14 mol % DMPC, the mid-point of the C_{16}-SPM phase transition decreases to approximately 33 °C (Lentz *et al.*, 1981) and hence a significant proportion of the SPM molecules will be in the relatively disordered, liquid crystalline state. Procollagen binding to liposomes made from 90 mol % SPM/ 10 mol % DMPC was restored when the temperature was lowered to 4 °C, well below the phase transition temperature of both lipids. The lack of binding to 100 % DMPC at 4°C showed that ordering of the phosphorylcholine groups, which are common to both SPM and PC, is not sufficient for the interaction with procollagen, and hence the interaction is specific for the gel crystalline phase of SPM. Low temperature binding was restored up to a DMPC concentration of 35 mol %. Lentz *et al.* (1981) have studied the temperature dependence of mixing of C_{16}-SPM and DMPC and concluded that these lipids mix freely at physiological temperatures. At 4 °C however, below the phase transition temperature of both lipids, there was evidence for lateral

phase separation at DMPC concentrations less than 40 mol % (Lentz *et al.*, 1981). This coincides with the abrupt change in the extent of procollagen binding to SPM/ PC mixtures at 4 °C (Fig. 5(b)). Therefore binding of procollagen to SPM appears to require discrete domains of SPM with the lipid in its gel crystalline state.

Cohen and Barenholz (1984) also found that binding of collagen-tailed acetylcholinesterase to SPM was markedly diminished by the presence of DMPC, though the temperature dependence of binding to SPM/ PC mixtures was not reported. The importance of phase transitions has been demonstrated in a number of protein-lipid interactions. For example, the binding of SP-A to 85 % DPPC/ 15 % DPPG was considerably increased when the temperature was below the phase transition temperature (King *et al.*, 1983), and similar observations were made with insulin binding to DMPC (Wiessner and Hwang, 1982).

Biological significance of the procollagen-SPM interaction - The dissociation constant for binding of procollagen I to SPM (K_d = 2.6 nM or 1.1 μg/ml) is likely to be lower than the procollagen concentration in secretory vesicles or in the immediate pericellular compartment (Birk *et al.*, 1991; Phelps *et al.*, 1985). Therefore, a significant interaction between SPM and procollagen or its released C-propeptides is possible *in vivo*. However, binding *in vitro* was only observed when the SPM concentration exceeded 60 % (w/w), and then only when SPM was in the gel crystalline phase and/or in discrete microdomains. SPM is a major component of plasma membranes, where it accounts for 5-20 % of total lipids depending on species and cell type (Gennis, 1989). Because of its relatively large head group, SPM is distributed asymmetrically in the membrane, where it occurs mostly on the extracellular surface (Gennis, 1989). Therefore the proportion of SPM in the outer leaflet will be greater than the SPM concentration as a proportion of total lipids. A similar situation exists in liposomes made from mixtures of SPM and DMPC, where again SPM is concentrated in the outer leaflet (Barenholz and Thomson, 1984; Kumar and Gupta, 1985). We conclude that the procollagen-SPM interaction may be important *in vivo*, but it requires relatively high concentations of SPM in discrete, ordered microdomains (Watkins *et al*, 1977; Cohen and Barenholz, 1984; Rintoul *et*

al., 1979).

An area in which procollagen-SPM interactions may well play a role *in vivo* is in biomineralisation, as mediated by matrix vesicles. Matrix vesicles are cell derived structures abundant in the extracellular matrix of mineralising tissues that appear to initiate the process of mineral deposition (Anderson, 1989; Ali, 1987). The membranes of matrix vesicles derived from chondrocyte microvilli during endochondral ossification are particularly rich in sphingomyelin (Hale and Wuthier, 1987; Wuthier, 1975), which accounts for up to 35 % of total lipid (and hence up to twice this amount in the outer leaflet). Vesicle contents include alkaline phosphatase, phospho-lipase A_2 and a number of annexin-like proteins that bind Ca^{2+} in the presence of acidic phospholipids (e.g. phosphatidylserine), thereby providing the necessary sink conditions for rapid Ca^{2+} influx (Genge *et al.*, 1989, 1990, 1991, 1992; Wu *et al.*, 1989, 1991b, 1991c).

Matrix vesicles bind collagens II and X (Wu *et al.*, 1989, 1991c). Anchorin CII is a collagen II binding protein and member of the annexin family (annexin V) that immunolocalises to chondrocyte plasma membranes (Pfaffle *et al.*, 1988). One of the matrix vesicle annexins is near identical to anchorin CII, and therefore it may play a role in linking the vesicle membrane to collagen fibrils (Genge *et al.*, 1990, 1992). Annexin V has also been found to be a voltage-regulated Ca^{2+} channel protein (Rojas *et al.*, 1990). A further factor is the presence of chondrocalcin, identified as the C-propeptide domain of procollagen II (van der Rest *et al.*, 1986) at the sites of initial mineral deposition (Poole *et al.*, 1984). Chondrocalcin binds strongly to hydroxyapatite and it may provide nucleation sites for calcification (Hinek *et al.*, 1987). In view of the strong homology between the C-propeptides of procollagens I and II (Dion and Myers, 1985), it is likely that procollagen II binding to SPM is also mediated by this domain.

The following hypothetical scenario can be envisaged for the role of SPM in cartilage calcification. Annexin mediated Ca^{2+} influx and binding to matrix vesicle acidic phospholipids (Genge *et al.*, 1991) leads to ordering and lateral phase separation of membrane lipid

microdomains (Haverstick and Glaser, 1987; Prigent-Dachary *et al.*, 1986), and hence SPM binding to extra-vesicular chondrocalcin. The increase in intraluminal Ca^{2+} activates phospholipase A_2 mediated breakdown of matrix vesicles (Wuthier, 1989), and then $Ca^{2+}$and Pi released into the matrix interact with chondrocalcin to initiate mineralisation. Fur-ther work is required to determine whether such a scenario operates *in vivo*.

Acknowledgements

We are grateful to Dr D.K. Apps for help with the radio-iodination, to Mr J.R.E. MacBeath for the lathyrtic rat skin collagen, to Dr L. Vuillard for help with dynamic light scattering, to Dr G L Atkins for analysis of the binding data, to Dr K E Kadler for supply of conditioned culture medium and to Drs J.H.P. Sizer, J. Perez-Castineira, J. Torbet, J.P. Bradshaw and R. Ashley for helpful discussions.

References

Ali, S.Y. (1987) *Fed. Proc.* **35**, 135-142.

Albelda, S.M. and Buck, C.A. (1990) *FASEB J.* **4**, 2868-2880.

Anderson, H.C. (1989) *Lab. Invest.* **60**, 320-330.

Barenholz, Y. and Thompson, T.E. (1980) *Biochim. Biophys. Acta* **604**, 129-158.

Barnes, M. (1985) *Collagen Rel. Res.* **5**, 65-97.

Birk, D.E., Silver, F.H. and Trelstad, R.L. (1991) in *Cell Biology of the Extracellular Matrix* (Hay, E.D., ed.), 2nd Ed, pp. 221-254, Plenum Press, New York

Chernousov, M.A., Fogerty, F.J., Koteliansky, V.E. and Mosher, D.F. (1991) *J Biol.Chem.* **266**, 10851-10858.

Cohen, R. and Barenholz, Y. (1984) *Biochim.Biophys.Acta* **778**, 94-104.

Curran, S. and Prockop, D.J. (1982) *Biochem.* **21**, 1482-1487.

Dehm, P. and Prockop, D.J. (1973) *Eur. J. Biochem.* **35**, 159-166.

Dion, A.S. and Meyers, J.C. (1985) *J. Mol. Biol.* **193**, 127-143.

Genge, B.R., Wu, L.N.Y. and Wuthier, R.E. (1989) *J.Biol.Chem.* **264**, 10917-10921.

Genge, B.R., Wu, L.N.Y. and Wuthier, R.E. (1990) *J.Biol.Chem.* **265**, 4703-4710.

Genge, B.R., Wu, L.N.Y., Adkisson, H.D. and Wuthier, R.E. (1991) *J. Biol. Chem.* **266**, 10678-10685.

Genge, B.R., Cao, X., Wu, L.N.Y., Buzzi, W.R., Showman, R.W., Arsenault, A.L., Ishikawa, Y. and Wuthier, R.E. (1992) *J. Bone Min. Res.* **7**, 807-819.

Gennis, R.B. (1989) *Biomembranes*, pp. 1-533, Springer-Verlag, New York.

Goldberg, B.D. and Burgeson, R.E. (1982) *J. Cell Biol.* **95**, 752-756.

Hale, J.E. and Wuthier, R.E. (1987) *J. Biol. Chem.* **262**, 1916-1925.

Haverstick, D.M. and Glaser, M. (1987) *Proc. Nat. Acad. Sci.* **84**, 4475-4479.

Hay, E.D., ed. (1991) *Cell Biology of the Extracellular Matrix*, 2nd Ed, pp. 1-468, Plenum, New York.

Hayashi, T., Curran-Patel, S. and Prockop, D.J. (1979) *Biochem.* **18**, 4182-4187.

Hinek, A., Reiner, A. and Poole, A.R. (1987) *J. Cell Biol.* **104**, 1435-1441.

Hojima, Y., van der Rest, M. and Prockop, D.J. (1985) *J.Biol.Chem.* **260**,15996-16003.

Hulmes, D.J.S. (1992) *Essays Biochem.* **27**, 49-67.

Hynes, R.O. (1990) *Fibronectins*, pp. 1-546, Springer-Verlag, New York.

Hynes, R.O. (1992) *Cell* **69**, 11-25.

King, R.J., Carmichael, M.C. and Horowitz, P.M. (1983) *J. Biol. Chem.* **258**, 10672-10680.

Krejci, E., Coussen, F., Duval, N., Chatel, J.-M., Legey, C., Puype, M., Vandekerckhove, J., Cartaud, J., Bon, S. and Massoulie, J. (1991) *EMBO J.* **10**, 1285-1293.

Kumar, A. and Gupta, C.M. (1985) *Biochem.* **24**, 5157-5163.

Kuroki, K. and Akino, T. (1991) *Biochem. Int.* **24**, 225-234.

le Lous, M., Boudin, D., Salmon, S. and Polonovski, J. (1982) *Biochim. Biophys. Acta* **708**, 26-32.

Lentz, B.R., Hoechi, M. and Barenholz, Y. (1981) *Biochem.* **20**, 6803-6809.

Limper, A.H., Quade, B.J., Lachance, R.M., Birkenmeier, T.M., Rangwala, T.S. and McDonald, J.A. (1991) *J. Biol.Chem.* **266**, 9697-9702.

Martinez del Pozo, A., Onaderra, M., Laynez, J. and Gavilanes, J.G. (1988) *Collagen Rel.Res.* **8**,133-144

Martinez del Pozo, A., Onaderra, M., Laynez, J. and Gavilanes, J.G. (1989) *Matrix* **9**,405-410.

Mauch, C., von der Mark, K., Helle, O., Mollenhauer, J., Pfaffle, M. and Krieg, T. (1988) *J. Cell Biol.* **106**,205-211

Mellor, S.J., Atkins, G.L. and Hulmes, D.J.S. (1991) *Biochem. J.* **276**, 777-784.

Miller, E.J. (1971) *Biochem.* **10**, 1652-1659.

Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) *Biochem.* **20**, 833-840.

Mollenhauer, J. and von der Mark, K. (1983) *EMBO J.* **2**,45-50.

Morris, N.P., Fessler, L.I., Weinstock, A. and Fessler, J.H. (1975) *J. Biol. Chem.* **250**, 5719-5726.

Mosher, D.F., Fogerty, F.J., Chernousov, M.A. and Barry, E.L. (1991) *Ann. N. Y. Acad. Sci.* **614**, 167-180.

Mould, A.P., Holmes, D.F., Kadler, K.E. and Chapman, J.A. (1985) *J.Ultrastruct.Res.* **91**, 66-76.

Mould, A.P. and Hulmes, D.J.S. (1987) *J. Mol. Biol.* **195**, 543-553.

Mould, A.P., Hulmes, D.J.S., Holmes, D.F., Cummings, C., Sear, C.H.J. and Chapman, J.A. (1990) *J.Mol.Biol.* **211**,581-594.

New, R.R.C., ed. (1990) *Liposomes*, pp. 1-301, IRL Press, Oxford.

Olsen, B.R. (1991) in *Cell Biology of the Extracellular Matrix* (Hay, E.D., ed.), 2nd Ed, pp. 177-220, Plenum, New York.

Olsen, B.R., Guzman, N.A., Engel, J., Condit, C. and Aase, S. (1977) *Biochem.* **16**,3030-3036.

Ozgunes, N. and Artvinli, S. (1988) *Biochem. Med. Met. Biol.* **40**, 269-275.

Pajean, M, Huc, A. and Herbage, D. (1991) *Int. J. Pharmacol.* **77**, 31-40.

Payne, K.J., King, A. and Holmes, D.F. (1986) *Biopolymers* **25**, 1185-1207.

Perez-Castineira, J.R. and Apps, D.K. (1990) *BiochemJ.* **271**, 127-131.

Pfaffle, M., Ruggiero, F., Hofmann, H., Fernandez, M.P., Selmin, O., Yamada, Y., Garrone, R. and von der Mark, K. (1988) *EMBO J.* **7**, 2335-2342.

Phelps, R.G., Martinez-Hernandez, A. and Goldberg, B.D. (1985) *Collagen Rel. Res.* **5**, 405-414.

Poole, A.R., Pidoux, I., Reiner, A. Choi, H. and Rosenberg, L.C. (1984) *J. Cell Biol.* **98**, 54-65.

Prigent-Dachary, J., Faucon, J.-F., Boisseau, M.R. and Dufourcq, J. (1986) *Eur. J. Biochem.* **155**, 133-140.

Pusey, P.N. (1989) in *Dynamic Properties of Biomolecuar Assemblies* (Harding, S.E. and

Rowe, A.J., eds), pp. 90-102, Royal Society of Chemistry, Cambridge.

Rintoul, D.A., Chou, S.M. and Silbert, D.F. (1979) *J. Biol. Chem.* **254**, 10070-10077.

Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C. and Burns, A.L. (1990) *J. Biol. Chem.* **265**, 21207-21215.

Ross, G.F., Notter, R.H., Meuth, J. and Whitsett, J.A. (1986) *J. Biol. Chem.* **261**, 14283-14291.

Ruoslahti, E. (1991) in *Cell Biology of the Extracellular Matrix* (Hay, E.D., ed.), 2nd Ed, pp. 343-363, Plenum, New York.

Smith, G.N., Hasty, K.A. and Brandt, K.D. (1989) *Matrix* **9**, 186-192.

Tall, A.R., Small, D.M. and Lees, R.(1978) *J. Clin. Invest.* **62**, 836-846.

Untracht, S.H. and Shipley, G.G. (1977) *J. Biol. Chem.* **252**, 4449-4457.

van der Rest, M., Rosenberg, L.C., Olsen, B.R. and Poole, A.R. (1986) *Biochem.J.* **297**, 923-925.

van der Rest, M. and Garrone, R. (1991) *FASEB J.* **5**, 2814-2823.

Watkins, M.S., Hitt, A.S. and Bulger, J.E. (1977) *Biochem. Biophys. Res. Commun.* **79**, 640-647.

Wiessner, J.H. and Hwang, K.J. (1982) *Biochim. Biophys. Acta* **689**, 490-498.

Wu, C.H., Walton, C.M. and Wu, G.Y. (1991a) *J. Biol. Chem.* **266**, 2983-2987.

Wu, L.N.Y., Sauer, G.R., Genge, B.R. and Wuthier, R.E. (1989) *J.Biol.Chem.* **264**, 21346-21355.

Wu, L.N.Y., Genge, B.R. and Wuthier, R.E. (1991b) *J.Biol.Chem.* **266**, 1187-1194.

Wu, L.N.Y., Genge, B.R., Lloyd, G.C. and Wuthier, R.E. (1991c) *J.Biol.Chem.* **266**, 1195-1203.

Wuthier, R.E. (1975) *Biochim. Biophys. Acta.* **409**, 128-143.

Wuthier, R.E. (1989) *Connect.Tissue. Res.* **22**, 653-659.

Footnotes

[1]This work was funded by the Wellcome Trust (Studentship to AAC and Research Leave Fellowship to DJSH) and by the Medical Research Council (equipment grant).

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[4]Abbreviations used: SPM, sphingomyelin; DMPC, dimyristoylphosphatidylcholine; [^{14}C]DOPC, 1-2-di [1-^{14}C] oleoyl L-3-phosphatidylcholine; DPPC, dipalmitoylphosphatidyl-choline; DPPG, dipalmitoylphosphatidylglycerol.

Figure Legends

Fig. 1. Centrifugation in discontinuous 0/30/40/60 % sucrose gradients of (a) ^{3}H-procollagen I alone (b) liposomes of DMPC with trace amounts of ^{14}C-DOPC (c) mixture of [^{3}H]procollagen I and DMPC liposomes and (d) mixture of [^{3}H]procollagen I and SPM liposomes. All gradients were in phosphate buffered saline, with samples initially in the 40 % sucrose region.

Fig. 2. Dependence of ^{3}H-procollagen I binding on SPM concentration. [^{3}H]pro-collagen I (2 nM) was incubated with increasing concentrations of SPM liposomes and then analysed by discontinuous sucrose gradient centrifugation. The SPM concentrations in the incubation mixtures and amounts of [^{3}H]procollagen I in the bound fractions (top half of the gradient) were (a) 12.5 μM, 41 % bound (b) 25 μM, 54 % bound (c) 50 μM, 77 % bound and (d) 100 μM, 85 % bound.

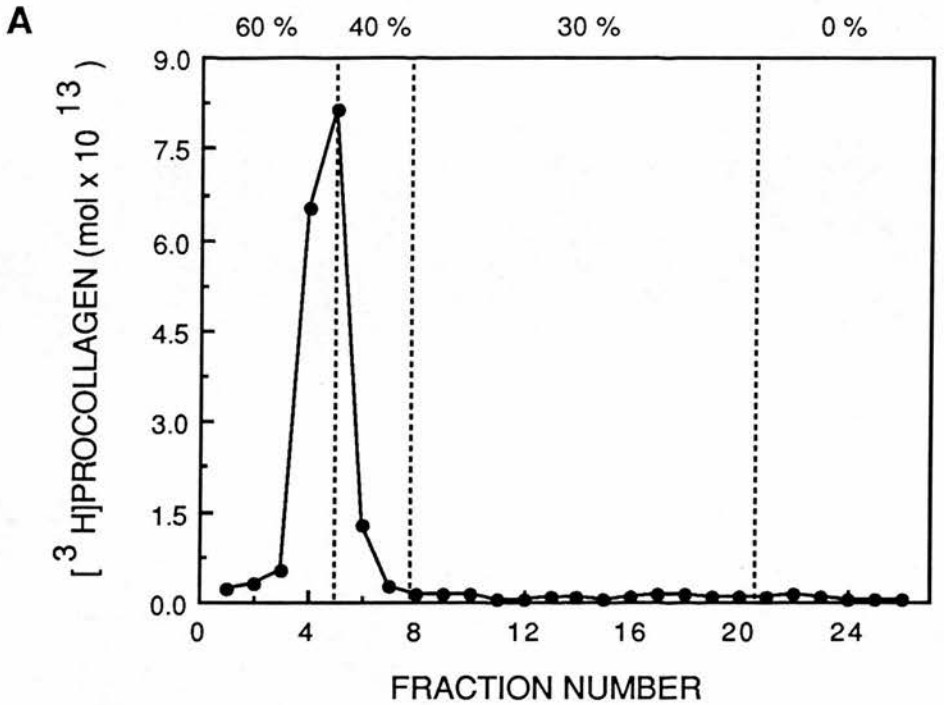
Fig. 3. Concentration dependence of [^{3}H]procollagen I binding to SPM liposomes. Increasing concentrations of [^{3}H]procollagen I were incubated with 0.1 mM SPM and the amount of procollagen in lipid-bound fractions determined by discontinuous sucrose gradient centrifugation. The curve shows the non-linear least squares fit to the data using a dissocation constant (K_d) of 2.55 nM and a saturation limit of 5.04 nM. Concentrations are expressed with respect to the volume of the original incubation mixture (450 μl).

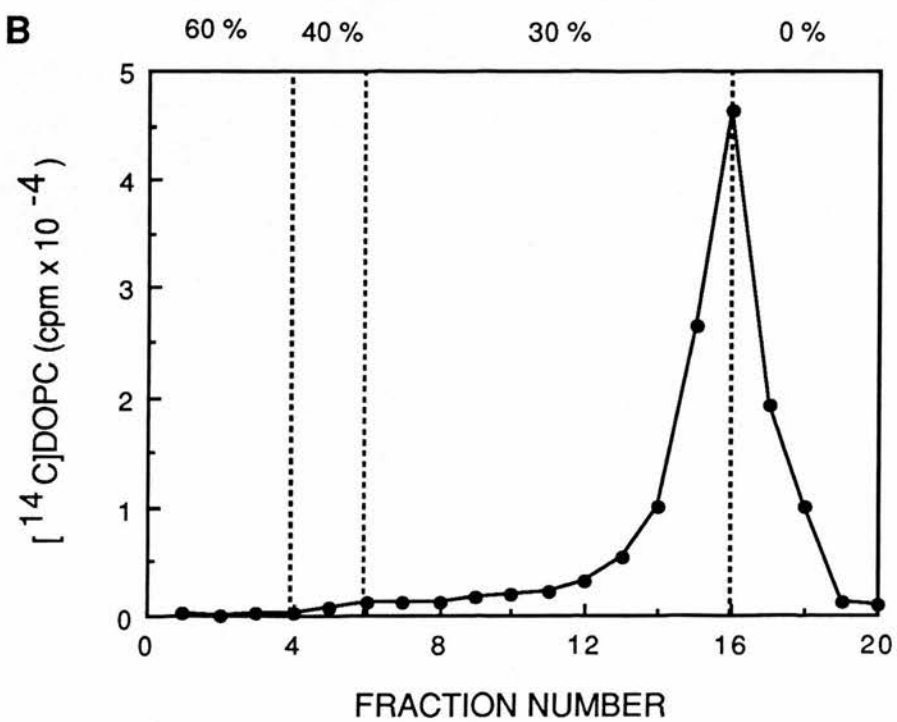
Fig. 4. Effect of temperature on [^{3}H]procollagen I binding to SPM liposomes. Bin-ding was assayed by discontinuous sucrose density gradient centrifugation in standard assay conditions (see text), and is expressed as the percentage of total ^{3}H cpm in the top half of the gradient. The binding of denatured [^{3}H]procollagen I at 35 °C is indicated by den(35). Error bars show standard error of the mean.

Fig. 5. Binding of [^{3}H]procollagen I to liposomes made from mixtures of SPM and DMPC. The percentage of ^{3}H cpm in the bound fractions was measured in standard assay buffer as a function of DMPC content using 2 nM [^{3}H]procollagen I and 0.1 mM total lipid concentration in the incubation mixture. Binding was measured at (a) 35 °C and (b) 4 °C. Error bars show standard error of the mean.

Fig. 6. Effect of unlabelled procollagen I and its various structural domains on the binding of [^{3}H]procollagen I to SPM. Binding was measured in standard assay conditions at 35 °C in the presence of added procollagen I (●), collagen I (▲), pepsinised collagen I (□) or procollagen I C-propeptides (○), and is expressed as the percentage of maximum binding in the absence of competitive inhibitors, with the molar ratio of inhibitor to [^{3}H]procollagen I on the horizontal axis.

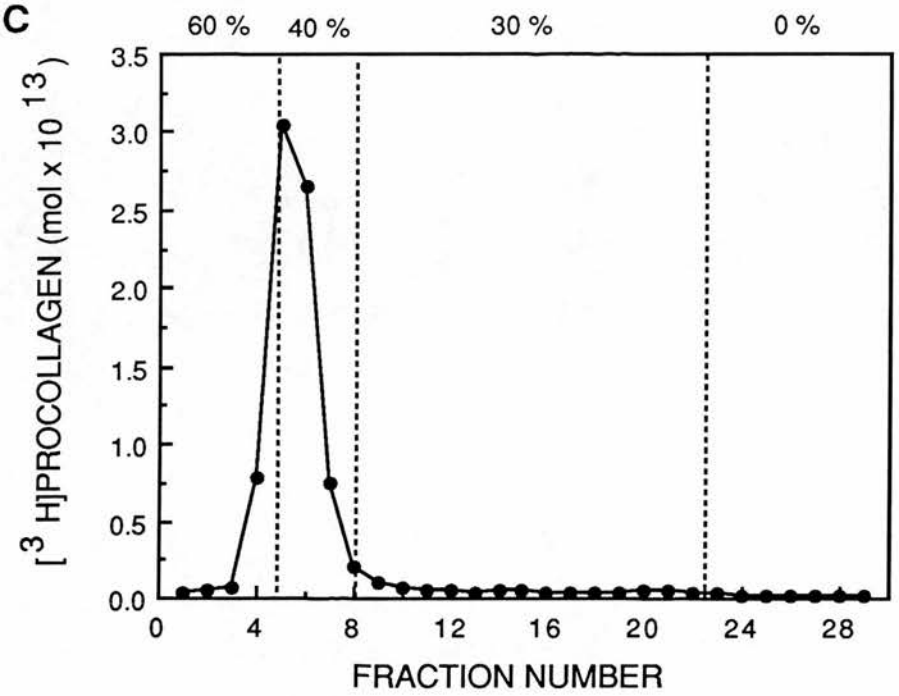
Fig. 7. Concentration dependence of the binding of ^{125}I-labelled procollagen I C-propeptides to SPM liposomes. Increasing concentrations of ^{125}I-labelled C propeptides were incubated with 0.1 mM SPM and the extent of binding was determined by discontinuous sucrose gradient centrifugation. The curve shows the non-linear least squares fit to the data using a dissocation constant (K_d) of 2.32 nM and a saturation limit of 25.6 nM. Concentrations are expressed with respect to the volume of the original incubation mixture (450 µl).



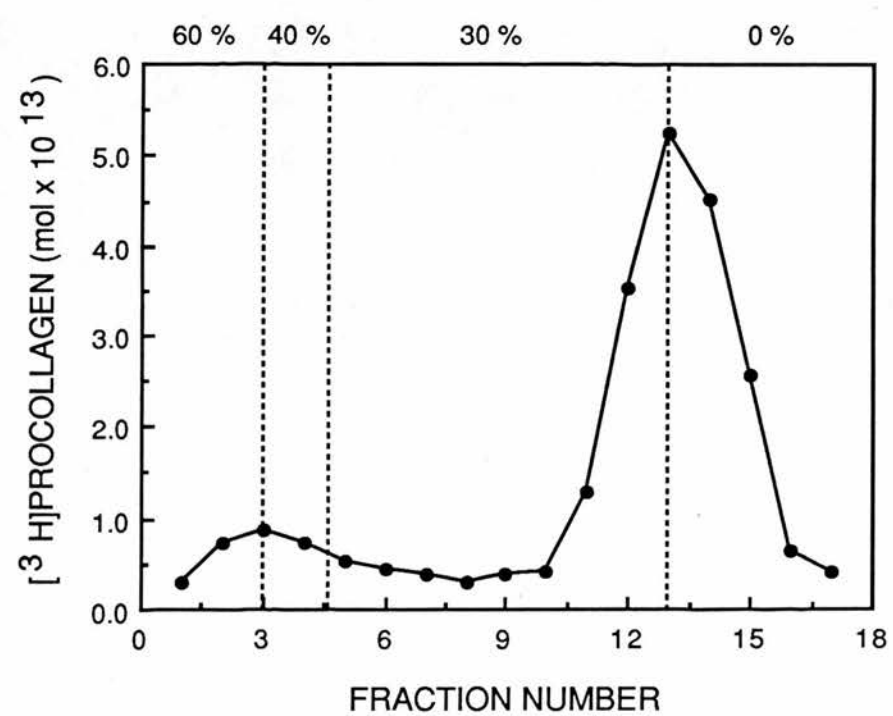


Chaplin et al Fig 1(a) + (b)

C

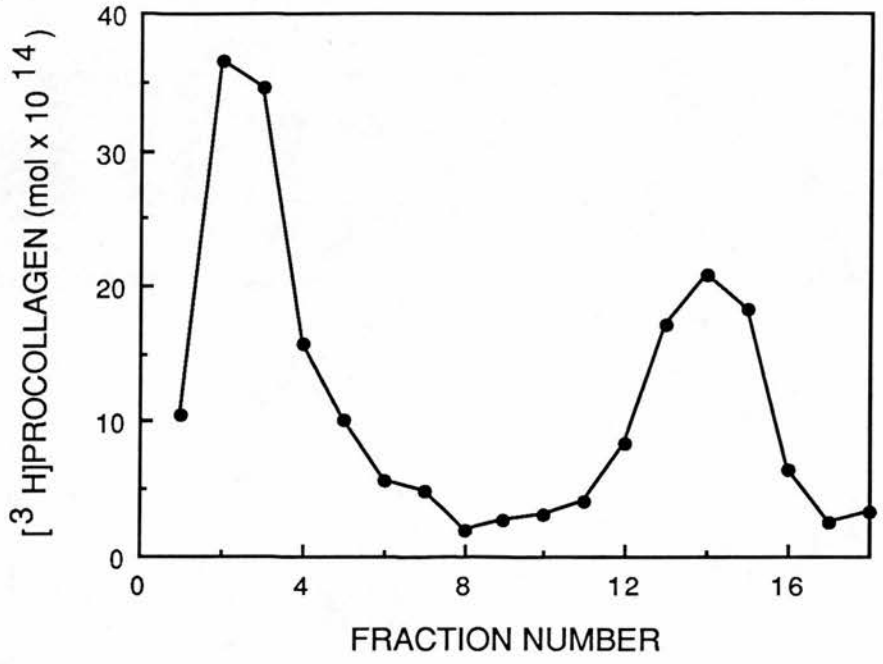


D



Chooling et al Fig 1 (c) + (d)

A



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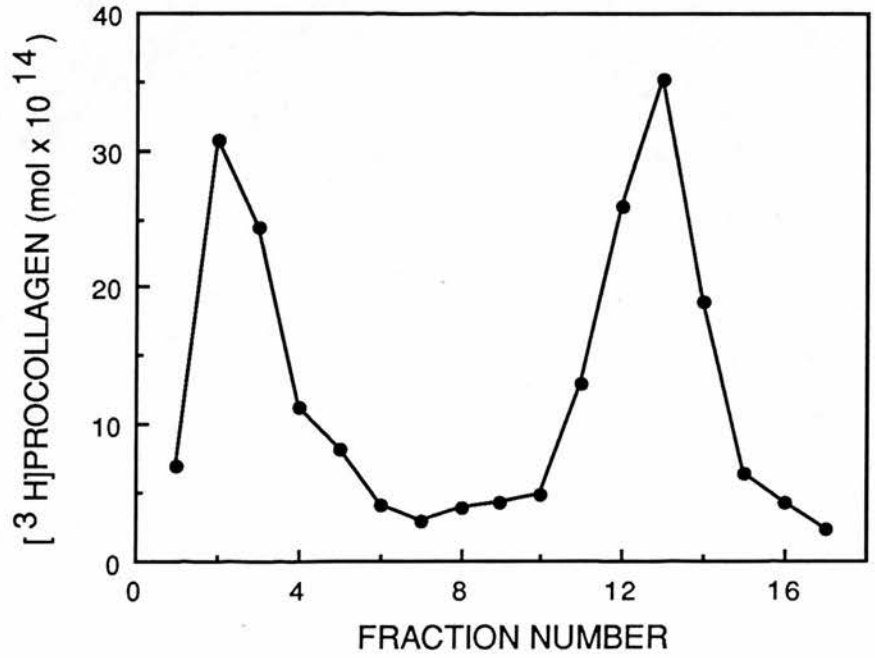
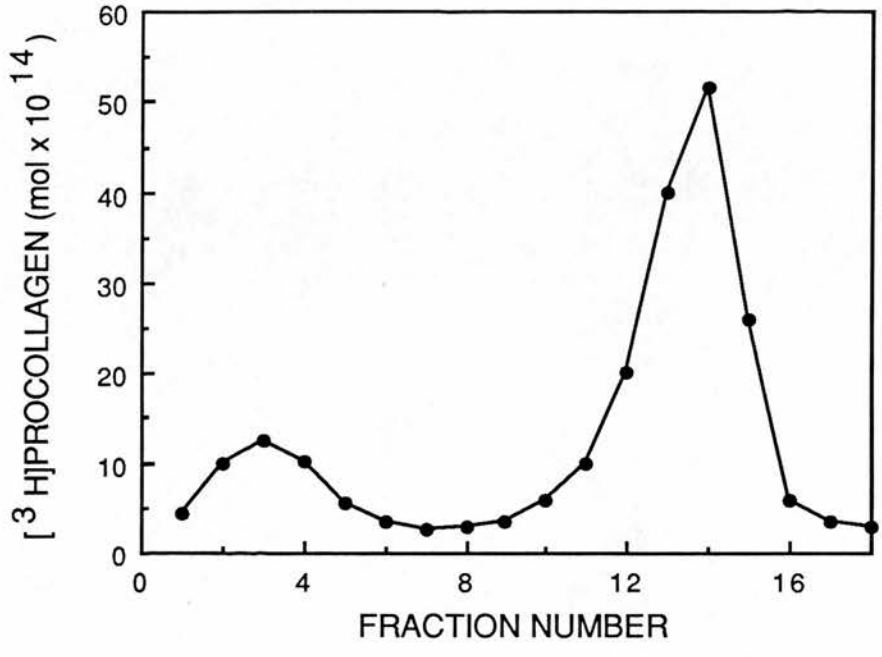


Chaglay et al Fig 2(a) & (b)

C



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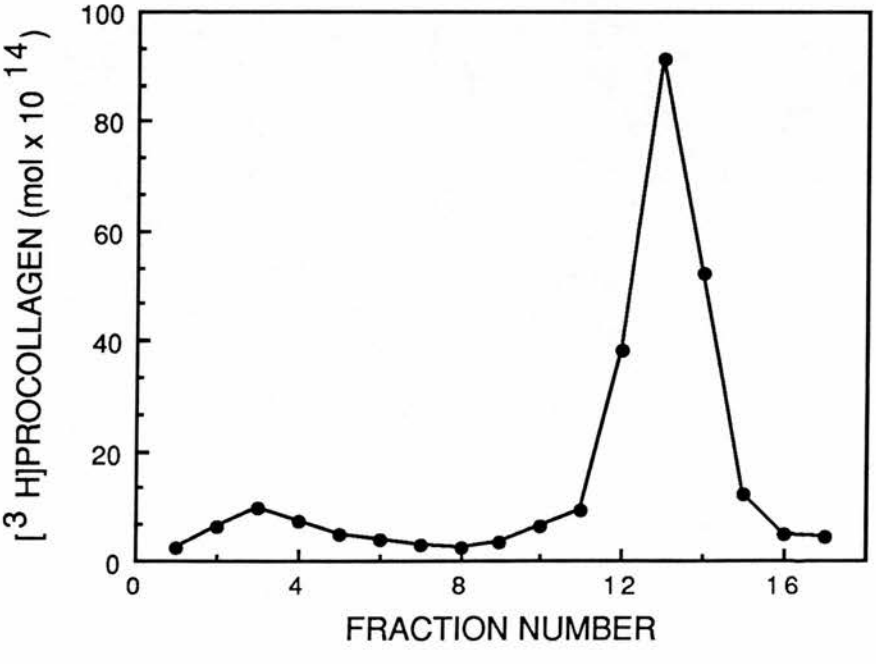

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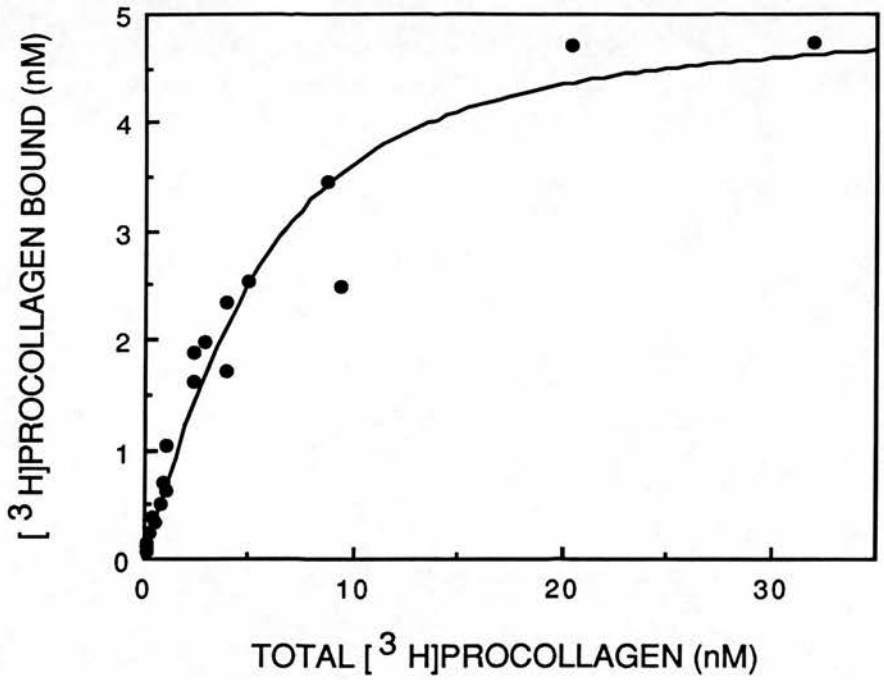

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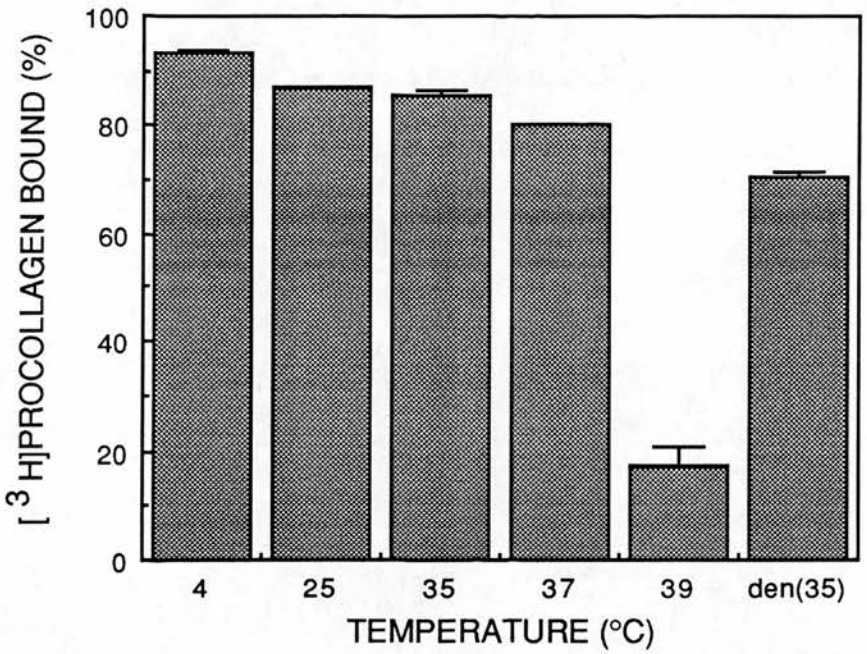

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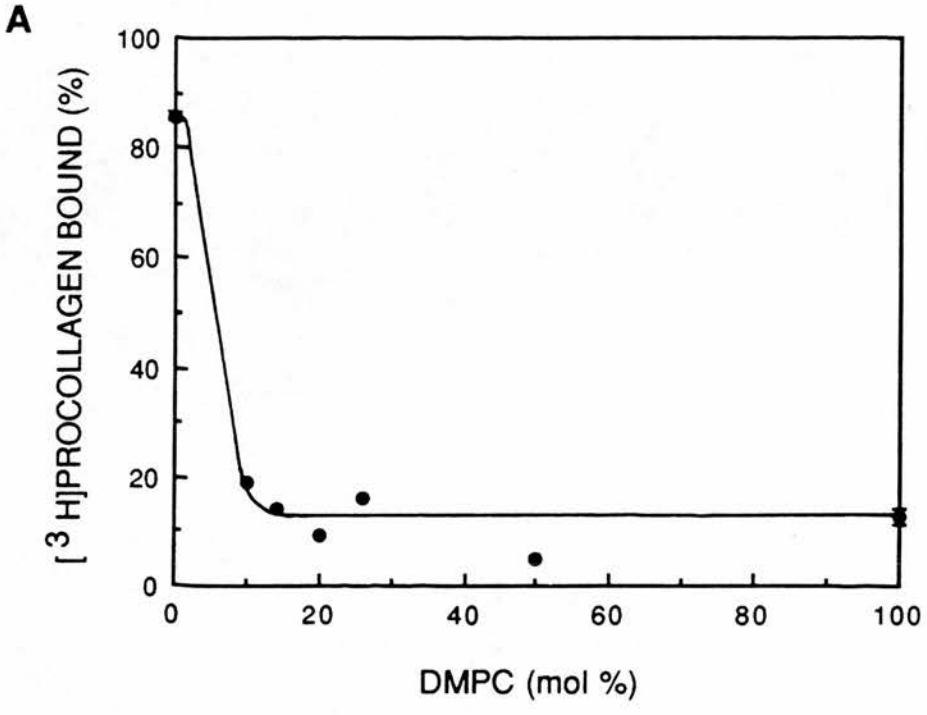
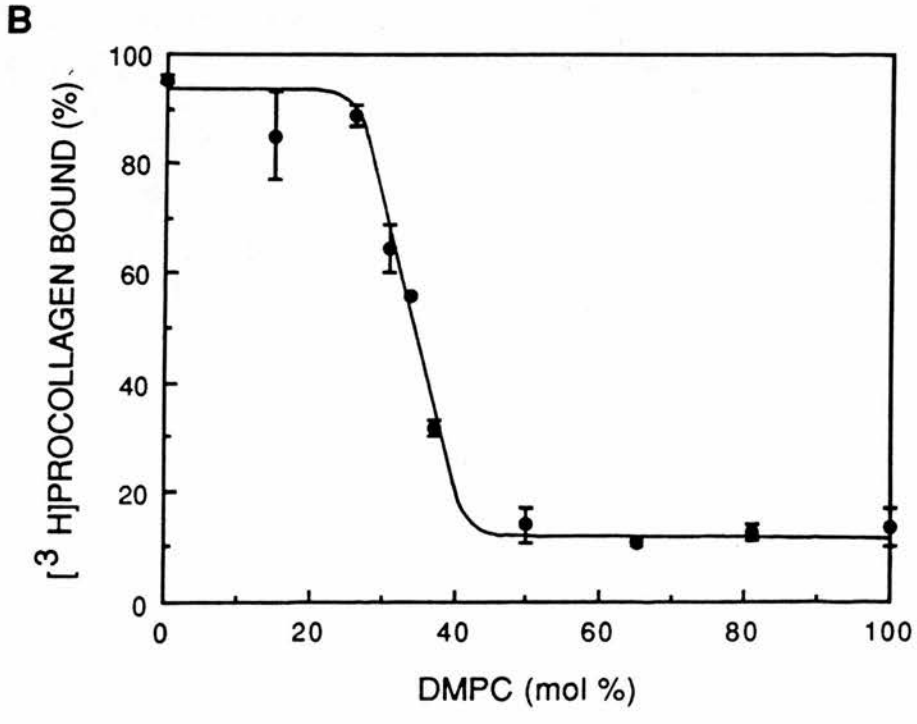
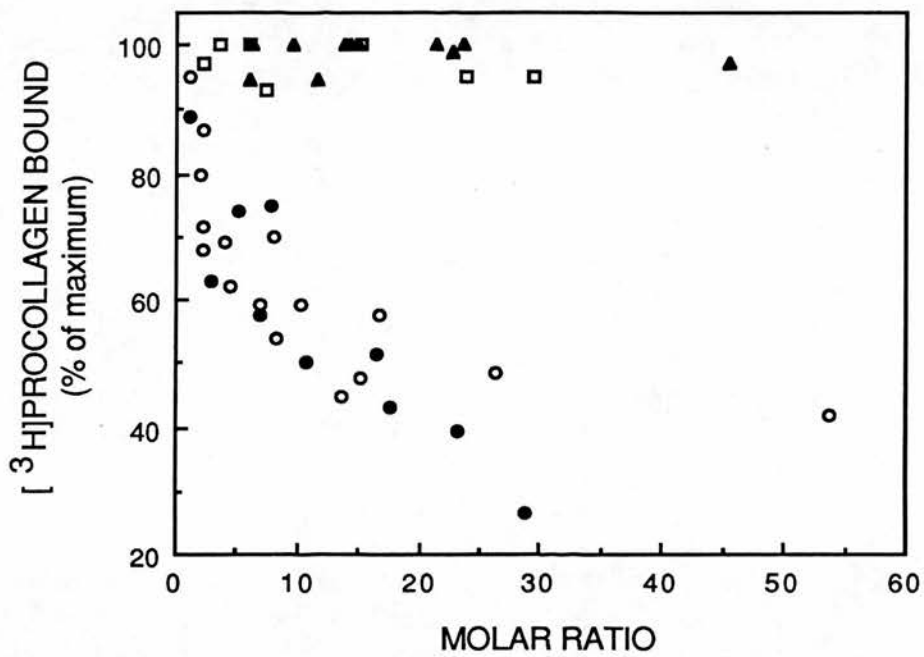




Chuelay et al Fig 5



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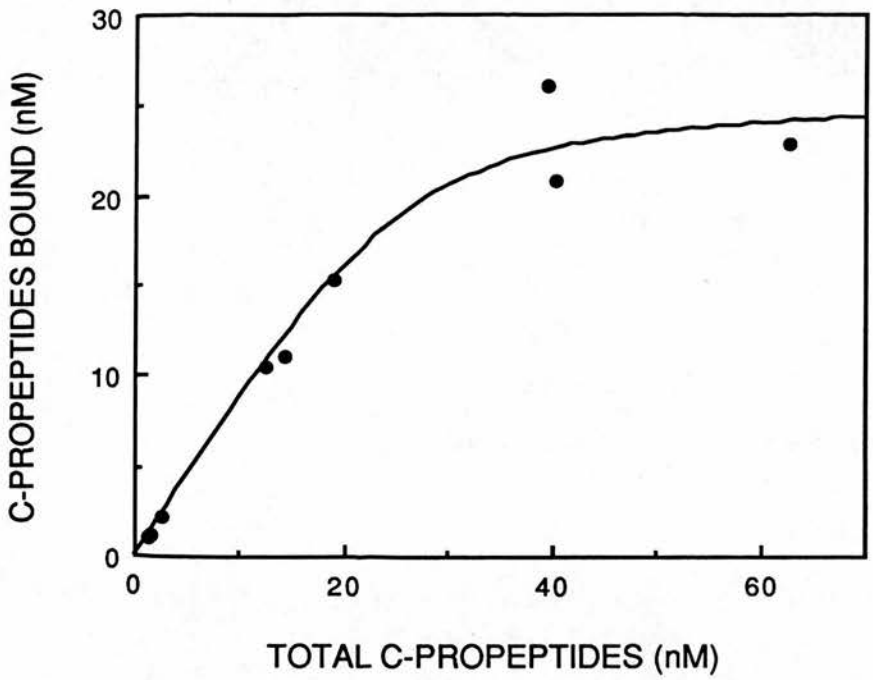

Chaglag et al Fig 7