THE ROLE OF COLLAGEN IN CELL-MATRIX INTERACTIONS

MODULATION OF COLLAGEN SYNTHESIS AND DEPOSITION IN CULTURED ENDOTHELIAL CELLS BY FIBROBLAST GROWTH FACTOR AND BY THE EXTRACELLULAR MATRIX

by

SCHEFFER CHUEI-GOONG TSENG

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

EXPERIMENTAL PATHOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date University Librarian

Degree Conferred: . . . . . JUN 2 8 1981 . . . . . .

To my dear father and mother

and to my loving wife

" You shall know the truth,

and the truth will make you free. "

- John 8:32

CONTENTS

ABSTRACT

The establishment of bovine vascular and corneal endothelial cells *in vitro* is facilitated by introducing fibroblast growth factor (FGF) into the culture media. In the presence of FGF, cells adopt the morphological characteristics of endothelium *in vivo* with extracellular matrix (ECM) elaborated exclusively on the subendothelial surface. In contrast, in the absence of FGF, vascular endothelial cells undergo structural and functional alterations different from their *in vivo* counterparts, and ECM is now deposited on all cell surfaces. However, plating cells on their own preformed matrix obviates the requirement for FGF.

The aim of this study is to explore the inter-relationship between FGF and ECM. The hypothesis is that FGF is responsible for modulating ECM production. Since collagen is the major component of ECM, the hypothesis was tested that FGF can modulate collagen synthesis. This was examined by the analysis of collagen synthesis and deposition. Quantitative assays of collagen were made by measuring radiolabeled hydroxyproline by Dowex 50W-X8 and by high voltage paper electrophoresis, and qualitative analyses of collagen types by a combination of DEAE-cellulose and CM-cellulose chromatographies, SDS-polyacrylamide gel electrophoresis, and CNBr-cleaved peptide mapping.

Collagen synthesis and deposition was compared in the cultured bovine corneal and vascular endothelial cells. In the presence of FGF and at the confluent stage, 1% and 0.5% of total protein synthesis was devoted to collagen synthesis by corneal and vascular endothelial cells respectively. Type III was the major interstitial collagen made by both endothelial cells, in addition to small amounts of type IV and V. Some type I collagen was synthesized by corneal endothelial cells. None was

found in vascular endothelial cell cultures. This indicated micro-heterogeneity in collagen type synthesis by endotheliums from different tissues. The preponderance of type III collagen in all cultures distinguished endothelial cells from fibroblasts.

Analysis was then performed in the absence of FGF. Twice the amount of collagen was synthesized by vascular endothelial cells in the absence of FGF than in its presence. In addition, there now emerged synthesis of type I collagen as well as changes in the monomers of type V collagen.

The changes were analyzed through the entire course of corneal endothelial cell cultures. At the both sparse and confluent stages, collagen was made at a rate two to three fold that made in the absence of FGF, and proportionately less was deposited into the cell layers. When FGF was added, deposition of collagen in the cell layers increased by a stepwise increment from the sparse to the confluent stage. This indicated an active role of FGF in organizing the collagenous content of ECM in cultured endothelial cells.

When ECM was provided as the natural substratum, collagen synthesis decreased to the range of one-half to one-third of the original level of the culture with FGF. This occurred even at the early sparse stage, indicating cells sensed and responded to the presence of collagenous matrix environment. This cell-matrix interaction provided by the natural substrata was important for maintaining cellular function, as shown by the evidence that cell surface polarity of elaboration of collagen was achieved in the sparse culture. Such an appropriate cell-matrix interaction by natural substrata also served as a feedback regulatory mechanism for collagen biosynthesis. Its loss resulted in an

increase in collagen synthesis and the failure to organize an appropriate collagenous ECM.

This study provides evidence for the hypothesis that FGF has an active role in modulating ECM production. The normal cell-matrix interaction thus obtained can further sustain the subsequent cellular functions.

ACKNOWLEDGEMENT

This thesis work was performed in the laboratory of Dr. Robert Stern, my research advisor. Under his continuous guidance, encouragement, and support, I was able to pursue the degree and work out this thesis project. To him I would like to express my deep thanks. Special thanks are given to Dr. Denis Gospodarowicz for providing the model system for this project. I am also indebted to the other two members of the thesis committee, Dr. Edward Smuckler and Dr. Montgomery Bissell, for their valuable advice and suggestions. There are a number of people to whom I wish to express my appreciation for their supportive efforts. These include Dr. Danute Nitecki, Dr. Naphtali Savion, Dr. Robert Siegel Dr. Joseph Fu, and Mr. Jackson Hall.

The research was carried out in a very intellectual environment. The critical mind of Dr. Zena Werb has been very helpful in the preparation of the manuscript. In the laboratory, the afternoon seminars have indeed broadened my understanding of basic science. For this, I appreciate Dr. Robert Stern for organizing these seminars, and the other participants, especially Jack Hall, Richard Kao, and Dr. Jim McKerrow, my colleagues in the laboratory.

In the end, I would like to thank Ms. Diane Jarest for the excellent assistance in the preparation of this thesis.

ABBREVIATIONS

ABAE-F	adult bovine aortic endothelial cell culture maintained in the absence of FGF
ABAE+F	adult bovine aortic endothelial cell culture maintained in the presence of of FGF
BAPN	β-aminopropionitrile fumarate
BCE	bovine corneal endothelial cell culture
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
cAMP	adenosine 3':5' cyclic phosphate
CM	carboxymethyl
CNBr	cyanogen bromide
DEAE	diethylaminoethyl
DMEM	Dulbecco's-modified Eagle's medium
ECM	extracellular matrix
ECMs	extracellular matrices
EDS	Ehlers-Danlos Syndrome
EGF	epidermal growth factor
FCS	fetal calf serum
FGF	fibroblast growth factor
PBS	phosphate-buffered saline, pH 7.4
PCMB	p-hydroxymercuribenzoate
PG	prostaglandins
PMSF	phenylmethylsulfonyl fluoride
RER	rough endoplasmic reticulum
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tris	tris (hydroxymethyl) aminomethane

CHAPTER I GENERAL INTRODUCTION

Collagen is the most abundant protein in the animal world. It provides an extracellular framework for all multicellular animals and is the major constituent of connective tissues. Collagen can take on a number of special forms or shapes such that each connective tissue is endowed with specific structural and functional characteristics: among such forms are ropes and strands (tendon and ligaments); woven sheets (skin and fascia); filtration membranes (glomeruli); supporting skeleton (bone and dentin), and others. Collagen serves not only as a major component of connective tissues, but also as a component of the pericellular substratum or extracellular matrix (ECM), onto which cells attach, proliferate, differentiate, and express their phenotypic characteristics.

This thesis addresses the general role of collagen in the interactions of cells with their ECMs. Specifically, collagen modulation in cultures of endothelial cells is examined as a model system for studying that interaction. The hypothesis addressed herein asks whether fibroblast growth factor (FGF), a soluble peptide factor, controls these cell-matrix interactions by modulating collagen synthesis and deposition.

In the beginning of this thesis, a general background is provided. This includes a brief review of collagen biosynthesis, collagen types and their tissue distribution, both quantitative and qualitative aspects of their regulatory mechanisms, and functional aspects of ECM and collagen. An attempt is made specifically to stress points relevant to this

thesis. Detailed information can be found in greater depth in the following reviews: Hance and Crystal (1975), Grant and Jackson (1976), Frietzek and Kühn (1976), Miller (1976), Fessler and Fessler (1978), Bornstein and Byers (1978), Prockop et al. (1976, 1979), and Bornstein and Sage (1980).

I. STRUCTURE AND BIOSYNTHESIS OF COLLAGEN

In terms of structure, extracellular collagen fibers are built of collagen molecules, which have a diameter of 1.4 nm and a length of 280 nm. These molecules consist of three polypeptide chains called α chains, coiled around one another in a unique rigid helical structure. The α chains contain about 1000 amino acid residues in a linear sequence with a molecular weight of 95,000 daltons. In each of the α chains, every third amino acid is glycine, and the polypeptide chain can thus be regarded as a polymer of tripeptide units $(Gly\text{-}X\text{-}Y)_n$, where n is equal to 338 for type I collagen. Characteristically, amino acid analyses demonstrate that in addition to glycine there is a high content of proline and lysine, their hydroxyl derivatives, and a relatively low content of tyrosine and cysteine, and the absence of tryptophan.

Biosynthesis of collagen follows the basic principles of protein biosynthesis. The main steps of biosynthesis and the corresponding structural features are summarized in Table 1 A & B. In brief, collagen is synthesized in a precursor form termed procollagen, which is composed of three precursor proα chains. Each proα chain is a primary product of transcription and translation, and possesses compositional features different from α chains. Non-helical extension propeptides are attached

at both N- and C- termini. Unique to collagen biosynthesis are a series of post-translational modification steps which occur to proα chains prior to their secretion in the form of procollagen. Firstly, hydroxylation of proline and lysine occurs through enzymatic reactions catalyzed by prolyl hydroxylase and lysyl hydroxylase. Both are dependent on the cofactors, Fe^{++},O_2, ascorbic acid, and α-ketoglutarate. This hydroxylation occurs only when proline or lysine are in the y position of the gly-x-y repeating triplet. Hydroxylation of proline is essential for triple helix formation and the stability of helical structure. Hydroxylation of lysine is essential for subsequent glycosylation reactions and extracellular crosslink formation, which will be described below. Secondly, after hydroxylation, certain hydroxylysyl residues in the helical portion of the proα chains are glycosylated by the addition of monosaccharide, galactose or the disaccharide, glucosylgalactose. These saccharide units are added sequentially through the enzymatic reactions of galactosyl transferase and glucosyl transferase. The function of glycosylation remains uncertain, although a number of possible functions have been suggested, including identification of the proα chain as a protein destined for secretion, and facilitation of crosslink formation. Thirdly, procollagen is formed by twisting three proα chains into a triple helical structure. This is preceded by interchain disulfide bond formation among extension propeptides. An additional requirement for proper triple helix formation is the presence of a certain number of hydroxyprolines which as aforementioned enhance the stability of the triple helix structure. After the above modifications, procollagen is ready for secretion. This takes place by translocation through the cisternae of the endoplasmic reticulum and finally secretion by the Golgi complex through the mechanism of exocytotic vesicle formation.

In the extracellular space, several unique processes work sequentially on the procollagen molecules which render them aggregated, insoluble, and cross-linked fibrous structures. Soluble procollagen molecules are initially converted into collagen by cleaving both the N- and C- terminal extension propeptides by specific peptidases. Collagen molecules then aggregate into collagen fibrils in a special fashion with a staggered arrangement. The mechanism of fibrillogenesis is still unknown. The collagen fibrils are finally stabilized through intra- and intermolecular crosslinks. Crosslinking is not necessary for fibril formation, but without crosslinks, the fibrils lack high tensile strength. Crosslinks are derived from lysyl or hydroxylysyl residues on neighboring chains. Their formation begins with oxidative deamination of the ϵ-amino group of lysine and hydroxylysine to the corresponding δ-semialdehyde, allysine ar hydroxyallysine. This step requires lysyl oxidase, a Cu^{++}-requiring enzyme, that acts on collagenous fibrils. The enzyme can be inhibited by Cu^{++} deficiency and by lathyrogens such as BAPN (β-aminopropionitrile) and D-penicillamine (Table 1B).

The ultimate ability of collagen to serve its supporting function requires the formation of strong collagen fibrils, which together with associated proteoglycans, glycosaminoglycans, elastin, and non-collagenous connective tissue glycoproteins, form the matrix for individual tissues.

Table 1A BIOSYNTHETIC STEPS OF COLLAGEN

STEPS	LOCATION	PROCESS AND FACTORS INVOLVED
	INTRACELLULAR	
1. Transcription	Nucleus	Gene selection, mRNA production
2. Translation	RER	PRO-αCHAINS
3. Post-translational modification	RER	
*(a) Proline hydroxylation		Ascorbic acid — Prolyl hydroxylase
*(b) Lysine hydroxylation		Iron, O_2 — Lysly hydroxylase
*(c) Glycosylation of hydroxylysyl residues		Gal or Galglu attached to Hylys — Galactosyl transferase, Glucosyl transferase
*(d) Chain folding & triple helix formation		Disulfide bond formation
		PROCOLLAGEN
4. Intracellular translocation & secretion	Golgi complex, Plasma membrane	Additonal glycosylation in non-triple-helical domains; Exocytosis
	EXTRACELLULAR SPACE	EXTRACELLULAR PROCOLLAGEN
*5. Conversion of procollagen to collagen		Procollagen peptidases → COLLAGEN
*6. Fibril formation		COLLAGEN FIBRILS (soluble)
*7. Aldehyde formation		Cu^{++} — Lysyl oxidase
*8. Cross-link formation		Cross-links → COLLAGEN FIBER (insoluble)

NOTE: 1. The upper table is compiled from the following references: Hance and Crystal (1975), Kivirikko and Risteli (1976), Fessler and Fessler (1978), Bornstein and Byers (1980).
2. * indicates a step unique to biosynthesis of collagen.

Table 1B DISTURBANCES IN THE BIOSYNTHETIC STEPS OF COLLAGEN

BIOLOGICAL SIGNIFICANCE	INHIBITORS	HEREDITARY OR METABOLIC DISEASES
1. Synthesis of mRNA	BrdU	Osteogenesis imperfecta
2. Synthesis of primary structure	Proline analogues	EDS type IVB
3.		
a) Essential for triple helix formation	Proline analogues Fe Chelator-- α,α' dipyridyl Homogentisic acid	Ascorbic acid deficiency EDS type VI Osteogenesis imperfecta
b) Essential for glycosylation reaction and cross-link formation		
c) Possibly affect fiber formation		
d) Facilitate folding & stability of helix		
4. Intracellular translocation	Anti-microtubular Agents: Colchcine Vinblastine Vincristine Local anesthetics	EDS type IVC
5. Limited proteolysis	EDTA, acidic pH, α-mercaptoethanol	EDS type VII Dermatosparaxis (cattle and sheep)
6. Molecular assembly, aggregation		Spondyloepiphyseal dysplasia EDS type IVD; I
7. Oxidative deamination	Semicarbazole Lathyrogen, BAPN	Cu^{++} deficiency, EDS type V Menkes' kinky hair syndrome Aneurysm-prone mice
8. Essential for stability of collagen fibrils	D-penicillamine	Homocystinuria

II. COLLAGEN TYPES AND THEIR TISSUE DISTRIBUTION

A. Collagen As Structurally Distinct Gene Products

Currently, collagen is recognized as a family of proteins of multigene products. There are at least 9 different α chains, each a distinct gene product. The structural uniqueness of each α chain is determined by differences in amino acid sequence, which in some cases have been determined by amino acid composition and peptide mapping following cleavage by CNBr or by particular proteases. These 9 different α chains are further grouped into 5 different types of collagen by their polymeric composition of the triple helix structure in vertebrate tissues. Table 2 summarizes the chain and molecular composition of these five collagen types.

Collagen types have been assigned Roman numerals according to the order in which each type was discovered. The corresponding chains are referred to as shown in Table 2. A decade ago, type I collagen was the only known molecular type of collagen in vertebrates, and since then it has been studied most intensively. The unique characteristic of type I collagen in molecular composition is that it is a heteropolymeric structure consisting of two different α chains in the form of $[\alpha 1(I)]_2\alpha 2$; despite the fact that a minor form of $[\alpha 1(I)]_3$, type I trimer, is also noted, as will be discussed below. The first evidence for genetic polymorphism of collagen in vertebrates was the discovery of α1(II) chain and its parent type II collagen molecules in hyaline cartilage (Miller and Matukas, 1969). This molecule is the product of a distinct gene as shown by the amino acid sequence (Francis *et al.*, 1978) and has the subunit composition $[\alpha(II)]_3$. Type III collagen, of the subunit composition $[\alpha 1(III)]_3$, was subsequently discovered in CNBr and pepsin

digests of human fetal skin and other tissue (Miller et al., 1971; Epstein, 1974; Chung and Miller, 1974). One of the distinct features of type III collagen is the presence of interchain disulfide bonds between cysteines at the carboxyl end of the helix.

A fourth type was assigned to collagen recovered from certain thick basement membrane tissues such as kidney glomeruli, lens capsule, and Descemet's membrane of the eye (Kefalides, 1973). Several compositional characteristics render the type IV collagen a unique structure. For example, the extension peptides of both N-and C- termini are retained in the extracellular space; in the helical portion, there are several globular regions which are susceptible to proteolysis. Much of the controversy concerning the structure of type IV collagen has resulted from the use of pepsin for its extraction (Daniels and Chum, 1975; Dixit, 1978; Tryggvason and Kivirikko, 1978; Bailey et al., 1979a; Glanville et al., 1979; Kresina and Miller, 1979; Sage et al., 1979). For some years, a single collagen type of subunit structure $[\alpha 1(IV)]_3$ has been a popular model (Kefalides, 1971). More recent studies have suggested that type IV procollagen contains two chains, although the stoichiometry of the chains in the molecules is presently unknown (Timpl et al., 1978; Crouch and Bornstein, 1979; Crouch et al., 1980). Nevertheless, the CNBr peptide profiles of both $\alpha 1(IV)$ and $\alpha 2(IV)$ chains have been obtained (Dixit and Kang, 1979;1980) and support the hypothesis that type IV procollagen contains at least two structurally distinct chains. The $\alpha 1(IV)$ and $\alpha 2(IV)$ chains have been isolated in a ratio of 2:1 (Kresina and Miller, 1979; Crouch et al., 1980), but there is also evidence that they may exist in separate molecules (Timpl et al., 1979a).

The type V collagen was first identified in pepsin-solubilized extracts of tissue that are rich in basement membranes (Burgeson et al., 1976; Chung et al., 1976). Initially, two chains α1(V) and α2(V), previously designated as αB and αA, were discovered and characterized. Subsequently a third chain, α3(V), previously designated as αC, was isolated from synovial membrane and placenta (Brown et al., 1978; Sage and Bornstein, 1979). The α1, α2, and α3 chains comprising type V collagen have been separated by ion exchange chromatography; subsequent characterization has shown that the pepsin-resistant portion does not contain disulfide bonds and has indicated a molecular weight approximating that of the α1(I) chain (Rhodes and Miller, 1978; Sage and Bornstein, 1979). Studies on the molecular organization of type V collagen has been controversial. Several laboratories have proposed the structure $[\alpha 1(V)]_2\alpha 2(V)$ based on the quantitative estimates of the two chains in the native molecules or on recoveries after chromatographic purification (Burgeson et al., 1976; Bentz et al., 1978; Davison et al., 1979; von der Mark and von der Mark, 1979; Hong et al., 1979; Madri and Furthmayer, 1979). Other evidence for the existence of these two chains in a single molecule has been provided by their similar characteristics with respect to salting out behavior (Burgeson et al., 1976), resistance to vertebrate collagenase (Hong et al., 1979), and production of only one segment-long-spacing (SLS) species (Davison et al., 1979; Hong et al., 1979). Thermal denaturation-renaturation studies have indicated that α2(V) chains are unable to form stable triple helical molecules independently, which suggests that the heteropolymer is the only form in which the α2(V) can exist *in vivo*, although $[\alpha 1(V)]_3$ molecules can be present as well (Bentz et al., 1978). In contrast, variable chain

Table 2

CHAIN AND MOLECULAR COMPOSITION OF COLLAGEN TYPES

Type	Chains	Molecular compsiton	Distinct Features			
			Hyl	Gly	Hyp	Others
I	α1, α2	$[\alpha1(I)]_2\alpha2(I)$	+	+	+	heteropolymer
I trimer	α1	$[\alpha1(I)]_3$				
II	α1	$[\alpha1(II)]_3$	++	++	+	glycosylation
III	α1	$[\alpha1(III)]_3$	+	+	+	interchain disulfides
IV	α1, α2	not established	+++	+++	+++ 3-Hyp	retain extension peptides extracellularly
V	α1,α2, α3	not established	++	++	++ 3-Hyp	

NOTE: 1. α1, α2, and α3 are currently recommended to replace αB, αA, and αC respectively (Bornstein and Sage, 1980).

2. Hyl, Gly, and Hyp connote levels of hydroxylation at lysyl residues, glycosylation at the hydroxylysyl residues, and hydroxylation at the prolyl residues, respectively.

ratios for type V collagen have also been reported in other laboratories (Rhodes and Miller, 1978; Brown et al., 1978; Jimenez et al., 1978; Sage and Bornstein, 1979a). With the discovery of α3(V) chain, the stoichiometry of the chains in type V collagen becomes even more complicated. The precise relationship will reside in the characterization of the precursor form(s) of type V collagen.

A new collagen type must contain chains that are coded for by genetic loci not previously assigned to any type. The possibility of the existence of new collagen types has received recent attention (Burgeson and Hollister, 1979).

B. Tissue Distribution

As a broad concept, the five collagen types can be segregated into two major categories, as judged by their structural role and extracellular location. The first category, interstitial collagens, comprises type I, II, and III collagens, which are known to be fibrillar and form the bulk of the extracellular fabric of connective tissues. Their native fibrils exhibit a characteristic 64 to 67 nm axial periodicity in electron micrographs (Table 3A). Type I collagens form large well-organized, fibrils and predominate in denser connective tissues where tensile strength is needed. The tissue distribution of type I is widespread, and, except in such hard tissues as bone and dentin, where only type I can be found, type I collagen usually exists in tissues intermixed with type III collagen (Miller, 1976). Type II collagen forms thinner fibrils which have a strong binding affinity with cartilage proteoglycans and glycosaminoglycans and thus together with them

constitutes the cartilageous matrix. It exists as the sole collagen type in hyalin cartilage (Miller, 1976), nucleus pulposis of the intervertebral disc (Eyre, 1979), notochord (Miller and Mathews, 1974), and vitreous body of the eye (Swann et al., 1972). Type III collagen is believed to be the collagen of the fine reticulin fiber previously described by histologists, an impression supported by electron microscopy using ferritin-labeled antibodies specific for type III (Gay, 1978). It predominates in tissues requiring higher levels of compliance (Bornstein and Traub, 1979) such as fetal skin, blood vessels, synovial membrane, uterus, spleen, granulation tissue, and reticular connective tissue in general. Type III collagen is predominant in embryonic and fetal tissue as well (Epstein, 1974; Jimenez et al., 1977).

The second category of collagens are those existing in lesser amounts, and are as yet poorly defined. They appear to be located in the pericellular environment (Table 3B). It consists mainly of type IV and V collagens. Type IV collagen is the sole collagenous component of basement membrane (Kefalides, 1973). This collagen is not fibrillar and is without periodicity when visualized under an electron microscope. The distinctive features of type IV collagen predispose it to form such a unique tissue component (Table 2). Whether type V collagen is a component of basement membrane structures is putative, though it was first discovered in basement-membrane-rich tissue (Burgeson et al., 1976; Chung et al., 1976). Recent evidence shows that fibroblasts and other mesenchymal cells are also surrounded by these collagens (Rhodes and Miller, 1978; Gay and Miller, 1978). The pericellular distribution and universal existence in a great number of tissues and cells (Burgeson et al., 1976; Chung et al., 1976; Duance et al., 1977; Mayne et al., 1978;

Table 3

STRUCTURAL CHARACTERISTICS AND TISSUE DISTRIBUTION OF NATIVE COLLAGENS

Type	Native Structural Characteristics	Predominant Tissue Distribution
A. Interstitial Collagen	Fibrillar, with periodicity	
I	large, well-organized fibrils	bone, tendon, dentin, skin, fascia.
II	thinner fibrils	cartilage, nucleus pulposis, notochord, vitreous body.
III	"reticulin fibers"	fetal skin, spleen, uterus, blood vessels, reticular connective tissue.
B. Basement Membrane Pericellular Collagen	Not fibrillar, without periodicity	
IV	basal lamina	kidney glomeruli, lens capsule, Descemet's membrane, basal laminae of all epithelial and endothelial cells.
V	unknown pericellular distribution, exoskeleton	widespread in epithelial and mesenchymal tissues

Jimenez et al., 1978 Rhodes and Miller, 1978) have led to the suggestion that type V collagen acts as an exoskeleton for all cells (Stenn et al., 1979; Gay et al., 1981).

III. REGULATION OF COLLAGEN BIOSYNTHESIS

It is evident that regulation of collagen biosynthesis exists, as marked changes are found in the rate of collagen synthesis during certain stages of development and in many diseases. During embryonic development, the collagenous matrix is dynamically remodeled at different stages of morphogenesis (Hay, 1973; Linsenmayer et al., 1973). However, in adult life, most of the collagen is metabolically stable (Lindstedt and Prockop, 1961), and normal cultured fibroblasts adopt a rigid control of collagen synthesis through most of the life span and cell cycle in vitro (Hance and Crystal, 1977). In diseased states, the net synthesis of collagen is either increased, as found in scar tissue, in pathologic fibrosis such as interstitial pulmonary fibrosis, liver cirrhosis, and atherosclerosis, as well as in hereditary connective tissue diseases; or decreased as seen in diseases such as scurvy, in poor wound healing, and in hypermetabolic states.

Changes in the regulation of collagen biosynthesis can occur at two levels: quantitative changes in the amount of collagen deposited, and quantitative changes in the types of collagen, the distribution of collagen fibers, their ultrastructural organization, and spatial relationship with other components of the ECM. Although the whole picture of regulation is complex and unable to be resolved into two separate events, for the convenience of discussion, the quantitative regulatory mechanisms will be discussed first, to be followed by a discussion of the qualitative aspects.

A. Quantitative Modulation of Collagen Synthesis

Quantitative changes of collagen synthesis, can be further divided into two functional compartments, intracellular and extracellular. As discussed in Section I, the biosynthesis of collagen consists of sequential steps of processing such that a certain amount of collagen is synthesized intracellularly, secreted, and deposited extracellularly (Table 1A). Among the two compartments, it is currently recognized that certain degrees of degradation occur within moments of synthesis. Intracellular degradation proceeds with a lysozomal enzymatic digestion of the intermediate procollagen molecules prior to secretion (Bienkowski et al., 1978 a&b); in normal cell culture it is estimated that in cultured fibroblasts 10 to 40% of total collagen synthesis is degraded in this manner. Extracellular degradation undergoes by another enzymatic reaction, collagenase concurrently with other proteases (Perez-Tamayo, 1978). The net amount of collagen deposition is comprised of these two opposite forces, synthesis and degradation, taking place in these two compartments.

Current knowledge about regulatory mechanisms is still limited and derived primarily from studies on experimental pathologic fibrosis. Nevertheless, it deserves more attention, since although the etiologies are multiple, the pathogenesis in diverse pathologic states are likely to be similar. In the following, an attempt is made to review the studies of regulatory mechanism. Factors which exert an effect on collagen biosynthesis are examined in an attempt to explore the underlying mechanism. After a review of the literature in this area, a list of factors is presented (Table 4). The action mechanisms of most of these factors are not known regarding the level of the regulatory

control or the specificity involved. According to the relative specificity of action on collagen biosynthesis, they are grouped into two categories. The first category consists of the factors which affect collagen synthesis by way of an action on general protein synthesis, and thus are less specific (Table 4A). Factors belonging to the first category include hormones, such as glucocorticoids (Cutroneo et al., 1971 & 1975; Krause et al., 1978), growth hormone, thyroxine, and parathyroid hormone (Kivikko, 1970); size of the amino acid pool (Rojkind and Kershenobich, 1975); pH (Nigra et al., 1973); cell density (Kao and Berg, 1979); prostaglandins (Blumenkrantz and Sandergaad, 1972); and cAMP (Baum et al., 1978). Each factor may act at certain levels in the pathway of collagen biosynthesis. For example, as shown by Krause et al. (1979), the basis for glucocorticoid-mediated decrease in tissue collagen in the system of mouse sponge-induced granuloma and in primary granuloma fibroblast culture may be a direct effect of hormone on the transcriptional or translational activities of the cell (Thompson and Lippman, 1974). This is contradictory to those studies performed by Cutroneo et al. (1971 & 1975) who claim that the action of glucocorticoids is through the suppression of the activity of prolyl hydroxylase, an enzyme specific for collagen synthesis in post-translational modification.

Many extracellular factors or stimuli are speculated to affect collagen synthesis by a common pathway through secondary mediators such as prostaglandins and cAMP. The prostaglandins, E_1 and $F_1\alpha$, stimulate collagen synthesis in cultures of chick embryo tibia (Blumenkrantz and Sondergaard, 1972). This may explain the mechanism by which anti-inflammatory drugs, such as aspirin, indomethacin, and sodium salicylate

inhibit collagen biosynthesis (Whitehouse and Bostrom, 1965; Nakagawa and Bently 1970; Kulonen and Potila, 1975), and why inflammation increases collagen accumulation. Most intriguing is the discovery of the effect of cAMP. Several different *in vivo* stimuli exert effects by altering cellular cAMP levels (Chlapowski *et al.*, 1975). These includes peptide hormones, prostaglandins, cholera toxin, and some pharmacologic agents. A number of peptide factors have been implicated to play a role of regulating collagen synthesis, and they were given at one time a vague name such as "connective-tissue-activating peptides" (Castor, 1975). Such peptide factors have been found in extracts from regenerating liver (McGee *et al.*, 1973), granulation tissue (Aalto and Kulonen, 1974), hyperlipidemic serum (Ronnemaa *et al.*, 1975), and in silica-treated macrophage (Aalto *et al.*, 1976). Although the identity of these factors is unknown, it is proposed that they probably exert an effect on transcription through binding with membrane receptors for prostaglandins and cAMP (Castor, 1975). Recent studies by Baum *et al.* (1980) suggest that the effect of raising levels of cAMP within fibroblasts causes an apparent decrease in collagen production by increasing rates of intracellular collagen degradation. This is not accompanied by the degradation of other proteins (Baum *et al.*, 1980). It appears to be an effect unique for collagen, since it is generally thought that all regulatory functions of cAMP operates at the transcriptional and translational levels (Chlapowski *et al.*, 1975; Ochoa and de Hara, 1979). By changing the extent of intracellular degradation but not affecting the synthesis of other proteins, collagen biosynthesis may be modulated in a unique way. This common mechanism involving cAMP may play a major role in regulating collagen production by various tissues.

Table 4

EXTERNAL FACTORS AFFECTING COLLAGEN BIOSYNTHESIS

Level of control Factors	CHANGES	COLLAGEN SYNTHESIS	REFERENCES
A. GENERAL EFFECT:			
1. Hormones e.g. glucocorticoids	↑	↓	Krause et al., 1978
2. Amino acid pool	↑	↑	Rojkind and Kershenobich, 1975
3. pH	alkaline	↑	Nigra et al., 1973
4. Cell density	high	↑	Kao and Berg, 1979
5. Prostaglandins	e.g. PG E_1 $F_1\alpha$	↑	Blumenkrantz and Sandergaard, 1972
6. cAMP	↑	↓	Baum et al., 1978 & 1980
B. SPECIFIC EFFECT:			
1. Transcriptional level			
a Viral transformation		↓	Adams et al.,1977&1979 Rowe et al., 1978 Sandmeyer and Bornstein, 1979.
2. Post-translational level			
a Enzyme activity	↑		
Ascorbic acid		↑	Peterkofsky,1972 Myllyla et al., 1978
Oxygen		↑	Bhatnagar et al., 1978 Yen et al., 1979
b Enzyme activity	↓		
Glucocorticoids		↓	Cutroneo et al., 1971 & 1975
α,α' dipyridyl		↓	Bhatnagar and Prockop,1978
c Proline analogues		↓	Rosenbloom and Prockop, 1971
3. Extracellular space			
a NH_2-terminal peptides		↓	Krieg et al., 1978 Wiestner et al., 1979
b Crosslinking inhibitors		↓	
BAPN			Nimni, 1980
D-penicillamine			Siegel, 1977
c Factors affecting collagenase		↓	Perez-Tamayo, 1978

NOTE: ↑ and ↓ represent increase and decrease respectively.

The second category contains factors which have a more specific effect on collagen biosynthesis (Table 4B). As one surveys the pathway of collagen biosynthesis presented in Section I, Table 1, one can speculate that specific action might occur at those steps which are collagen-specific. Among these are the selection of particular collagen genes at the transcriptional level, collagen-specific post-translational modifications, and extracellular processing (Table 1, steps with an *).

Viral transformation serves as an excellent example of how a single factor, a virus, can modulate collagen synthesis at a precise transcriptional level. Viral transformation is known to cause several changes in normal cells including altered shape and adhesive properties. Several studies demonstrate that transformation of chick embryo fibroblasts by Rous sarcoma virus results in severely decreased synthesis of proα1 and proα2 chains of type I collagen (Green et al., 1966a; Levinson et al., 1975; Hata and Peterkofsky, 1977). Recent studies have further indicated that this decrease is due to a reduction in translatable mRNAs coding for these collagen polypeptides (Adams et al., 1977;1979; Rowe et al., 1978; Howard et al., 1978; Sandmeyer and Bornstein, 1979). The above investigators also observe a dramatic decrease in fibronectin production. This effect seems to be more specific for collagen, since the decrease of collagen and that of fibronectin appears to be uncoupled (Parry et al., 1979). It appears that these two extracellular macromolecules are controlled by different mechanisms.

At present it is not known whether any of the post-translational modification steps are rate-limiting, although this has been suggested. The activity of prolyl hydroxylase parallels changes in rates of collagen biosynthesis in a number of situations, such as in aging,

embryonic development, wound healing, and in granuloma development. On the basis of these findings this enzyme activity has been invoked as the rate-limiting reaction for collagen synthesis.

Exogeneous addition of ascorbic acid (Peterkofsky, 1972; Myllyla et al., 1978) or increases in the atmospheric concentration of oxygen (Bhatnagar et al., 1978; Yen et al., 1979) can increase the amount of collagen synthesized in culture as well as the degree of hydroxylation of prolyl and lysyl residues in collagen molecules. These results suggest that the action is collagen-specific and works at a post-translational level by enhancing the activity of prolyl and lysyl hydroxylases.

Collagen molecules can be synthesized in an underhydroxylated state in the absence of ascorbic acid or in the presence of an iron chlator, α,α' dipyridyl (Bhatnagar and Prockop, 1966); or in a defective state by incorporating proline analogues, such as cis-hydroxy-L-proline (Rosenbloom and Prockop, 1971; Chvapil et al., 1974), cis-4-fluoro-L-proline, and L-azetidine2-carboxylic acid (Takeuchi and Prockop, 1969; Takeuchi et al., 1969). Such molecules are prone to be degraded intracellularly (Berg et al., 1978). They are unable to be secreted, and are thermally unstable when they are present extracellularly (Jimenez and Rosenbloom, 1974; Uitto and Prockop, 1974).

In the extracellular space, the understanding of regulatory mechanisms is limited. It has been postulated that there must be feedback control systems in the extracellular space such that the amount of extracellular collagen can be regulated. One of such mechanisms may be through the extension peptides of the procollagen molecules, which are cleaved extracellularly. In a feedback-control fashion, the N-terminal

extension acts as an inhibitor to suppress the intracellular collagen synthesis (Lichtenstein et al., 1973; Krieg et al., 1978; Wiestner et al., 1979). Paglia and coworkers (1979) have demonstrated that a specific inhibition of procollagen synthesis by these N-terminal peptides occurs at the translational level. Lathyrogens, such as BAPN and D-penicillamine, can inhibit the crosslinking of the extracellular collagens (Nimni, 1980; Siegel, 1977). The amount of collagen deposited extracellularly becomes decreased and more extractable. The mechanism by which collagen synthesis is affected by crosslinking is unknown. Part of the explanations may be that non-crosslinked collagens are more susceptible to collagenase digestion (Vater et al., 1979). In order to generate a clear picture, more studies are needed to resolve the relationship between crosslinking, degradation, and feedback controls in the extracellular space.

Finally one should also bear in mind that the regulatory system can be altered together with the degree of cellular differentiation. A wide range in the ability to synthesize collagen is observed among different cells types; more in mesenchymal cells, less in epithelial cells, and negligible levels in lymphocytes (Green et al., 1966b; Langness and Udenfried, 1974). Even among a single group of fibroblastic cell lines, wide differences are noted (Green and Goldberg, 1965). When normal fibroblasts are transformed by viruses, some expected effects on collagen synthesis by environmental stimuli are lost and occasionally the opposite is observed. For example, Furcht et al. (1979) find that glucocorticoids induce SV40 transformed human fibroblasts to make more collagen. Cyclic AMP becomes a stimulator of collagen synthesis in Kirsten sarcoma virus-transformed BALB 3T3 cells (Peterkofsky and Prather, 1974); and effects of ascorbic acid on hydroxylation are lost

in the aforementioned system (Evans and Peterkofsky, 1976). It is conceivable that some regulatory mechanisms of collagen biosynthesis are unique for each cell type, despite a common biosynthetic pathway.

B. Qualitative Modulation of Collagen Type Synthesis

Based on this background knowledge that different collagen types exist and that there are unique features in tissue distribution and structural characteristics (Table 3), one can speculate that the substitution of one type of collagen for another or an anatomic redistribution of collagen types will be of major consequences in pathophysiology. These qualitative changes in collagen types have been observed in several instances both *in vivo* and *in vitro*. The work in this area is to be reviewed here.

Modulation of collagen type synthesis *in vivo* has been described in the following developing systems, based on immunofluorescence studies. In the development of the chick embryo, certain types of collagen becomes apparent in certain tissues at a particular stage of development (Linsenmayer *et al.*, 1973; Johnson *et al.*, 1974; von der Mark *et al.*, 1976b). During morphogenesis of a particular tissue or organ, a dynamic change in collagen types and distribution can also be noted. For instance, in the development of the long bones of the chick, (Oohira *et al.*, 1974; von der Mark *et al.*, 1976a; von der Mark and von der Mark, 1977) endochondral bone formation apparently begins with deposition of type III collagen by bone marrow-derived cells in lacunae of hypertrophic chondrocytes. This is followed by deposition of type I collagen, a constituent of endochondral osteoid, and then by type II collagen in calcifying cartilage. It is of interest that the sequence

of appearance of type III, I, and II collagens is also observed in the enchondral bone formation that follows subcutaneous implanatation of demineralized rat bone matrix into allogeneic recipients (Reddi et al., 1977). During the development of the chick eye, type I collagen is detected at stage 19 (the earliest stage examined) in the corenal stroma, vitreous body, and along the lens surface; type II collagen is first observed at stage 20 in the primary corneal stroma, neural retina, and vitreous body; type III is observed transiently in corneal subepithelial layer but is prominent in the fibrous sclera, iris, and other tissue of the fully developed eye (von der Mark et al., 1977). In addition to these changes, alterations in collagen types are also revealed in such developing systems as the rat palate during shelf elevation (Hassell and Orkin, 1976) and the developing tooth (Becker et al., 1976; Lesot et al., 1978; Thesleff et al., 1979). Changes are also noted in a tissue such as skin. Development here extends into adult life. The proportion of type III collagen in human skin decrease progressively as a function of age from the embryo to approximately age 15 and beyond (Epstein, 1974).

In addition to the above development systems, modulation of collagen types has also been demonstrated in such pathologic situations as inflammation, wound healing, and pathologic fibrosis. Enhanced type III collagen synthesis and deposition together with alterations in the degree of hydroxylation as well as crosslinking is found in a variety of inflammatory states and in the early phase of wound repair in response to diverse injurious agents. These findings raise the possibility that during the inflammatory response and in the early phase of would healing, fibroblasts of skin or other tissues may revert to an embryonic

stage, with a synthetic pattern similar to that of fetal fibroblasts, in which more type III collagen is produced. The conditions which have been analyzed to date are dermal scars (Barnes et al., 1976), keloids (Bailey et al., 1975; Hayakawa et al., 1977; Weber et al. 19978), Dupuytren's contractures (Bailey et al., 1977; Bazin et al., 1977), rheumatoid arthritis (Adam et al., 1976; Weiss et al., 1975), liver injury caused by carbon tetrachloride (Kent et al., 1976) and gingiva affected by periodonitis (Narayanan and Page, 1976). As a chronic stage is reached, there is a conversion to a preponderance of type I collagen in the affected tissue. This is true for situations such as chronic pulmonary and hepatic fibrosis and the atheromatous plaque in atherosclerosis (McCullagh and Balian, 1975).

From these results there emerges a common pattern, that substantial changes in the distribution of collagen types in the morphogenetic events or pathologic fibrosis are associated with a change in cellular differentiation.

To study the mechanism by which collagen type-specific synthesis is modulated, in vitro cell culture system are usually employed. It is recognized that cells have a wide repertoire of collagen type-specific synthesis. As observed in clonal growth of bovine dermal fibroblasts, both type I and III collagens are synthesized (Church et al., 1973). Furthermore, each individual human fibroblast has been demonstrated by immunofluorescence to synthesize both type I and III procollagens simultaneously (Gay et al., 1976). As a result, one need not attribute changes in collagen type synthesis to changes in cell populations. A more likely way to explore the regulatory mechanism will be to seek factors which affect the phenotypic expression of collagen synthesis and

ask questions as to how it is influenced. Despite the fact that detailed studies in the mechanism of regulating collagen type synthesis are still lacking, one can speculate that it can occur potentially at several levels in the biosynthetic scheme, as shown in Table 1. Noteworthy is the example of type IV Ehler-Danlos syndrome patients whose tissues have a diminished level of type III collagen and whose fibroblasts do not secrete significant amounts of that protein (Pope et al., 1975). It was proposed originally that the defect lies in the secretory mechanism. No intracellular accumulation of type III procollagen is observed (Gay et al., 1976). This suggests that the defect is transcriptional or a gene deletion. To verify the level of control, technical progress in the preparation of specific cDNA probes for different procollagens is required.

With a limited understanding of the regulatory mechanisms of collagen type-specific synthesis, external environmental factors have been sought which can modulate expression of collagen type synthesis. A survey of the literature relevant to this subject is summarized in Table 5. To date, the majority of such studies have been performed with cultured chondrocytes or with cartilage (Table 5). Unlike fibroblasts, in which the collagen phenotype is under rigid control (Hance and Crystal, 1977), the phenotype of chondrocytes is not at all stable and lends itself easily to the study of the modulation of collagen type synthesis. Under a variety of conditions, the capacity of making cartilage-specific type II collagen is lost and replaced by type I, αI trimer, III and minor species, the X, Y chains, which are now recognized as α1(V) and α2(V) chains (Table 5). Such change in phenotypic expression of collagen types are observed in chondrocyte cultures, after several

population doublings (Cheung et al., 1976; Benya et al., 1977;1978), after even longer culture time (von der Mark et al., 1977c), and to senescence (Mayne et al., 1976a). This can also be made to occur with the introduction of a number of factors into the culture system, such as embryo extract (Mayne et al., 1976b), 5-bromo-2'-deoxyuridine, (Mayne et al., 1975), fibronectin (West et al., 1979) or Ca^{++} and changes in the pyrophosphate concentration, or with cyclic AMP, and with calcitonin (Deshmukh et al., 1976 a&b, 1977 a&b, 1978). The normal phenotype of chondrocytes is preserved as long as the cells grow in a detached floating state or in an adherent spread state in which case they are relatively sessile and have a polygonal, or epithelioid morphology (Holtzer and Abbott, 1968). Thus the phenotypic change in collagen type can be induced by changing from a suspension to a monolayer culture (Müller et al., 1977; Norby et al., 1977) and occurs as normal cell-cell and cell-matrix interactions are lost (Müller et al., 1977). The basis for this phenomenon, which leads to the appearance of cells resembling fibroblasts and is sometimes called dedifferentiation, is not known. A complex interplay of several forces might determine a cellular phenotype.

The experience with cell types other than chondrocytes is less extensive (Table 5). Fibroblasts change their collagen production by increasing the ratio of type III to type I in the presence of higher concentration of serum (Narayanan and Page, 1977) or when cultured at higher cell density (Abe et al., 1979). In the case of smooth muscle cells, a higher proportion of type III collagen to type I is made, compared to that of fibroblasts. It has been suggested that the proportion of type I collagen synthesized by smooth muscle cells increases

with increasing passage number (Mayne et al., 1978). In one study of vascular endothelial cells, type III procollagen and fibronectin are the predominant secreted product in culture (Sage et al., 1979b). At confluence, over the typical monolayer of polygonally shaped cells, a focal growth consisting of elongated cells in a mycelial pattern develops. This phenomenon is called sprouting (Gospodarowicz and Mecher, 1978; Schwartz, 1980). However, unlike typical endothelial cells, sprout cells resemble fibroblasts. They secret predominantly type I procollagen and less fibronectin (Cotta-Pereira et al., 1980). The appearance of sprout cells is of interest in that they represent phenotypically altered endothelial cells rather than a contaminating cell population (Schwartz, 1978), and this phenotypic switching is reversible (Cotta-Pereira et al., 1980).

The preceding review on the modulation of collagen phenotypes introduces several intriguing hypothetical questions. First, does the collagen phenotype reflect a cellular phenotype and does this become altered with the differentiated state? Secondly, how do external factors exert their effect on cellular phenotype? This will be discussed in more detail in the following section.

Table 5

FACTORS MODULATING COLLAGEN TYPE SYNTHESIS IN VITRO

Cell Type	Factors/and Processes	Collagen type change	References
1. CHONDROCYTES		II→I, I trimer, III, V	
	↓ Population doublings		Cheung et al., 1976 Benya et al.,1977;1978
	↑ Time in culture		von der Mark, et al., 1977
	In vitro senescence		Mayne et al., 1976a
	Bromodeoxyuridine		Mayne et al., 1975
	Embryo extract		Mayne et al., 1976b
	Fibronectin		West et al., 1979
	↑ Ca, pyrophosphate		Deshmukh et al., 1976
	↑ cAMP, Calcitonin		a&b, 1977 a&b, 1978
	Suspension culture v.s. monolayer culture		Norby et al., 1977
	Cell-cell and cell-matrix interactions		Muller et al., 1977
2. FIBROBLASTS		III/I ↑	
	↑ Serum		Narayanan and Page, 1977
	↑ Cell density		Abe et al., 1979
3. SMOOTH MUSCLE CELLS		I/III ↑	
	↑ Passage number		Mayne et al., 1978
4. ENDOTHELIAL CELLS		III→I	
	Sprouting		Cotta-Pereira et al., 1980
5. MYOBLASTS			
	Differentiation and myotube formation		Bailey et al., 1979b

IV. FUNCTIONAL ROLE OF COLLAGEN IN CELL-MATRIX INTERACTIONS

A. Collagen as the Major Component of the Extracellular Matrix (ECM)

As described previously, collagen is the major constituent of connective tissue. Extracellularly, together with glycosaminoglycans, proteoglycans, and non-collagenous glycoproteins, it forms a pericellular matrix or microexudate for each cell. There are at least five types of collagen, I through V (see Section II), eight types of glycosaminoglycans (see review by Lindahl and Hook, 1978), several proteoglycans (see review by Muir, 1977), and three kinds of glycoproteins, namely fibronectin (Stenman and Vaheri, 1978), laminin (Timpl et al., 1979b), and chondronectin (Hewitt et al., 1980). Without going into a detailed compositional analysis, one can surely appreciate the existence of various ECMs. The variety is even more diverse when the three-dimensional and temporal organization of these three components is considered. The cumulative overall characteristics constitute the uniqueness of the ECM of each cell and tissue. Not only the quality of ECM is characteristic, but the capacity for making an ECM for different cell types also varies. The capacity for synthesizing collagen is ubiquitous, covering a wide range of different cell types (Green et al., 1966 a&b). It also indicates that such a capacity can correlate with the state of cellular differentiation.

This contrast can be further demonstrated by comparing two widely different cell types, mesenchymal and epithelial cells. By summarizing current information (Table 6), a simplified scheme is introduced which demonstrates the differences between mesenchymal cells and epithelial cells, and their differences in the capacity for making their own unique ECMs in vitro.

Table 6 COMPARISON OF ECM MADE BY MESENCHYMAL AND EPITHELIAL CELLS

	Mesenchymal Cells	Epithelial Cells
Major Collagen Type	Interstitial Collagens I, II, III	Basement Membrane Collagen IV
Major glycoprotein	Fibronectin	Laminin
Capacity for making ECM *in vitro*	High	Low

NOTE: Difference in glycosaminoglycans and proteoglycans is not established yet.

This raises the following questions. How does a cell interact with its matrix environment? Is there specificity in the interaction with the ECM? And, what is the role of collagen in such an interaction?

B. Functional Roles of Collagen

Although the exact nature and composition of ECMs are still to be elucidated, a variety of cell-matrix interactions have been explored. The ECM produced by cells is the natural substratum upon which cells attach, migrate, proliferate, and differentiate *in vivo*. The interactions between a cell and its matrix can thus be grossly classified into these four aspects: attachment, migration, proliferation, and differentiation. However it is difficult or may be impossible to divide these into four separate and discrete events, since one may be closely associated with another. For convenience of discussion, the role of collagen played in these four cell-matrix interactions will be reviewed below.

For many years it has been known that normal cells must attach to a solid substratum in order to grow *in vitro*. This is termed "anchorage dependence" (MacPherson and Montagnier, 1964; Stoker *et al.*, 1968). Collagenous surfaces represent a major substratum for cell attachment to occur both *in vivo* and *in vitro*. The role played by collagen in cell attachment is essential to understanding all cell-matrix interactions. The attachment of cells to a substratum is a complex process requiring one or more glycoproteins, e.g. fibronectin or cold-insoluble globulin, cations, and metabolic energy (Klebe, 1975). Many laboratories have been using a collagen-coated substratum for promoting cell attachment *in vitro* (Linsenmayer *et al.*, 1978; Schor and Court, 1979). Part of the mechanism operates by way of the cell surface protein, fibronectin (Klebe, 1975; Linsenmayer *et al.* 1978; Pearlstein, 1978; Grinnell and Minter, 1978; Kleinman *et al.*, 1979). This has been verified by the presence of a strong binding affinity between different types of collagens and fibronectin *in vitro* in a number of studies (Kleinman *et al.*, 1978a; Engvall and Ruoslahti, 1977; Engvall *et al.*, 1978; Ruoslahti *et al.*, 1978). A number of cells including human fibroblasts, Chinese hamster ovary cells, 3T3, and periosteal cells, attach equally well to type I through IV collagens (Kleinman *et al.*, 1978b). In contrast to fibroblasts, guinea pig epidermal cells (Murray *et al.*, 1979) and mammary epithelial cells (Wicha *et al.*, 1979) attach preferentially to type IV collagen. An epithelial cell line PAM212 prefers to utilize laminin as a promoting agent for attachment to type IV collagen (Victor *et al.*, 1980). This indicates a preferential requirement of a certain kind of collagenous matrix for cell to anchor to its environment. Furthermore, the evidence of Schor and Court (1979) indicates that cell attachment to

denatured collagen films occurs by a mechanism different from that mediating cell attachment to native collagen fibers. This allows one to speculate that cellular ability to attach to the substratum can be affected by a change in the collagenous matrix in the following possible manners: in a change of collagen types, in the nature of the collagen fibers, or in the arrangement or organization with other matrix components, and also the presence or absence of adhesive glycoproteins. In a similar way, one can also extrapolate that the ability of cell attachment is correlated with the cellular capacity for making its own ECM, when a certain type of cell is cultured *in vitro*. in a matrix-free, plastic or glass environment (see Table 6).

After attachment, cells *in vitro* begin to become oriented and to migrate. The cellular orientation and spreading has been shown to be under the influence of the substratum provided. Contact guidance, as first conceived by Weiss (1959), the tendency of cells to follow oriented fibrous pathways in the substratum, is derived from his early observations on cell orientation and guidance *in vitro* on aligned, naturally fibrous substrata such as stressed plasma clots and the collagenous lamellae of fish scales. This phenomenon has since been observed for various cells and nerve axons (Elsdale and Bard, 1972; Ebendal, 1974; 1976; Maxwell, 1976). Several studies have strongly suggested that the orientation of cells is a direct response to the three dimensional shape of the substratum (Curtis and Varde, 1964; Rovensky *et al.*, 1971). A recent study by Dunn and Ebendal (1978) further indicates that the three-dimensional structure of collagen fibers preserved in the

hydrated state (collagen gel) is critical in directing cellular alignment, and the effect is lost when collagen is air-dried. Three postulated mechanisms invoked by different investigators to explain such cell-matrix interaction have been reviewed (Ohara and Buck, 1979). As yet no one mechanism has been entirely accepted. Overall, it is a complex process involving interactions among intracellular structures such as bundles of microfilament, membrane adhesion proteins, and the extracellular fibrillar matrix.

Cell spreading is also a complex process and possibly involves the interactions described above for cell attachment in a very similar fashion. An experiment by Pouyssegur and Pastan (1979) reveals that the adhesiveness provided by the substratum is a major determinant _in vitro_ controlling directional locomotion of fibroblasts. It serves to stabilize the leading edge and to promote the organization of microfilaments into bundles, which is the prerequisite for the persistence of directional motion. It will be of interest to see what degree and quality of adhesiveness can achieve such a cell-matrix interaction. Studies by Stenn _et al_. (1979) suggest that migrating epithelium synthesizes type V collagen at the leading membrane edges which is necessary for the continous movement by these cells in culture. This is the first demonstration of a possible function of type V collagen. The ability of type V collagen to direct or guide cellular spreading is of interest and compatible with its ubiquity and its pericellular location. Fisher and Solursh (1979) demonstrate that mesenchyme from neural crest, somite, and limb bud all spread most extensively on tadpole tail basal lamina, a type IV basement membrane collagen matrix, less on type I interstitial collagen gel, and least on a collagen gel mixed with

hyaluronic acid, a type of glycosaminoglycan. An interesting observaton in the above experiment is that the supportive effect varies with the collagen type, as well as with the presence of other components, such as glycosamioglycans. In the embryo, cell migration is an event critical for morphogenesis. An important example is given by Hay (reviewed in 1980). In the development of the vertebrate cornea, the presumptive fibroblasts (keratocytes) invading the acellular primary stroma of avian cornea at stage 27-28 from the peripheral mesenchyme seem to use the collagenous, hyaluronic acid-rich hydrated ECM as substrata which has been prepared especially by the preceding endothelium. All these examples support the notion that the behavior of embryonic mesenchyme, here cell migration, is determined largely by the collagenous properties of the available substratum.

Proliferation and differentiation are two cellular events which are difficult or impossible to separate one from the other. These may be associated cellular functions. Both are also the events occuring predominantly during embryonic morphogenesis. The role of ECM in the control of cell behavior during embryogenesis was a concept first introduced by Grobstein in 1953, and has been explored by Grobstein (1967,1975) and others (Bernfield et al., 1972; Slavkin et al., 1977; Lash and Vasan, 1977). The view put forth by these investigators is that ECM does not function merely as an inert structural support but contributes to the control of cellular functions, especially in the interactions between embryonic epithelium and mesenchyme. Because the mesenchyme can provide the bulk of collagenous matrix to the epithelio-mesenchymal junction, it could in turn modulate the proliferation and differentiation of the epithelium. It has been given the name,

secondary embryonic induction, to indicate such a phenomenon, and emphasizes that close cell-cell contact is not involved. Direct evidence that cell-cell contact is not involved in these interactions is derived from those experiments substituting killed matrix for living inducers (Konigsberg and Hauschka, 1965; Dodson, 1963; Dodson and Hay, 1971; Newsome, 1976). However the role of collagen in these cell-matrix interactions was not verified until collagen-coated substrata were used in the culture systems or when collagenous materials were removed from the matrix by collagenase treatment in experiments on morphogenetic development. These studies are summarized in Table 7A. The effect observed is without exception, that cellular proliferation and differentiation is promoted in the presence of collagenous substrata, as compared to its absence (Table 7A). The effect is not limited to cells or tissues of embryonic origin. The collagenous substrata also support cell growth and preserve the differentiated phenotype for adult cells and tissues in culture systems (summarized in Table 7B). Noteworthy is that the effect has been shown not only for mesenchymal but also for epithelial cells and tissues, though for some fully-differentiated epithelial cell lines, such as hepatocytes and mammary epithelial cells, a floating collagen gel is shown to be superior to a collagen film. Supplement of collagen to the culture system seems to have replenished the need of matrix components which are missing when cells are dissociated from tissues in vivo. In this regard, the collagenous matrix does support cell growth and differentiation in vitro; and could be implicated as the inducible matrix molecule indicated in the formulations of Grobstein and others.

It is intriguing to ask whether collagen is the molecule sine qua non mediating these cell-matrix interactions. It is not clear as yet,

though evidence indicates that other matrix components can exert similar effects. Glycosaminoglycans and proteoglycans are shown to modulate chondrogenesis of the chick embryonic somites (Kosher et al., 1973; Toole et al., 1972), and to stimulate corneal epithelium to produce glycosaminoglycan but not collagen (Meier and Hay, 1974b). This is different from the inductive effect of collagen (Meier and Hay, 1974a). These components have also been visualized in the interface between two interacting tissues in the presence of nucleofilters by electron microscopy (Bernfield et al., 1972; Hay, 1977). For some of the cells of epithelial origin, supplementation of collagen alone cannot achieve the full state of phenotypic expression. This has been discussed by Reid and Rojkind (1979) in the culture of hepatocytes. Recently, a series of observations by Gospodarowicz and his coworkers has further demonstrated that different types of collagens or fibronectin alone are unable or are insufficient to support cellular proliferation in vitro of bovine adrenal cortical cells, granulosa cells (Gospodarowicz et al., 1980), vascular endothelial cells, and corneal endothelial cells (Gospodarowicz and Ill, 1980). It suggests that an intact ECM is crucial in exerting such an effect for some cell types. The cells examined in their experiments are mainly epithelial or epithelial-like cells.

Finally, in contrast to the ECM, an insoluble complex, there are various circulating or diffusable factors in the extracellular milieu which conceivably can exert effects on cell behavior. This gives rise to a complex and complicated picture as one attempts to formulate a hypothetical mechanism involving both soluble and insoluble factors simultaneously. Whereas, based on the fact that biosynthesis of collagen can be modulated by a host of factors (see review in Section III of this

Chapter), one is able to formulate a working hypothesis that some of these factors may exert effects on cell behaviors such as attachment, migration, proliferation, and differentiation by modulating collagen biosynthesis. Since collagen is one of the major components of ECM, the change in the amounts and types of collagen synthesis will presumably modify the original properties of the natural substrata. This will in turn alter the normal cell-matrix interactions. The change in cell-matrix interactions subsequently creates abnormalities in phenotypic expression and in responsiveness to other external stimuli. A study exploring the role of collagen in such interactions will shed insights to the understanding the mechanism of how cells interact normally and abnormally with their extracellular environments. This will also serve as an approach to understand the regulation of collagen biosynthesis, the abnormality of which may represent the basis of many connective tissue disorders.

This thesis has been developed with such an aim. This rationale forms the basis of its approach, and will be pursued in a detailed way in the remainder of the thesis.

Table 7A EFFECT OF COLLAGEN SUBSTRATA ON CELL PROLIFERATION AND DIFFERENTIATION

SYSTEM	TESTED MATERIAL	PROLIFERATION	DIFFERENTIATION	REFERENCES
A. Embryonic Tissues or Cells:				
I. MESENCHYMAL				
1. Fibroblast	collagen	+ (long-term culture)	N.T.	Ehrmann & Gey, 1956; Gey et al., 1974
2. Myoblast	collagen	+	+ (myotube formation)	Hauschka & Konigsberg, 1966
3. Somite	collagen & procollagen	N.T.	+ (chondrogenesis)	Kosher & Church, 1975
II. EPITHELIAL				
1. Salivary gland	collagenase treatment	-	- (branching)	Grobstein & Cohen, 1965
2. Lung, ureteric bud, pancreas gland	collagenase treatment	-	- (branching)	Wessells & Cohen, 1968
3. Skin epidermal basal cell	frozen-thawed dermis, collagen	+ (with chick embryo extract)	+ (columnar cell shape)	Dodson, 1963 Wessels, 1964
4. Cornea epithelium	collagen	N.T.	+ (↑ collagen & GAG synthesis)	Meier & Hay, 1974a

NOTE:
1. Chick is the species used in the above experiments.
2. Collagen represents collagen-coated substrata, unless otherwise indicated.
3. " + " and " - " represent " promote " and " inhibit " respectively
4. " ↑ " and " ↓ " represent " increase " and " decrease " respectively.
5. N.T. indicates that the item is not tested by the author(s).

Table 7B EFFECT OF COLLAGEN SUBSTRATA ON CELL PROLIFERATION AND DIFFERENTIATION

SYSTEM	TESTED MATERIAL	PROLIFERATION	DIFFERENTIATION	REFERENCES
B. Post-embryonic or Adult Tissues:				
I. MESENCHYMAL				
1. Rat subcutaneous fibroblast	collagenous bone matrix	+	+ (Endochondral ossification & hematopoiesis)	Reddi & Anderson, 1976; Rath & Reddi, 1979
2. BALB/c mice connective tissue cells	↑collagen by cis-hydroxy proline	-	N.T.	Liotta et al., 1978
3. Rat schwann cell	collagen	+	+ (myelination)	Bunge & Bunge, 1978
4. Mice spleen lymphocytes	collagen	N.T.	+ ? (↑IgG production)	Kemshead & Askonas, 1978
II. EPITHELIAL				
1. Rat hepatocyte	collagen/ floating collagen gel	(↑viability)	(maintain in vivo some phenotypes)	Michalopoulos & Pitot, 1975; Lin & Sondgras, 1975 Sirica et al., 1978
2. Rabbit skin epithelial cells	collagen	(↑viability)	N.T.	Liu & Karasek, 1978
3. Bovine cornea epithelial cells	collagen	+ (responsive to EGF)	+ (cuboidal cell shape)	Gospodarowicz et al., 1978
4. Mouse mammary epithelial cells	floating collagen gel	+	+ (glandular structure & casein secretion)	Emerman et al., 1977 a & b Yang et al., 1979

NOTE: The symbols used in this table are explained in Table 7A.

CHAPTER II EXPERIMENTAL MODEL SYSTEM AND WORKING HYPOTHESIS

I. INTRODUCTION OF THE EXPERIMENTAL MODEL SYSTEM

This thesis addresses the question of the mechanism of interaction between cells and their ECMs. The experimental model employed is a cell culture system of endothelial cells, derived from bovine endothelium, from both blood vessels and cornea. This culture system, developed in the laboratory of Dr. Denis Gospodarowicz, is well characterized. Aspects of this culture system relevant to the present study and the advantage of this cell culture in the model system are described below.

In vivo, anatomically, endothelia are chiefly, if not exclusively, found at the interface between a fluid and a tissue. In the cases of vessels and cornea, endothelium lines the innermost layer of the tissue and serves as a barrier separating the tissue proper from the blood stream and aqueous humor respectively. It is a single cell monolayer consisting of closely-apposed, flattened, cuboidal cells. Functionally, vascular endothelium serves as a selective permeability barrier for the circulating nutrients in the blood stream. The surface facing the lumen is metabolically engaged in active transport and maintenance of homeostasis. As for corneal endothelium, the cell surface is actively functioning as a sodium pump such that water is constantly transfered from the stroma proper into the anterior chamber. By doing so, the cornea is maintained in a permanent state of deturgescence and the transparency of the cornea is thus insured. One of the major morphological characteristics of the differentiated phenotypes of the endothelium is the cell surface polarity. The apical cell surface, facing the lumen or

cavity, is devoid of any attachment to or association with the underlying ECM. Histologically, ECM is only visualized underneath the basal surface of the endothelium, and is recognized as a distinct basement-membrane-like structure. In the case of the cornea, this basement membrane is termed Descemet's membrane. The cell surface polarity and cell-matrix relationship is essential for the ability of the endothelium to express its physiological functions, and to maintain its non-thrombogenic property.

In vitro, establishment of vascular and corneal endothelial cells in a culture system has been facilitated by introducing fibroblast growth factor (FGF) to the culture medium (Gospodarowicz et al., 1977 a&b). When exposed to this mitogen, sparse cultures have a high mitotic index and upon reaching confluence, adopt the morphological organization characteristic of endothelium in vivo, a well-organized monolayer of contact-inhibited, flattened, cuboidal cells (Gospodarowicz et al., 1979a & 1980b). There is also a cell surface polarity such that the ECM is elaborated subendothelially. The asymmetrical distribution of the ECM has been confirmed by immunofluorescence studies using antibodies against fibronectin, a matrix component, and by visualization with a transmission electron microscope (Birdwell et al., 1978). It endows the apical cell surface of confluent endothelial cells with non-thrombogenicity, a characteristic phenotype of endothelium in vivo, which is further verified by platelet binding experiments (Gospodarowicz et al., 1979b). In addition to the cell surface polarity described above, at confluence, the cultured endothelial cells also exhibit other in vivo differentiated characteristics, such as active production of prostacycline (PGI_2) and formation of an efficient barrier against a

receptor-mediated uptake of low density lipoprotein (Fielding *et al.*, 1979; Vlodavsky *et al.*, 1978). Thus most, if not all, of the expression of the differentiated phenotype of endothelium *in vivo* can be preserved successfully *in vitro*. However, the chief advantage of using this cell culture system is in the study of the asymmetrical deposition of ECM, and the attendant cell-matrix interaction. This has been difficult to study *in vivo*.

In contrast, in the absence of FGF, the cultured vascular endothelial cells undergo, within two to three passages, structural and functional alterations that are incompatible with their typical *in vivo* morphological appearance and physiological functions (Vlodavsky *et al.*, 1979). The cells become highly vacuolated, proliferate slowly, and quickly become senescent. At confluence, a monolayer consisting of large polygonal cells appears. These cells lose contact-inhibition and overlap one another. Most striking is that the polarity of cell surfaces is also lost, and the ECM is found covering all of the cell surface. The altered phenotype of vascular endothelial cells can be restored to the original normal configuration as FGF is exogeneously added back into the culture media. These results indicate that FGF is not merely a mitogen able to stimulate the proliferation of endothelial cells, but is also an agent able to control their differentiation and phenotypic expression. That the phenotype of endothelial cells can be modulated by a single peptide factor, FGF, *in vitro*, lends itself an excellent model system to study the controlling mechanism of cellular proliferation and differentiation, and the possible association between altered phenotype and the anomalous deposition of ECM.

Finally, another advantage of using this cell culture system is that one can readily obtain the ECM in vitro. As aforementioned, at confluence ECM is deposited only underneath the basal cell surface of endothelial cells, as the culture is exposed to FGF. The production of ECM is further augmented when dextran is added together with FGF. The cell monolayer can then be removed by treating the culture with an isotonic saline solution containing a detergent (to be discussed in the Methods section, chapter VI). The underlying ECM is preserved intact on the plastic culture plates. The ECM prepared in this manner from corneal endothelial cultures has been shown able to support cell proliferation and differentiation of a number of cell types including corneal and vascular endothelial cells (Gospodarowicz and Ill, 1980), and other cells (Gospodarowicz, et al., 1980a). Thus, in the presence of this preformed natural substrata, the exogenous addition of FGF is no longer required. It raises several questions: What is the mechanism of action for this natural substrata? Is there any relationship between the action of FGF and that of ECM in modulating cell growth and differentiation in vitro?

In summary, as described above, the model system using the culture of endothelial cells furnishes several advantages in addressing questions regarding cell-matrix interactions. First, by changing the culture conditions, the in vitro system can be manipulated easily and accurately. Second, in this cell culture system, the growth of endothelial cells is comparable to the differentiated phenotype of their counterparts in vivo. Third, because of the asymmetrical deposition of ECM and cell surface polarity in this model system, the operational scheme of cell-matrix interactions is simplified into a one-dimensional

fashion. Fourth, by addition or withdrawal of a single peptide factor, FGF, one is able to modulate the cell-matrix interactions with respect to proliferation and differentiation. Fifth, the underlying ECM can be readily isolated *in vitro* and utilized as a natural substratum. Sixth, it provides a good model system to study the relationship between a soluble and diffusable factor, FGF, and the insoluble substratum, ECM, since both exert comparable effects on cell behavior.

II. WORKING HYPOTHESIS

The model system described above portrays a phenomenon distinctive for the dramatic modulation of cell behavior. Both proliferation and differentiation can be controlled by either a soluble factor or by the ECM to which cells are exposed. The mechanism underlying this phenomenon is the chief concern herein. The growth and phenotypic expression of cultured endothelial cells is modulated by the addition or removal of a single peptide FGF. Noteworthy is that the phenotypic changes are manifested not only in morphological configuration but also in the pattern of deposition of ECM and in cell surface polarity. This suggests that FGF is involved with the control mechanism for the production of ECM, the insoluble matrix. However, when a natural substratum, ECM prepared from the same cell type is supplemented, cell growth and differentiation is facilitated even in the absence of FGF. Thus ECM is able to substitute for the exogeneous growth factor.

Based on this phenomenon, a working hypothesis is formulated. One, if not all, of the mechanisms of action of FGF is by directly modulating the production of ECM in cultured endothelial cells. The ECMs produced by cells are the natural substrata upon which cells attach, migrate,

proliferate, and differentiate. ECM has been implicated by Grobstein and other investigators as a permissive factor for such cell-matrix interactions (see Section IV, Chapter I). Therefore, production of the appropriate ECM in the presence of FGF can indirectly support cell growth and differentiation. In the absence of FGF, the alteration of ECM in the model system interferes with normal cell-matrix interactions. As a result, cell growth and differentiation are affected.

Collagen is the major protein component of the ECM, and has also been postulated as the primary molecule in the ECM mediating the interactions between cell and matrix (see the Section IV, Chapter I). It is reasonable to believe that the integrity of ECM is dependent on the normal amounts and types of collagen. One possible way to generate an altered ECM is by affecting the normal scheme of collagen biosynthesis. Thus the working hypothesis mentioned above can be restated in the following manner. The mechanism of action of FGF is specific towards collagen production. The mechanism of action of FGF is by modulating collagen synthesis in cultured endothelial cells. This thesis will test this working hypothesis. Based on such a hypothesis, the experimental design and approach is formulated and developed in the subsequent chapters.

CHAPTER III DESIGN OF A NEW RAPID METHOD FOR QUANTITATING COLLAGEN BIOSYNTHESIS

I. INTRODUCTION

The first step in approaching the working hypothesis proposed in Chapter II is to design a rapid method of quantitating collagen biosynthesis. This is the aim of this chapter.

In studies of collagen biosynthesis and metabolism, it is essential to measure the specific activities of radiolabeled proline and its two hydroxyl derivatives, 4-hydroxyproline and 3-hydroxyproline. Hydroxyprolines are found in vertebrate tissues almost exclusively in collagen. The two known exceptions are the C1q subcomponent of the complement system (Porter and Reid, 1978), and the enzyme acetylcholinesterase (Rosenberry and Richardson, 1977). A collagen-like sequence is found in these two quite unrelated proteins. In collagen synthesis, hydroxylation is a post-translational event. Peptide-bound proline is a substrate for two separate enzymes, either prolyl-4-hydroxylase or prolyl-3-hydroxylase (Risteli et al., 1977; Tryggvason, 1977). The form and extent of hydroxylation is different for the different types of collagens. Interstitial collagens, type I, II, and III, contain more 4-hydroxyproline than 3-hydroxyproline; about one residue of 3-hydroxyproline per α chain in type I and III and two residues per α chain in type II (reviewed by Hall and Jackson, 1976). However, basement membranes containing type IV collagen, are characterized by a high 3-hydroxyproline content, ranging from 10 to 15 residues per α chain (reviewed by Kefalides, 1973; Fessler and Fessler, 1980). Type V collagen

contains 3 to 5 3-hydroxyproline residues. The contents of proline and the two derivatives of hydroxyproline of different types of α chains are summarized in Table 1. The determination and quantitation of these three imino acids should offer valuable information not merely on the synthesis of collagenous versus noncollagenous protein, but also the proportion of interstitial collagen to basement membrane collagen synthesis, in various organ and cell culture systems using radiolabled proline (Man and Adams, 1975).

Currently, no single method can achieve these three measurements simultaneously. The method of Rojkind and Gonzalez (1974), a modification of the procedures of Peterkofsky and Prockop (1962) and Switzer and Summer (1971), is limited to determinations of [^{14}C]proline and [^{14}C]hydroxyproline. In addition, this method contains tedious steps of oxidation and extraction. Some concentration-dependent and time-dependent correction factors are also involved. An alternative method employed by most investigators is a time-consuming ion-exchange column chromatographic technique (Bienkowski et al., 1978; Cutroneo et al., 1972). Each sample requires a separate column chromatographic run and more than three hours to complete. In addition, free proline cannot be quantitated in the presence of labeled protein on the ionexchange column, and integration of the peak area is required to obtain the total radiolabeled counts. Furthermore, the method does not separate 3-hydroxyproline from 4-hydroxyproline unless an amino acid analyzer is used (Porter and Reid, 1978; Grant et al., 1972; Lembach et al., 1977).

Herein a rapid, simple, multi-sample method is described for quantitating simultaneously these three labeled imino acids, based on high voltage paper electrophoresis, and which yields excellent resolution.

Table 1 PROLINE AND HYDROXYPROLINE CONTENTS IN DIFFERENT TYPES OF COLLAGEN

Residue	α1(I)	α2(I)	α1(II)	α1(III)	α1(IV)	α1(V)	α2(V)
3-Hyp	1	1	1	2	12	5	3
4-Hyp	91	87	95	119	128	87	89
Total Hyp	92	88	96	121	140	92	92
Pro	138	117	121	107	65	129	96
Hyp/Pro+Hyp (%)	0.40	0.43	0.44	0.53	0.68	0.42	0.49
3-Hyp/Total Hyp (%)	1.1	1.1	1.0	1.7	8.6	5.4	3.3

NOTE: 1. The data digested above are all from bovine species.
2. The number of each residue is measured on the basis of 1000 total amino acid residues.
3. The data are summarized from the following studies: Clark and Veis, 1972, Fujii and Kühn, 1975, Herbage et al., 1977, Dehm and Kefalides, 1978.

The problem of counting efficiency on a heterogenous solid support was solved by one-step extraction of the sample, which was thus converted into a homogeneous system. It is a reproducible system with a high recovery rate and sensitivity.

II. MATERIALS

Standard Imino Acid Solutions: Standard solutions of L-proline and 4-hydroxyproline (Calbiochem) were prepared with triply distilled water at a concentration of 5 mg per ml; 3-hydroxyproline (trans-3-L-hydroxy-proline) was courteously provided by Dr. J. P. Borel, and was used at the same concentration. The characterization of 3-hydroxyproline has been reviewed by Szmanovicz et al. (1979).

Radioactive Proline Standards: [^{14}C]proline standards were obtained from two sources. Amersham L-[U-^{14}C]-proline, CFB-71, batch 64, with a specific activity of 285 mCi/mmol, was used in most studies. New England Nuclear, NEC-285, batch 1164-215, with specific activity of 291 mCi/mmol was a kind gift of Dr. R. Bhatnagar. Additional purification of Amersham [^{14}C]proline was achieved using cationic exchange column chromatography (Cutroneo et al., 1972). Pooled peak fractions of proline were evaporated, reconstituted with distilled water and used as the radioactive proline standard. A [^{3}H]proline standard was purchased from Amersham, L-[5-^{3}H]-proline, TRK 323, batch 20, with a specific activity of 14 Ci/mmol and was used in the experiment of establishing the counting system for tritiated samples.

Electrolyte Buffer: The buffer was prepared by mixing 75 ml of glacial acetic acid with 27.5 ml formic acid (98%) and diluting with distilled water to 1l. The pH of the final solution used for high voltage electrophoresis was 1.85.

High Voltage Paper Electrophoresis: The tank of Savant Co., model LT 48A, was used for the electrophoretic separation connected to a 5,000 volt/300 ma power supply. Whatman No. 1 filter paper (46 x 57 cm) was used as the supporting medium throughout.

Isatin Staining Reagent: The reagent was made by dissolving isatin (indole-2,3-dione), 1 g, (Fisher Scientific); collidine, (2,4,6-trimethylpyridine), 2.7 ml, (Aldrich); anhydrous glacial acetic acid, 1.8 ml; and absolute ethanol, 100 ml.

Microcapillary pipets (Kimble) were used to apply sample aliquots to the paper. The volume ranged from 1 µl to 100 µl. Scintillation fluid, Aquasol, New England Nuclear, NEF-934, was used for liquid scintillation counting. RPMI 1640 medium, PBS, pH 7.4, and FCS were purchased from Grand Island Biological Company. [^{14}C-methyl]-BSA was obtained from New England Nuclear.

III. METHODS

Electrophoretic Separation of the Imino Acid Standards: Sample aliquots of standard imino acid solutions were applied with the aid of the microcapillary pipets to the origin line on the Whatman No. 1 paper, which was marked 8.3 cm from one end. Fifteen samples can be applied easily to one sheet of paper in a single electrophoretic run. To achieve optimal visualization and separation, 10 µg of proline and 20 µg of hydroxyproline were found sufficient. A hair dryer with gentle heating was used to facilitate the spotting. After wetting evenly with the electrolyte buffer, pH 1.85, the sheet was pressed as dry as possible. The paper was transferred to the electrophoretic tank with the origin close to the anode. Each electrophoresis was run routinely for

30 minutes at 5,000 volts (88 volts/cm). The sheets were dried in a heated oven at 90° for 10 minutes and sprayed with isatin staining reagent. Bluish spots developed on the yellowish background after being reheated for 10 minutes.

Liquid Scintillation Counting: It was performed in a Beckman LS-100C counter, with maximal counting efficiency of 96.3% for the ^{14}C wide window, 75.9% for the narrow window, and 60.3% for the ^{3}H, under unquenched conditions. Each counting was set to allow 1% error. Quenching due to ethanol-water solutions was corrected by the internal standard method, and counting efficiencies (E) for ^{14}C and ^{3}H under such serial quenching effect were obtained by the formula:

$$E(\%) = \frac{\text{observed cpm}}{\text{actual dpm}} \times 100$$

All determinatons were performed in triplicate and reported as the mean value.

Ion Exchange Chromatography on Dowex 50W-X8: This was performed as described by Cutroneo et al. (1972).

IV. RESULTS

A. Electrophoretic Separation of the Three Imino Acids

The isatin reagent was used for color development because it provided specific coloration for imino acids; deep blue for proline and light blue for hydroxyprolines; other amino acids appeared pink on the yellowish background (Kruze et al., 1967). Relative electrophoretic mobilities are shown in Fig. 1.

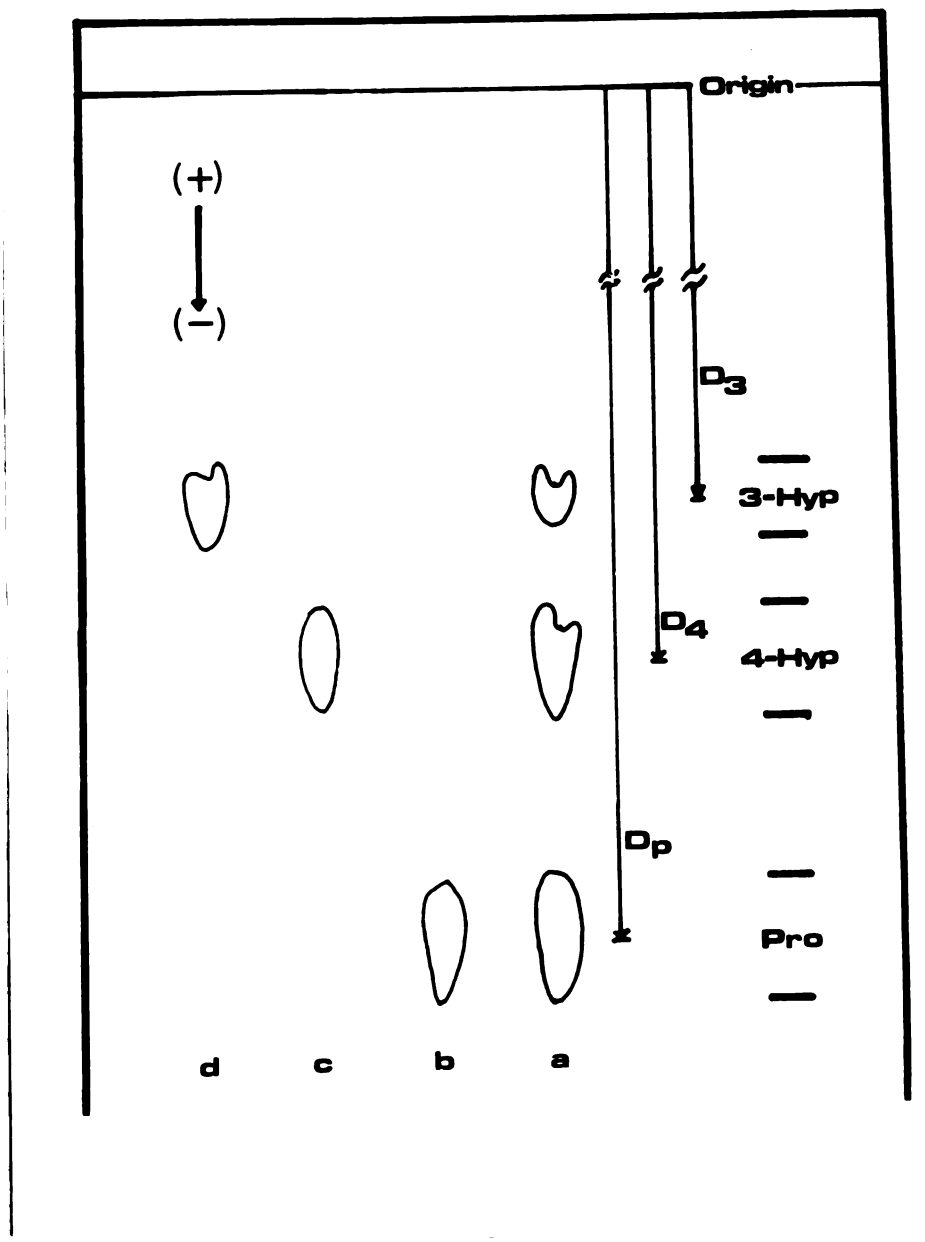


Figure 1:

Electrophoretic pattern of imino acid standards. The paper was stained and the spots were outlined; (d), 3-hydroxyproline; (c), 4-hydroxyproline; (b), proline; (a), mixture of all three; 10µg of proline and 20 µg of hydroxyprolines were used.

Three imino acids were widely separated, with the length of the spot less than 3.0 cm. Such separations were highly reproducible at different times and offered sufficient spacing for cutting out the desired areas. Relative electrophoretic mobility coefficients (E) for hydroxyprolines were obtained and shown in Table 2, using proline as the reference. The values were calculated as the ratio of the distance of migration of the hydroxyprolines to that of proline and denoted as E_p^3 for 3-hydroxyproline and E_p^4 for 4-hydroxyproline.

Salt, acid, alkali, amino acids, and proteins were added to the standards, in increasing amounts, to investigate their influence on electrophoretic mobility and the size of the spots. The electrophoretic mobility and length of the proline and 4-hydroxyproline spots were not altered as equal volumes of PBS, pH 7.4, and different concentrations of NaCl were added at the origin and run simultaneously. A salt concentration could be tolerated up to 4M (Fig. 2a-d). As shown in Fig. 2e, these values remained unchanged in the presence of 6 N HCl, which is the usual condition for the acid hydrolysis of proteins. However, spots became retarded and elongated in the presence of alkali, 1 N and 4 N NaOH (Fig. 2f and g). The presence of other free amino acids did not influence the mobility and size of the spots, when an equal volume of RPMI 1640 medium was added which contains biological concentrations of several amino acids (Fig. 2h).

Table 2 RELATIVE ELECTROPHORETIC MOBILITIES AND SPOT LENGTHS

	Relative Electrophoretic Mobility Coefficients (E)	Spot Lengths (cm)[a]
Proline (Ep)	1.0	2.67 ± 0.46 (16)[b]
4-Hydroxyproline (E_p^4)	0.77 ± 0.01 (13)	2.25 ± 0.38 (16)
3-Hydroxyproline (E_p^3)	0.62 ± 0.02 (4)	1.38 ± 0.19 (8)

NOTE:

a) Initial spot length, as applied at the origin, was on the average, one-fourth of this size. The center of the spot was used to derive Ep. No special attempt was made to control the temperature or to standardize these values by correcting for electro-osomotic flow by the use of dyes (Werum et al., 1960).

b) Number of experiments.

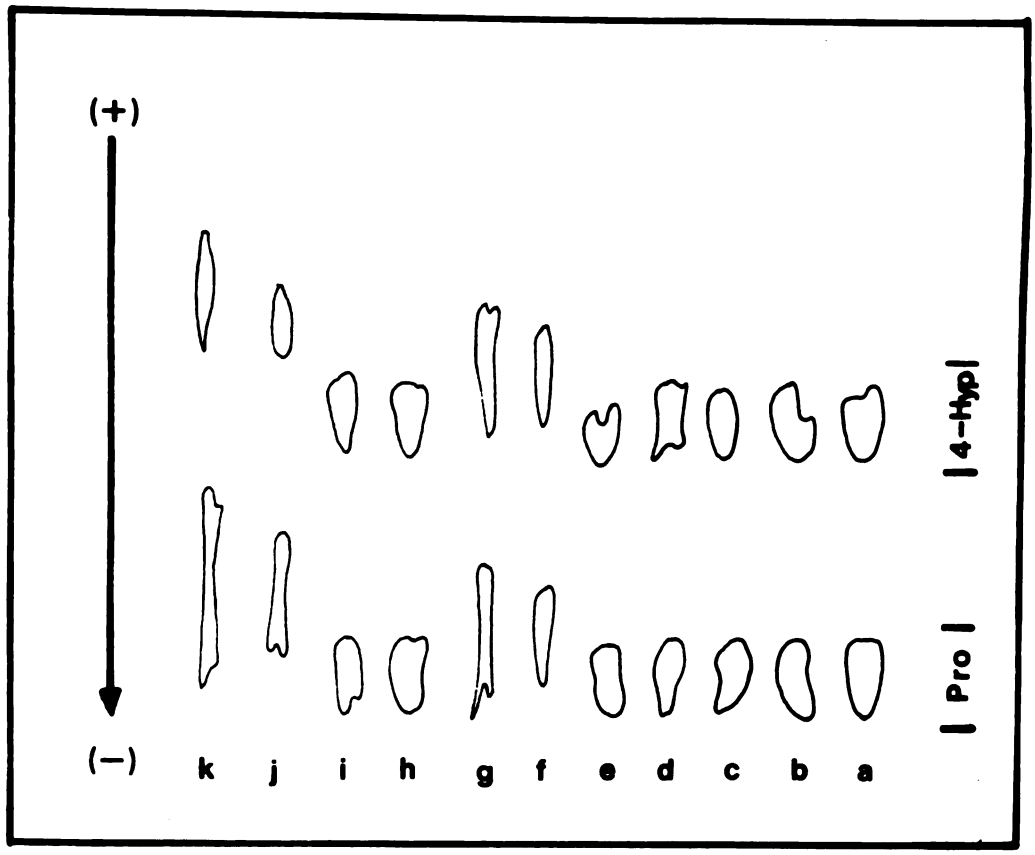


Figure 2:

Influence of various materials on the electrophoretic pattern of standard proline and 4-hydroxyproline. Control mixture in water, (a), and in the presence of (b), PBS, pH 7.4; (c) 1 M NaCl; (d), 4 M NaCl; (e), 6 N HCl; (f), 1 N NaOH; (g) 4 N NaOH; (h), RPMI 1640 medium; (i), RPMI 1640 medium containing 10% FCS; (j), undiluted FCS; (k), 2-fold volume of undiluted FCS. Each application was 2 µl and repeated in triplicate.

This result agrees with earlier findings (Atfield and Morris, 1961). Even when 10% FCS in medium was added to the imino acid solutions (Fig. 2i), the spots were not distorted. Interestingly, in the presence of high concentrations of protein (Fig. 2i and k), although retardation and elongation of the spots did occur, imino acid standards could still be separated. 3-hydroxyproline was not tested in the above and following experiments due to scarcity of this material.

B. Establishment of Maximal Conditions for Extraction and Counting

One of the problems encountered in calculating counting efficiency, was that the radioactive samples were counted on solid supports and it was difficult to evaluate the extent of dissociation of the material from the support into the scintillator solution. If the samples remained on the support, many factors would have to be considered, such as the magnitude of β-energy, the size of the sample molecule (Bransome and Grower, 1970; Long et al., 1976). Self-absorption is the key event which compromises counting efficiency and which is nearly impossible to calculate on a theoretical basis.

A series of experiments was performed to establish maximal conditions for extraction of the sample from the electrophoresis paper and for optimizing counting efficiency. Standards of [^{14}C] and [^{3}H]proline, were prepared in four groups as follows. Group (a) control: [^{14}C]-proline, 20 µl, containing 49,300 dpm and 10µl of [^{3}H]proline containing 97,000 dpm were added directly to counting vials containing 1 ml of various concentrations of ethanol in water, to which 10 ml of Aquasol

was added. Group (b) counting of paper with extraction: identical aliquots of [^{14}C]proline and [^{3}H]proline standards were spotted directly on paper rectangles, 3 cm x 3.5 cm, the same size cut from electrophoresis sheets. The dried paper strips were then cut into 8-12 small pieces and transferred together into a single vial containing the various concentrations of ethanol in water. The vial was mixed with a Vortex mixer for 30 seconds and 10 ml of Aquasol added. The paper strip remained in the counting vial. Group (c) counting of the supernatant following extraction of the sample from paper: the same procedure described in (b) was repeated in test tubes containing 2 ml of the various concentrations of ethanol-water. After mixing, the sample was centrifuged at 25,000 rpm for 3 minutes. One ml of the supernatant was transferred to a counting vial and 10 ml of Aquasol added. Group (d) counting of paper strips with labeled standards immersed directly in Aquasol:labeled standards were spotted on paper strips, dried, and immersed directly in counting vials containing 10 ml of Aquasol without attempting to extract radiolabeled material.

In order to examine the spectrum of energy distribution in the [^{14}C]-containing samples, the radioactivity of each sample in group (b) was counted in the presence of paper, using both wide and narrow windows (Fig.3). Only the high energy portion of ^{14}C nuclides were detected using the narrow window. There was a difference between these two measurements, as compared to the control samples (group a) which contained no paper strip in the vial. The difference was more pronounced in samples containing 80 to 100% ethanol. The reason for the increased differences at the higher concentrations of ethanol was that, as will be shown below, the efficiency of extraction of radiolabeled samples from

the paper was decreased. The column on the right of Fig. 3 indicates the count rate of samples without extraction from paper (group d).

Using simple one-step extraction (group c), it was possible to obtain 100% recovery of the count rate with a range of ethanol concentrations of 20 to 50% (Fig. 4). There was no difference in counting using either the wide or narrow window, indicating it was identical to the homogeneous system. Under such conditions, quenching can be corrected for and counting efficiency can be calculated. On the other hand, extraction with water or 10% ethanol yielded a recovery rate of less than 100% (Fig. 4). This might be due to self-aggregation of proline molecules in the presence of Aquasol (Peng, 1977). Higher concentrations of ethanol extracted less than 80% of the radioalabeled material from the paper (Fig. 4). The presence of the paper strip apparently can prevent penetration of β particles.

In the case of [^{3}H]proline (Fig. 5), the extraction curve was similar to that of [^{14}C]proline. A comparison of the two isotopes over the range of ethanol concentrations demonstrated no difference in extractability (compare Fig. 4 and 5). Yet the presence of the paper strip caused a more dramatic decrease in count rate recovery of [^{3}H]proline (Fig. 5), the extraction curve was similar to that of [^{14}C]proline (Fig. 5, solid line) at the higher ethanol concentrations, compared to [^{14}C]proline (Fig. 3, solid line). This indicated that paper prevented penetration of β particles more effectively in the case of ^{3}H which has a lower energy emission. Without extraction (group d), the counting efficiency was low in the cases of both [^{14}C] and [^{3}H]proline count rates (Fig. 3 and 5 respectively, right hand columns). Only 10% of the original count rate could be detected in the case of [^{3}H]proline, without extraction as compared to 70% for [^{14}C]proline.

Even in the case of [^{3}H]proline, a recovery rate of over 98% was achieved in the range of 0 to 30% ethanol, regardless of the presence of the paper strip in the counting vial (Fig. 5), indicating that extraction from the paper is the critical factor.

C. Quantitation of the Specific Activity of Radioactivity Imino Acids

Calculations for the determination of specific activity are dependent upon rates of recovery of the individual imino acids as well as determination of the counting efficiency under the experimental condition.

Electrophoresis of labeled imino acids was performed and two lateral lanes were used for non-radioactive standards. These two strips were then removed and sprayed for color development. The remaining lanes were cut for extraction of labeled smaples, using the two guide lines, 0.5 cm above and below the margins of the developed standard spots. Since there is no 3-hydroxyproline commercially available, the reference of 3-hydroxyproline was derived from the E^{3}_{p} of 3hydroxyproline (Table 1).

Thirty percent ethanol in water was chosen as the extraction solution since it provided near 100% recovery (Fig. 4 and 5). After extraction, one-half of the supernatant was removed for liquid scintillation counting and a subsequent correction of two was made. The counting efficiency at this concentration was 73.8% for ^{14}C using the wide window, and 46.7% for ^{3}H.

The recovery rate of the [^{14}C]proline was determined. The New England Nuclear and the Amersham [^{14}C]prolines were used initially in electrophoresis. However, both samples contained impurities (Fig. 6). After column purification, the Amersham [^{14}C]proline had a single radioactive area coinciding to the proline spot (Fig. 6c). If ten smaller

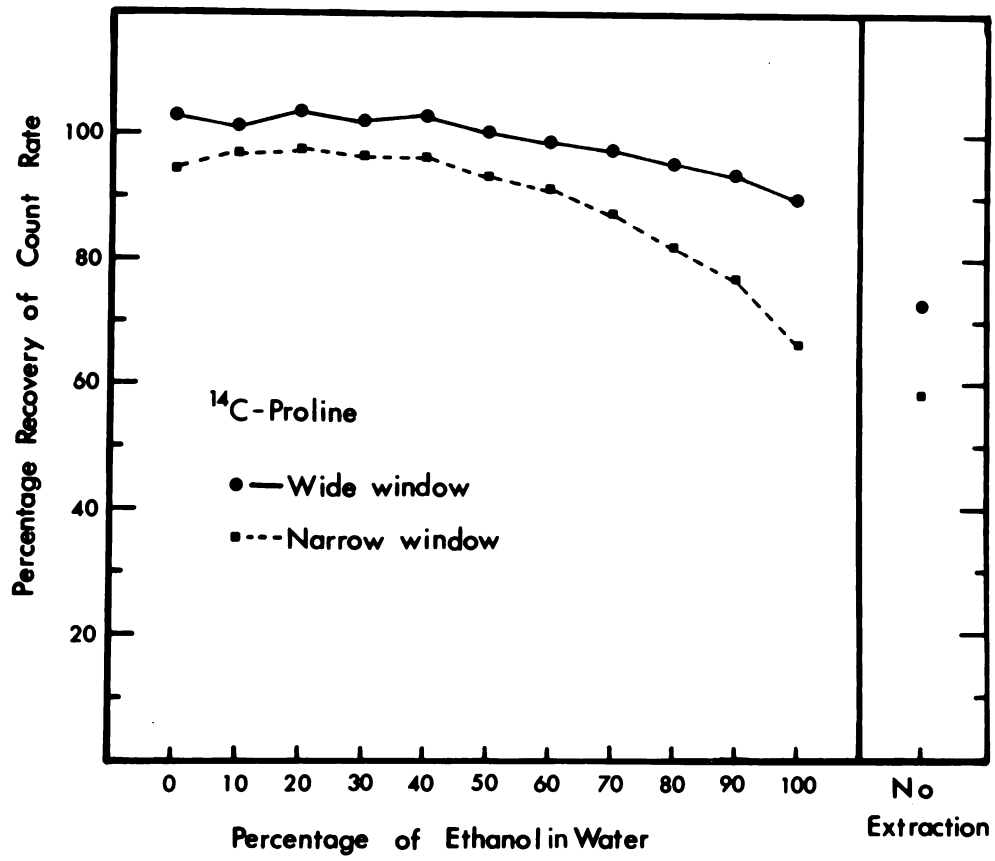


Figure 3:

Extraction of [^{14}C]proline into ethanol-water solutions, as described in group (b); counted in presence of the paper strip. The percentage of recovery of count rate was based on control counts in group (a). Each point on the graph represents the mean of three separately prepared samples.

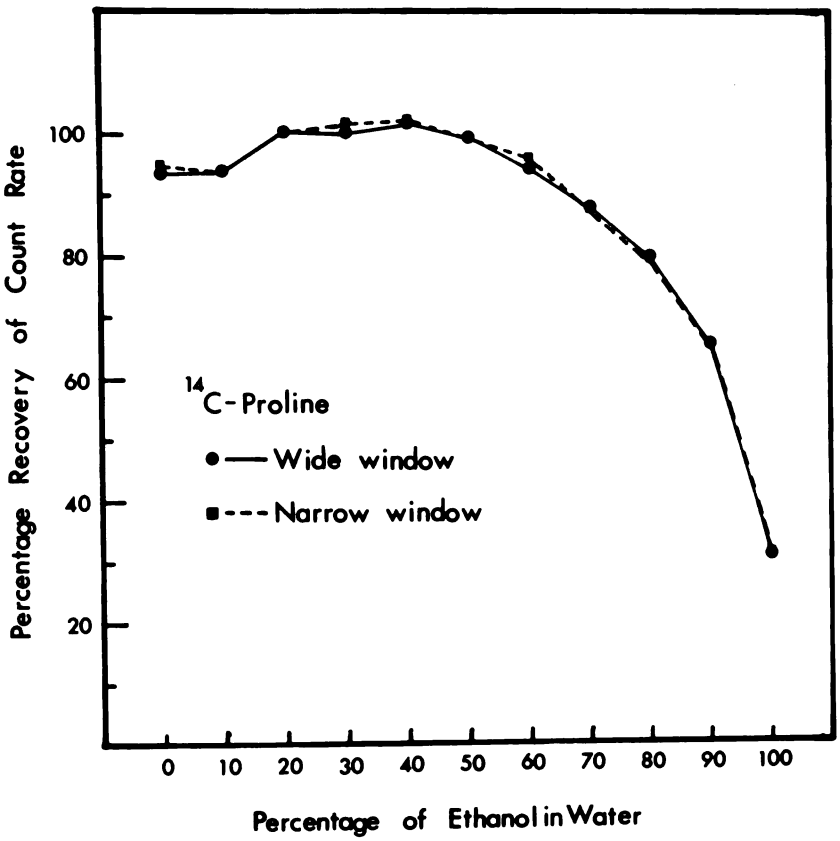


Figure 4:

Extraction of [^{14}C]proline into ehtanol-water mixture as described in group (c); the percentage recovery of count rate was based on control counts in group (a). Each point on the graph represents the mean of three separately prepared samples.

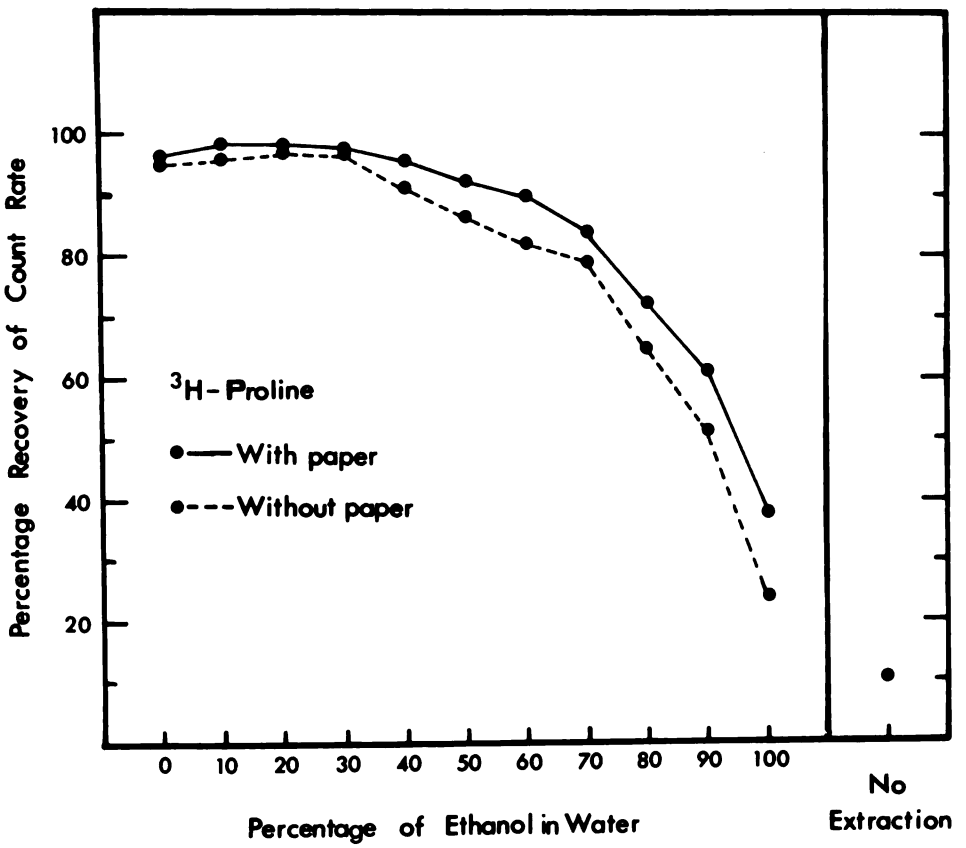


Figure 5:

Extraction of [^{3}H]proline into ethanol-water mixutre. Counting was performed in presence of paper in the vial (group b) and in supernatant only (group c). Percentage recovery was based on control group (a). Each point on the graph represents the mean of three separately prepared samples.

strips were cut from the 5 cm proline spot area, it was found that 99% of the radioactivity was limited to a 1.5 cm region (Fig. 6e). The proline spot in Fig. 6c was removed and re-electrophoresed on a separate sheet of paper. The same profile as Fig. 6c was obtained (not shown), further confirming the identity of the radioactive material as proline.

The recovery rate was measured as the ratio of counts obtained from the proline spot following electrophoresis, to the original number of counts. When radioactive proline standard purified by column chromatography was used, 97.8% of the count rate was recovered on the proline spot after electrophoresis and extraction (Table 3). This was almost identical to the recovery rate, 98.1% of an unpurified standard (Table 3), within an acceptable 1% error. Both experiments confirmed that the present method had a high recovery rate. There is no reason to expect different behaviors for determinations of specific activities or recovery rates of 3-hydroxyproline and 4-hydroxyproline. The influence of unlabled carrier (20 μg per spot) upon the recovery rate was also investigated. No effect was observed (Table 3). Therefore, imino acid carriers, either unlabeled proline or hydroxyproline were not necessary using the current method, since recovery rates remained the same in their absence.

The effect of other agents was also examined. Common cell culture medium, RPMI 1640, containing 10% FCS or [^{14}C]-BSA (1734 dpm), were added in equal volume to the radioactive proline standard before electrophoresis. In the presence of protein, either non-labeled or labeled, 97-98% of the count rate could still be recovered in the proline spot (Table 3), based on the recovery rate of proline standard electrophoresed without added protein.

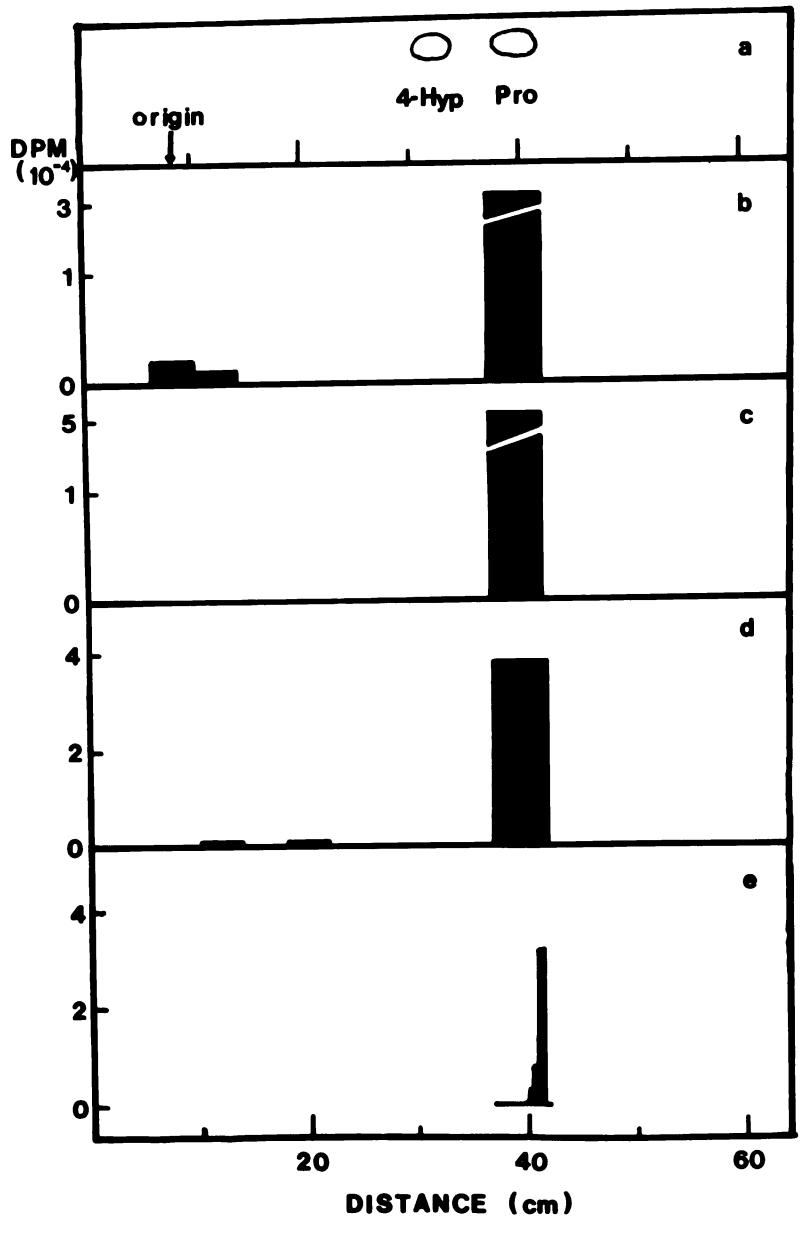


Figure 6:

Electrophoretic profiles of proline and 4-hydroxyproline standards visualized by isatin (a). Other strips, after cutting out proline area (5 cm), were divided int 4 cm long pieces and counted by routine procedure described in method C. Results: (b), New England Nuclear NEC-285, diluted 40x; (c), column purified Amersham CFB-71; (d), Amersham CFB-71, unpurified, diluted 20x; (e), same as (d), with proline spot area further divided into ten pieces to show the distribution of radioactivity.

Table 3

RECOVERY RATES OF THE RADIOACTIVE PROLINE STANDARDS

[^{14}C]-proline standard in 30%[a] ethanol	dpm	Extraction from the proline spot after electrophoresis	dpm	% Recovery
1. Reconstituted, column purified	62,604[b]		61,224	97.8
2. Undiluted, unpurified	126,887	(A) No additives	124,448	98.1
		(B) With cold proline carrier[c]	124,174	97.9
		(C) Mixed with RPMI medium containing 10% FCS	121,298	95.6 (97.5)[d]
		(D) Mixed with ^{14}C-BSA[e]	121,820	96.1 (97.9)[d]

NOTE:
a) Amersham standard CFB-71 was used throughout
b) All numbers represent the mean of experiments performed in triplicate
c) 20 µg per spot
d) when based on the dpm in 2A
e) 1734 dpm

The effect of serial dilution was examined next. The radioactive [^{14}C]proline standard was serially diluted into two separate groups, up to 1:10,240. The control dilutions were spotted into a paper square (3 x 4 cm) followed by routine extraction and counting as described above. The experimental group was electrophoresed, the proline spots cut out, and counted in the same way. The counting efficiency and recovery rate from high counts to low counts was investigated, and the limit of detection of the method was determined (Fig. 7). Following serial dilution of the [^{14}C]proline standards, a linear relationship was found to exist through the entire range of dilution. After electrophoresis and extraction there was a linear recovery count rate. The average recovery rate was 92%. This indicated that the method is suitable over a wide range of radioactivity. The theoretical limit of detection of this method, the minimal concentration which can be detected (approximately twice the level of background noise) was 3.4×10^{-5} of the original concentration equal to 6×10^{-6} pmol for the case of [^{14}C]proline.

V. DISCUSSION

The method of separation of the three imino acids described in this chapter and their quantitation is reproducible, rapid, and convenient. Fifteen samples can be separated on one sheet and measured within two hours. Column chromatographic separation of these same imino acids is much more tedious. This method can be used to determine the specific activities of the individual imino acids in the presence of proteins and hydrolysates of proteins in the biological specimens. The results will be presented in Chapter IV, Methods section. It demonstrated definitively that there was no difference in recovery rates of these three

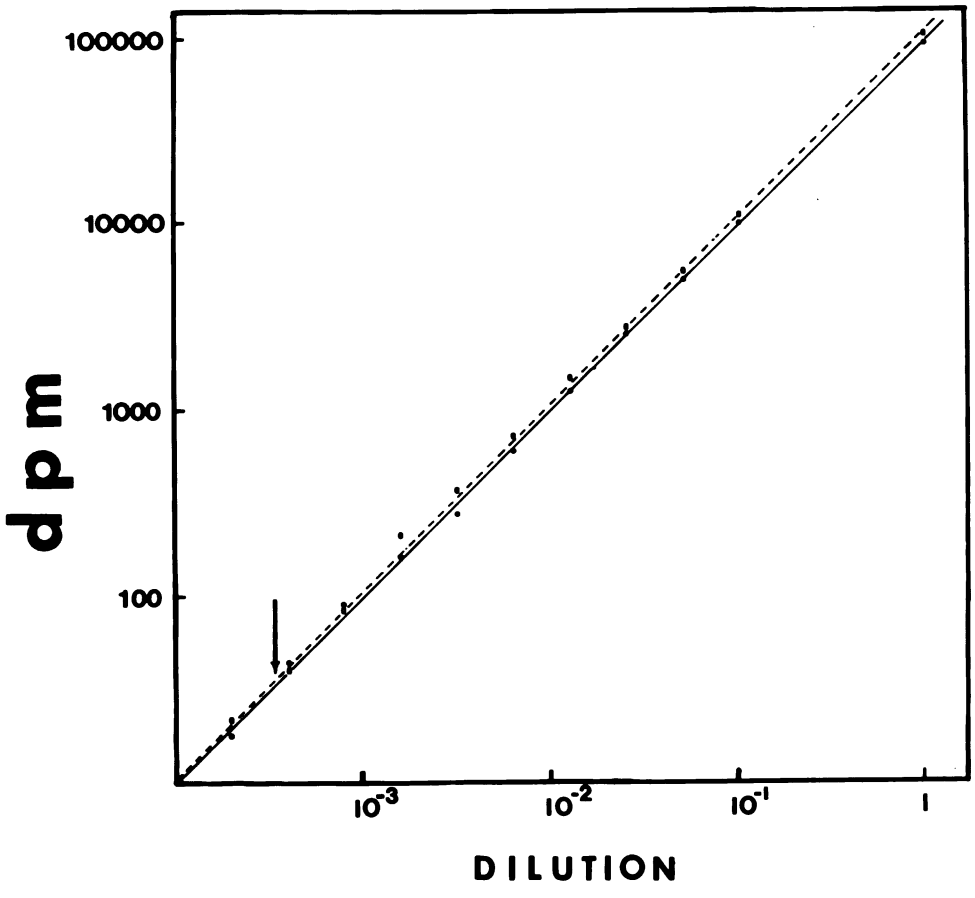


Figure 7:

Serial dilution of [^{14}C]proline standard CFB-71. Count rates were measured before electrophoresis (O-------) and after electrophoresis of the proline spot (●———), by the routine procedure using 30% ethanol extraction and counting without the paper. Means of triplicates of each group are plotted on logarithmic axes. The background of the counter for ^{14}C wide window was 35 cpm. Arrow indicates the statistical detection limit of the method = 2x background.

imino acids, and thus the specific activity of each imino acid can be derived in the same manner. The sensitivity of the method allows analysis of the pool size of different cellular compartments, measurement of enzyme activities of both 4-prolyl-hydroxylase and 3-polyl-hydroxylase simultaneously (Risteli et al., 1977; Tryggvason, 1977), and quantitation of synthesis and degradation of interstitial collagens and basement membranes (Man and Adams, 1975). The problem of counting in a heterogeneous system has been resolved by extraction. The recovery rates are high, 89-96%, and remain linear over a wide range of counts (Fig. 7). This allows investigation of biological samples wherein both high and low counts are expected to occur in the simultaneous determination of proline and the two hydroxyprolines.

Modification of this method may also be applicable to the quantitation of unlabeled imino acids using colorimetry following extraction from the electrophoresis paper.

CHAPTER IV CHARACTERZATION OF COLLAGEN SYNTHESIS BY CULTURED ENDOTHELIAL CELLS

I. INTRODUCTION

As described in the experimental model system in Chapter II, endothelial cells in culture can preserve most, if not all, the differentiated phenotype of their *in vivo* counterparts as long as a mitogenic peptide, FGF, is introduced into the media. One of the major morphological characteristics of this cell culture is that there is a cell surface polarity and that the deposition pattern of ECM is asymmetrical. Since collagen is one of the major components of ECM, characterization of collagen synthesis under such a condition can serve as baseline information toward understanding the collagenous composition and property of the ECM.

In this chapter, collagen synthesis with respect to quantitative and qualitative aspects is analyzed in the cultured endothelial cells in the presence of FGF. This is done in an attempt to understand how cultured endothelial cells make their collagenous matrix, and whether production of the collagenous matrix can serve as a marker for the cell phenotype. The data presented in this chapter concern primarily the characterization of collagen synthesis by corneal endothelial cells, together with a comparison between corneal and vascular endothelial cells. Part of the result of characterization of collagen synthesis by vascular endothelial cells is presented in Chapter V where a comparison of collagen synthesis is made with and without FGF.

II. EXPERIMENTAL PROCEDURES

A. Materials

FGF was purifed from bovine brains as previously described (Gospodarowicz et al., 1978b). Brain FGF yielded a single band on polyacrylamide gel electrophoresis at pH 4.5 on an isoelectric focusing column (total volume 110 ml, pH range 3.4 to 11.0). All the activity focused within an isoelectric range of 9.2 to 9.6. Bovine calf serum, DMEM, type H-16, and glutamine were purchased from Gibco. Tissue culture dishes were from Falcon plastics. L-[2,3,4,5,-^{3}H] proline (80-110 Ci/mmole) was obtained from Amersham, L-ascorbic acid was from Calbiochem. BAPN, PMSF, and PCMB were the products of the Sigma Co. and benzamidine of the Eastman Kodak Co. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were from Whatman. Pepsin A was from Worthington and Aquasol from New England Nuclear.

B. Methods

1. Preparation of collagen standards

Unlabeled collagen standards were prepared as follows:

(a) Type I Collagen was extracted from lathyritic rat skin according to the procedure described by Bornstein and Piez (1966). In brief, newly-weaned Sprague-Dawley rats were put on a BAPN diet (0.3% w/w BAPN in ground rat chow) for 2 to 3 weeks. Subsequent extraction procedures were performed at 0-4°C. After sacrifice, the skin was removed and ground in a meat grinder which was pre-cooled by grinding dry ice through it. The ground skin was first extracted with 1M NaCl, 50 mM Tris/HCl, pH 7.5, with occasional stirring for 2 days. The extraction was then filtered through a laboratory towel. A similar extraction was performed on the residue and the filtrate was combined with

the first one. Celite was added to the combined filtrates to facilitate the filtration through a Buchler funnel with a filter paper. NaCl was added to the filtrate to make a final concentration of 15% (w/v). The pellet was collected by centrifugation (10,000 rpm, 30 min) after stirring gently overnight. The pellet was then dissolved into distilled water in a sufficient amount with stirring, and followed by dialysis versus 1 M NaCl, 50 mM Tris/HCl, pH 7.5, with several changes. The supernatant was collected after centrifugation (12,000 rpm, 30 min). Glacial acetic acid was subsequently added into this supernatant to make a final concentration of 0.5 M, and NaCl was added to a final concentration of 7% (w/v). The pellet was collected by centrifugation (15,000 rpm, 30 min) after stirring gently overnight. The pellet was redissolved in 0.5 M HAc, with gentle stirring. If it was unclear, the insoluble material was removed by filtration and the filtrate was dialyzed against 0.02M Na_2HPO_4, pH 7.4. A cloudy appearance developed. The pellet was collected by centrifugation (15,000 rpm, 30 min), redissolved in 0.5 M HAc dialyzed exhaustively versus 0.1M HAc, and finally lyophilized.

(b) Type II collagen was purified from a Swarm rat chondrosarcoma which was a gift from Dr. George Martin (NIH), according to the procedure described by Smith et al. (1975). In brief, a tumor cell suspension was made by mincing the rat chondrosarcoma in a sterile PBS solution. An aliquot of 1 ml tumor cell suspension was subcutaneously injected bilaterally into the ventral abdominal surface of each newly-weaned Sprague-Dawley rat. They were then put on a BAPN diet as described above for 2 to 3 weeks. A tumor mass of 4 x 5 cm size developed

in 3 to 4 weeks, and was removed by excising carefully from the surrounding host fibrous capsule. All subsequent work was performed at 0-4°C. Tumor masses were minced by polytron at low speed in two volumes of 1 M NaCl, 50 mM Tris/HCl, pH 7.5. The first extraction was gently stirred for 2 days. The extract was removed by spinning out the insoluble materials (12,000 rpm, 30 min). The residue might be re-extracted with the same buffer solution. NaCl was added to the combined supernatant to a final concentration of 20% (w/v). After stirring gently overnight, the pellet was collected by centrifugation (12,000 rpm, 30 min). The pellet was purified repeatedly by dissolving in 20% NaCl, 50 mM Tris/HCl, pH 7.5 and spinning. The clean pellet was dissolved in 0.2 M NaCl, 50 mM Tris/HCl, pH 7.5, and dialyzed versus 0.02 M NaCl, 50 mM Tris/HCl with frequent changes. NaCl was added to make a final concentration of 20% (w/v) and pellet was collected by centrifugation (12,000 rpm, 30 min). The pellet was redissolved in 50 mM Tris/HCl, pH 7.5, and NaCl was added to 0.2 M by the measurement of a conductivity meter. By passage through a DEAE-cellulose column which had been preequilibrated with 0.2 M NaCl, 50mM Tris/HCl, pH 7.5, type II collagen was separated from associated proteoglycans. The unabsorbed fractions were pooled and dialyzed extensively versus 0.02 M Na_2HPO_4, pH 7.5. The flocculated pellet was then collected by centrifugation (12,000 rpm, 30 min), redissolved in 0.5 M HAc, dialyzed versus 0.1 M HAc, and lyophilized.

(c) Type III collagen was extracted from human fetal skin by using the method of limited pepsin digestion described by Byers et al. (1974). In brief, human fetal skin was obtained from fresh human abortus by carefully dissecting skin from the subcutaneous fat tissue. The

dissected skin was stored in -70°C before use. To use, skin was finely minced, extracted overnight with chloroform-methanol (2:1, v/v) and then with methanol for 6 hours at 4°C. The defatted tissue was suspended in 0.5M HAc at a concentration of 20 gm/100 ml. Pepsin A was subsequently added at a concentration of 1 mg/ml (Chung and Miller, 1974). The pH was adjusted to 2.5 by adding concentrated HCl. The mixture was then stirred for 24 hours at 8°C. The pepsin digest was centrifuged (12,000 rpm, 30 min, 4°C), and the skin residues were redigested overnight with additional pepsin at the same concentration, and centrifuged as before. The supernatants were pooled and added with NaCl to a final 0.9 M . Pellet collected by centrifugation (12,000 rpm, 30 min, 4°C) after stirring gently overnight, was redissolved in 50 mM Tris/HCl, pH 7.5 buffer of sufficient volume, and dialyzed extensively versus a solution of 1 M NaCl, 50 mM Tris/HCl, pH 7.5 after several changes. The dialysate was subsequently increased NaCl concentration to a final 1.7 M. The insoluble material collected by centrifugation (12,000 rpm, 30 min, 4°C) was dissolved in 0.5 M HAc, dialyzed versus 0.1 M HAc, and lyophilized. This fraction was denoted crude Type III collagen standard which has been shown to contain more than 60% type III by the criteria of elution profile by CM-cellulose chromatography (See Fig. 4). Further purification was achieved by molecular-sieve chromatography (Bio-Gel A 5m, 100-200 mesh, Bio-Rad Lab.). Fractions corresponding to the elution position for r trimeric form of collagen were pooled and regarded as pure type III collagen.

(d) Type IV collagen was prepared from bovine anterior lens capsule obtained from either Pel-Freeze Co. or a slaughter house.

The procedure used was according to Dehm and Kefalides (1978). In brief, anterior lens capsules were first removed from lenses and cleaned by rinsing several times with PBS. They were then subjected to limited pepsin digestion as described above for the type III collagen preparation. The pepsin digest was added with NaCl to a concentration of 1.8 M. The pellet developed after gentle stirring overnight at 4°C was collected by centrifugation (12,000 rpm, 30 min, 4°C), dissolved in 0.5 M HAc, dialyzed exhaustively versus 0.1 M HAc, and lyophilized.

(e) Type V collagen was isolated from a whole human placenta by the method described by Sage and Bornstein (1979). The detailed procedure has been well described by the above authors.

Each collagen was verified for its purity by SDS-PAGE. The results are shown in Fig. 1. Each band represented a specific type of collagen chain. The chain composition of each collagen standard was further analyzed by CM-cellulose chromatography (see below). The distinctive elution profile of each type of collagen standard is presented in Fig. 2-6 and their elution peaks were further analyzed by SDS-PAGE as shown in the inset picture of each figure respectively. A detailed discussion about their purity and composition, can be seen in the legend of each representative profile (Fig. 2-6). The validity of each collagen standard was confirmed finally by the unique pattern in CNBr-cleaved peptide mapping (Fig. 7).

2. CNBr-cleaved peptide mapping

The technique of CNBr-cleaved peptide mapping (CB peptides) has been used in the study of collagen biochemistry by taking the advantage

of the presence of less than 10 methionine residues per α chain. Since collagens constitute a family of distinct gene products, different CB peptide mappings can be presumably generated for different types of collagen. This technique has been used for identification and quantitation of different types of collagen. In the experiment, the procedure for CNBr cleavage was performed in the following ways.

One mg of collagen standard was initially subject to reduction to decrease the quantity of methionine sulfoxide which will interfere with the later cleavage. The reduction was performed by incubating the above collagen standard solution in 500 ul of 0.02M HAc containing 10% 2-ME (v/v). Reduction proceeded at 37°C for 24 hr. The samples were then diluted 1:5 (to final 2.5ml) with dearated water and lyophilized. CNBr cleavage proceeded by adding CNBr, two to four fold excess in weight compared to the weight of collagen samples, in the form of 10 mg/ml in 70% formic acid, prepared freshly each time. This was then flushed with N_2, and sealed with a screw-cap. The reaction mixture is incubated at 30°C for 4h. The reaction was terminated by adding distilled water 10 to 20 volumes and lyophilized. To remove CNBr and formic acid, additional amounts of distilled water were added and lyophilized again. The generated CB peptides were then analyzed by 15% SDS-PAGE.

3. Cell cultures

Bovine corneal endothelial cell cultures (BCE) were established from steer corneas (Gospodarowicz et al., 1977b). Stock cultures were maintained in DMEM (H-16) supplemented with 10% fetal calf serum and 5% calf serum. Endothelial cells were passaged weekly at a 1:64 split ratio and FGF (100 μg/ml) was added every other day until the cultures became subconfluent.

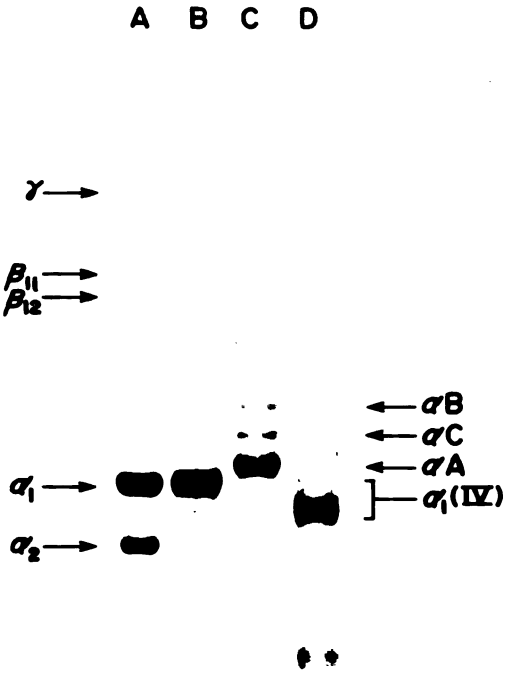


Figure 1:

SDS polyacrylamide gel patterns of (A) type I collagen from lathyritic rat skin, (B) type II collagen from the rat chondrosarcoma, (C) type V collagen from human whole placenta containing A, B, and C chains, and (D) type IV collagen from bovine anterior lens capsule. All collagen standards were prepared as described under Methods, dissolved in the sample buffer, and heated to 60°C for 30 min prior to application to the gel. After electrophoresis, the slab gels were stained with 0.1% Coomassie blue, followed by destaining in acetic acid (7%) and drying between dialysis paper sheets.

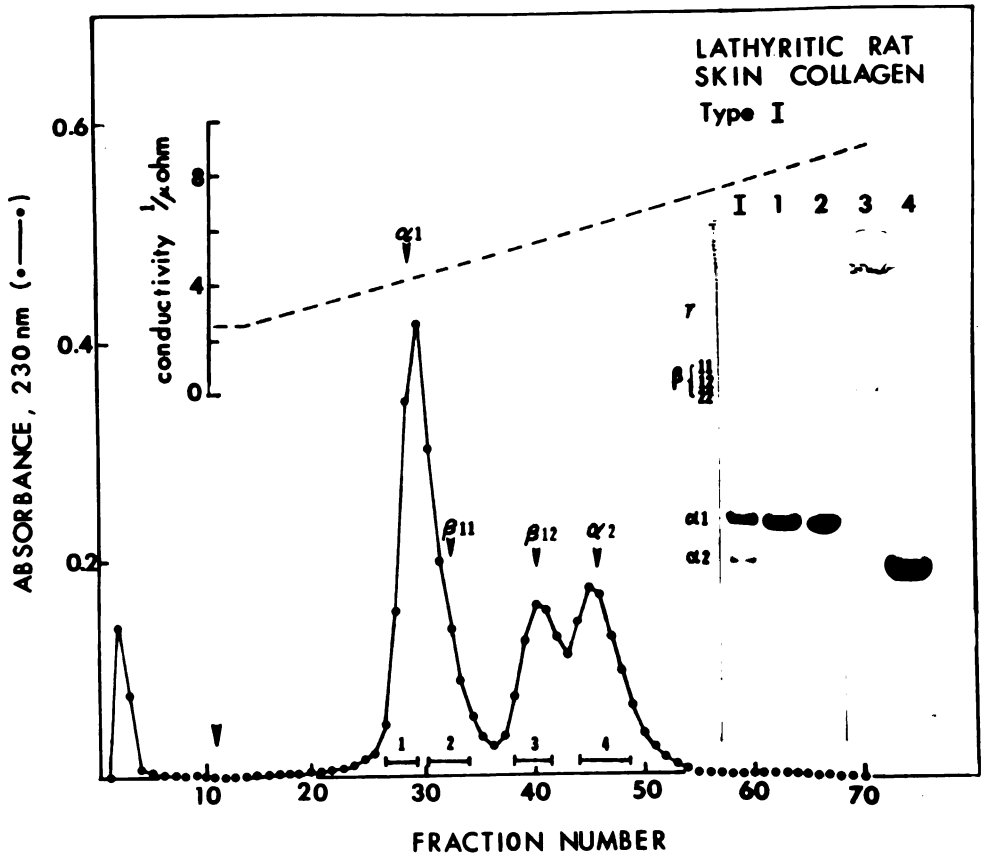


Figure 2:

CM-cellulose chromatogram of type I collagen standard purified from rat skin after BAPN treatment as described in the Methods. The standard, about 5 mg, was chromatographed according to the procedure described in the Method and yielded three distinctive elution peaks by NaCl gradient. The fraction pooled as indicated was further identified by SDS-PAGE (see inset picture). The purify as type I collagen was confirmed. The elution position of each different chain was indicated by an arrow.

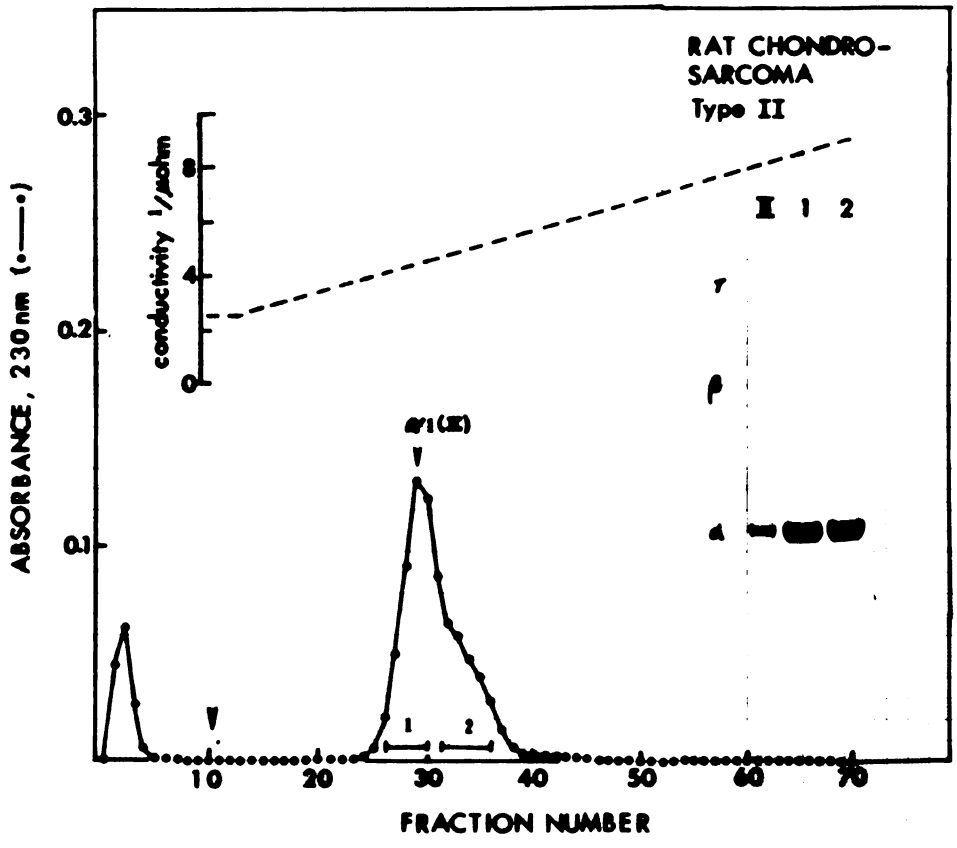


Figure 3:

CM-cellulose chromatogram of type II collagen standard purified from a rat chondrosarcoma as described in Methods. The chromatography was performed with 5 mg material according to the procedure described in the Method and yielded a single elution peak corresponding to that of α1(I) and a shoulder, but distinctive from that of type I collagen (Fig. 2). .The pooled fraction as indicated was further identified by SDS-SPAGE (see inset picture). The purify as type II collagen was then confirmed.

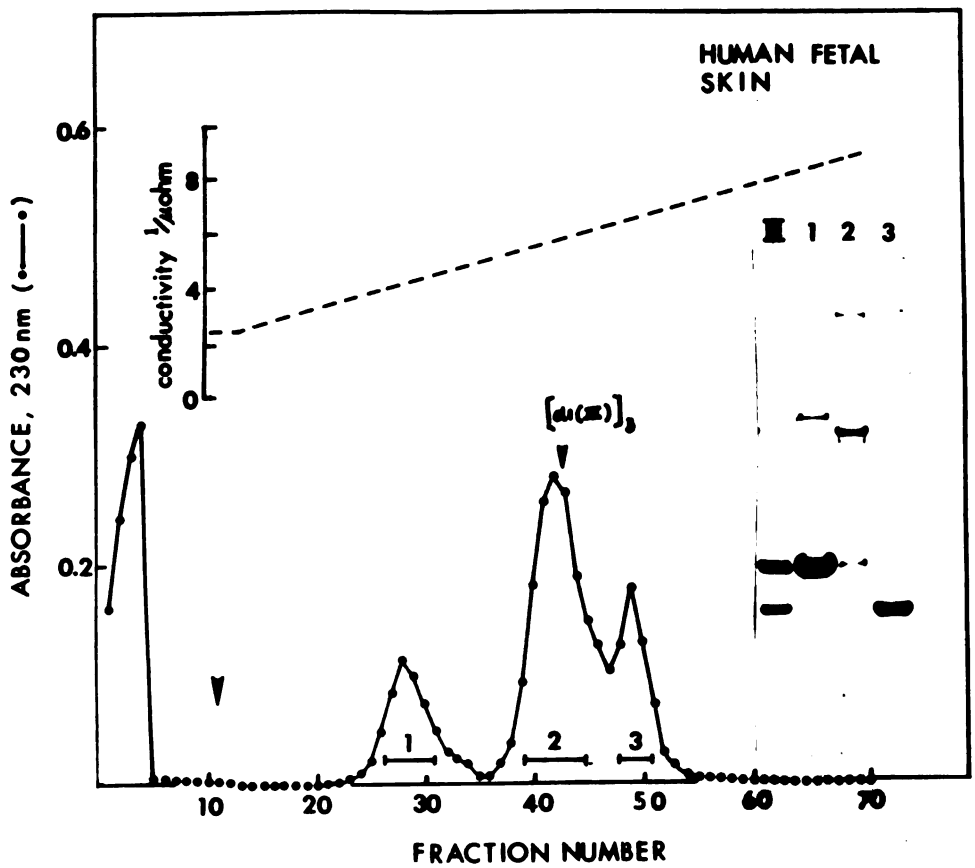


Figure 4:

CM-cellulose chromatogram of human fetal skin collagen extracted by limited pepsin digestion and neutral salt precipitation at 1.7 M NaCl as described in Methods. The chromatography was performed according to the procedure described in the Method, and yielded an elution profile similar to that of type I standard (see Fig. 2), instead a prominent second peak is noted. The pooled fraction as indicated by the underlying bar was further studied by SDS-PAGE (see inset picture). The existence of type III collagen was identified by an arrow.

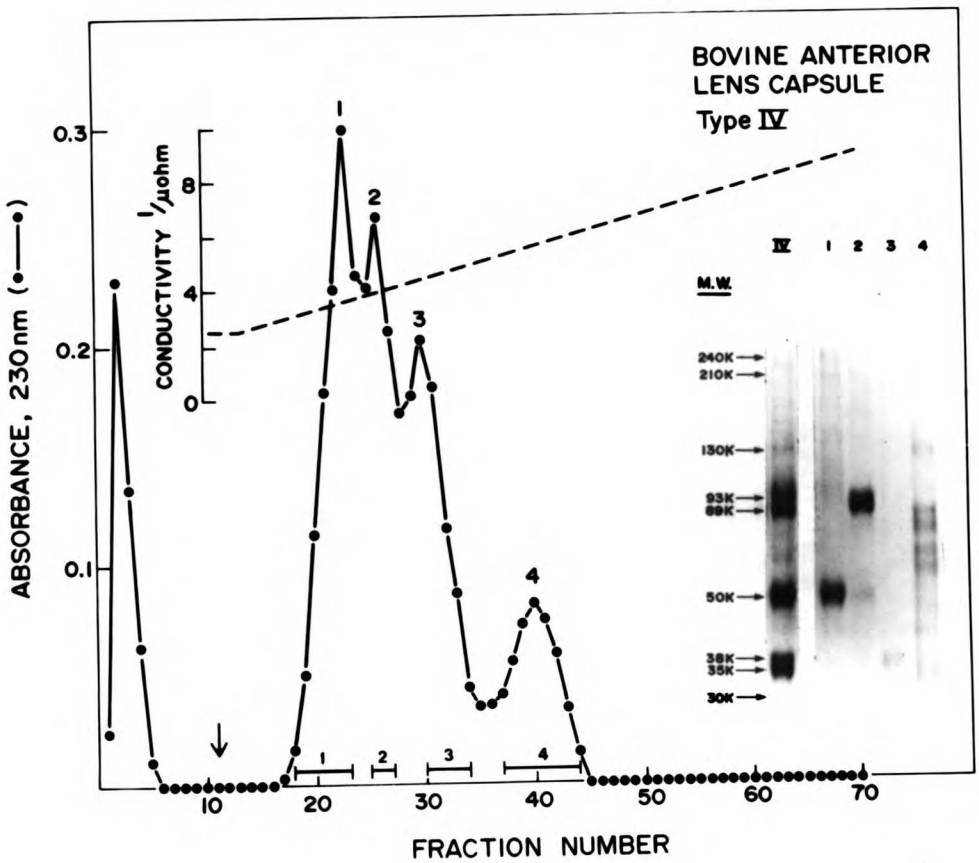


Figure 5:

CM-cellulose chromatogram of type IV collagen standard prepared from bovine anterior lens capsule. The chromatography was performed according to the procedure described in the Method, and yield a unique elution profile with four identifiable peaks. The first three peaks were of the similar position to that of α1(I) chain (Fig. 2). The pooled fractions were further analyzed by SDS-PAGE. It indicated that each elution peak corresponded to one of the several bands of type IV collagen.

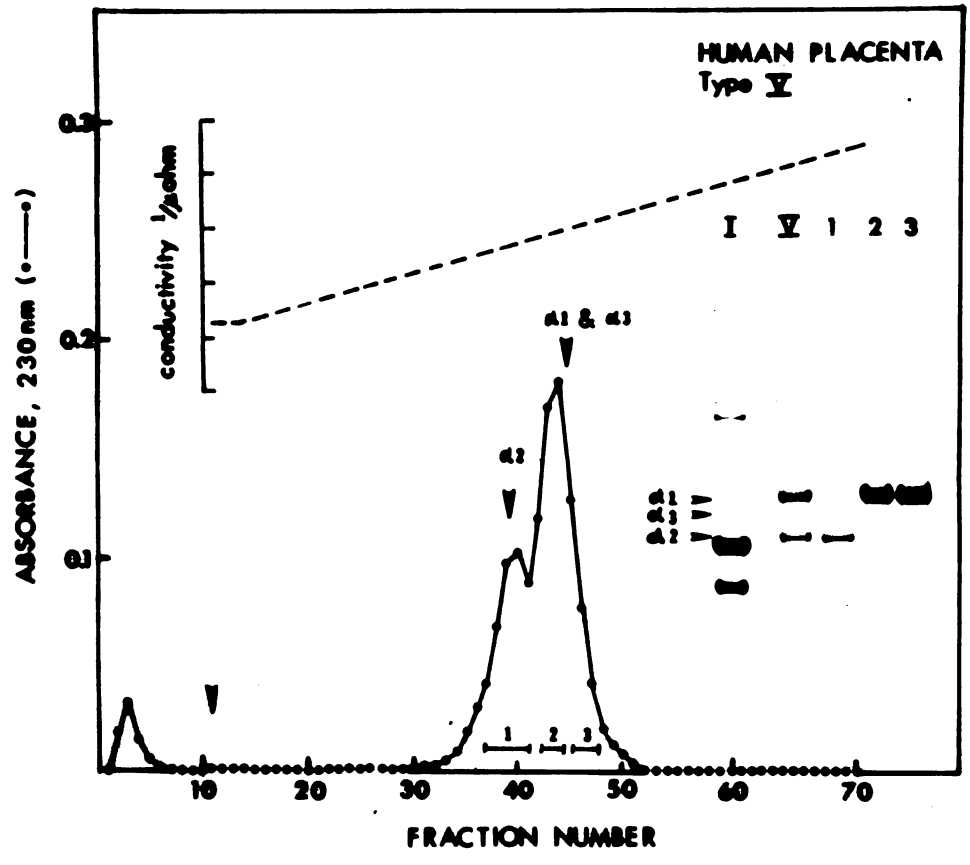


Figure 6:

CM-cellulose chromatogram of type V collagen standard prepared from human whole placenta. The chromatography was performed according to the procedure described in the Methods and yield a distinctive elution profile with two peaks in the position between that of α1(I) and α2(I) (Fig. 2). The pooled fraction was further identified by SDS-PAGE (see inset picture) and the purity was confirmed.

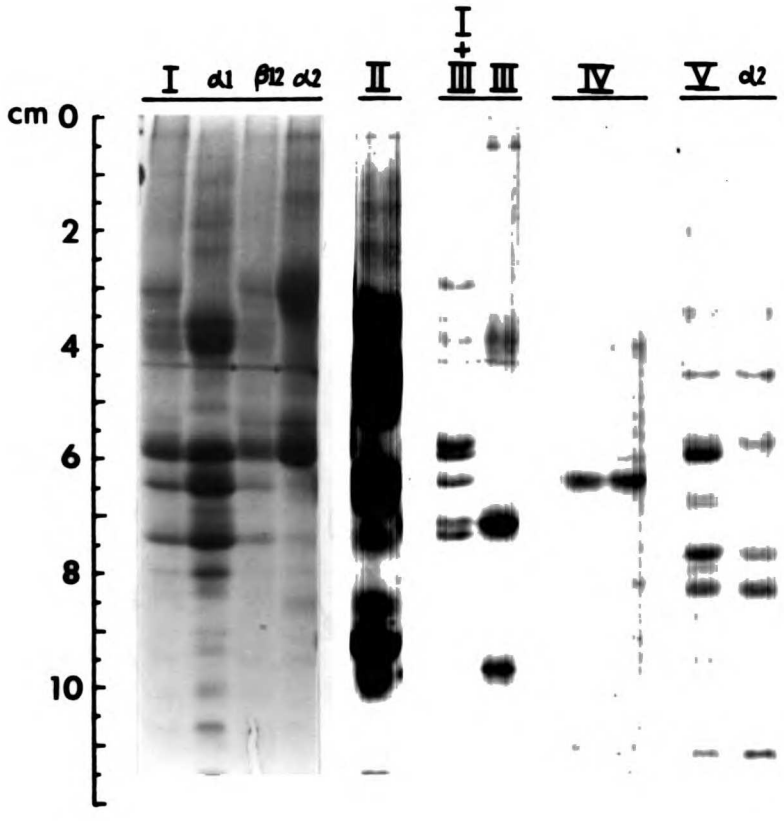


Figure 7:

CNBr peptide mapping of collagen standards after fractionation by SDS-PAGE on a 15% gel. Each gel was identified by a Roman numeral as indicated. The CNBr peptides (CB fragments) and their relative mobility were as follows:

α1(I)		α1(II)	
CB2-4-5-8-3	37-42 mm	CB10-5 (31,000)	43-48 mm
CB7 (24,000)	58-61 mm	CB11 (25,000)	63-68 mm
CB8 (24,000)	65-66 mm	CB8 (13,600)	85-88 mm
CB6 (18,000)	74-76 mm	CB9 (10,800)	92-95 mm
CB3 (13,600)	80-81 mm	CB7-12 (8,000)	98-100 mm
α2(I)		α1(III)	
CB3-5 (29,000&29,500)	28-32 mm	CB5-9 (20,000&22,000)	40-43 mm
CB4 (31,000)	58-62 mm	CB8-3 (10,600&7,050)	73-74 mm
		CB6 (8,200)	96-98 mm

Bovine vascular endothelial cells (ABAE) derived from aortic arch were routinely cultured in DMEM (H-16) supplemented with 10% bovine calf serum, 2.5 µg/ml Gentamycin. FGF (100 µg/ml) was added every other day until the cultures became confluent. Cell stocks were passaged weekly at a split ratio of 1:64.

4. Metabolic labeling

Subconfluent cultures of bovine corenal or vascular endothelial cells were preincubated for 24 h in glutamine-free DMEM (H-16) supplemented with 10% bovine calf serum and ascorbic acid (25 µg/ml). The cultures were then exposed (24 h, 37°C) to ascorbic acid (25 µg/ml) and L-[2,3,4,5-^{3}H] proline (40 µCi/ml). BAPN (80 µg/ml) was added to prevent collagen cross-linking. After 24 h, the medium containing the secreted procollagen was removed and the cultures were washed three times with cold (4°C) PBS. The plates were then stored at -70°C. Culture medium and the first wash were then combined. To inhibit further proteolysis, Tris-HCl (pH 8.0), EDTA, PMSF, PCMB, and benzamidine were added at a final concentration of 50, 20, 0.1, 1, and 0.1 mM respectively. The media were then centrifuged (800 xg, 10 min) to remove cell debris and the supernatants were stored at -70°C.

5. Quantitation of collagen synthesis by the determination of radiolabeled proline , 4-hydroxyproline ,and 3-hydroxyproline

To quantitate the radiolabeled proline and two isomers of hydroxyproline, 3-hydroxyproline and 4-hydroxyproline, the samples of media and the cell layers were processed as follows. The media were dialyzed stepwise against 1 M NaCl, 0.2 M NaCl, and distilled water to remove the free radiolabeled proline. The cell layers which contained the cells

and the ECM were scraped into a hypotonic solution containing 10mM Tris/HCl, 10mM NaCl, 3mM $MgCl_2$, 0.5% Triton X-100, pH 7.5, homogenized, and dialyzed in the same fashion as described for the media. After dialysis, both samples were lyophilized and hydrolyzed with 6 N HCl, at 110°C, under vacuum for 24 h. The hydrolysates were then evaporated to dryness with air, reconstituted with distilled water, and filtered (Millipore, 0.45μ). Aliquots were taken for the determination of total incorporation, and the remainder was subjected to Dowex 50W-X8 cationic exchange chromatography to quantitate radiolabeled proline and hydroxyproline (Cutroneo et al., 1972). An example of a profile showing the separation and resolution of proline and hydroxyproline is given in Fig. 8. The recovery rate exceeded 97% for both, with the use of radiolabeled proline and hydroxyproline standards respectively. To separate and quantitate the two isomers of hydroxyproline, samples were applied to high-voltage paper electrophoresis, as described in Chapter III. A representative profile showing the resolution and quantitation of proline, 4-hydroxyproline, and 3-hydroxyproline is given in Fig. 9. The ratios of both [^{3}H] hydroxyproline/[^{3}H]proline and [^{3}H] 3-hydroxyproline/total [^{3}H] hydroxyproline are presented as such without correction for the loss of radioactivity which occurs when proline residues become hydroxylated.

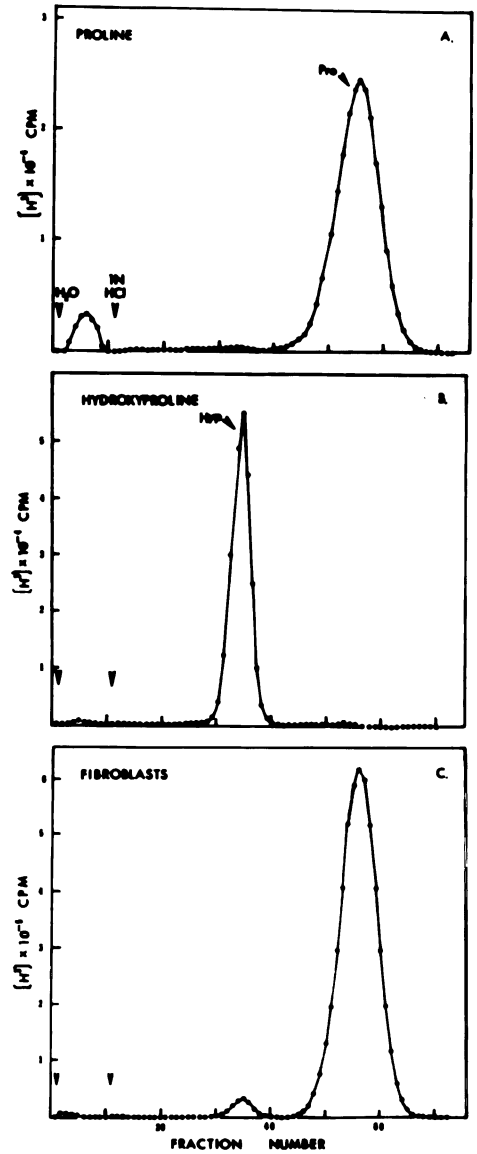


Figure 8:

Dowex 50W-X8 cationic chromatograms of (A) radioactive proline standard (L-Proline [2,3-^{3}H], specific activity 22.7 Ci/mmole, 60 NCS from Amersham) (B) radioactive hydroxyproline standard (L-4-[^{3}H(G)] hydroxyproline, specific activity 5.45 Ci/mmole) (C) acid hydrolysate of the media of human foreskin fibroblasts labeled with radioactive proline. The chromatography was run on a column (0.7 x 7 cm) preequilibrated with triply distilled water with a flow rate 2.5 ml/10 min and fraction of 1.25 ml was collected. It was first eluted with triply distilled water for 10 tubes and changed to 1 N HCl for another 60 tubes. Hydroxyproline was eluted between the 30th and 40th tubes and proline was subsequently eluted between the 50th and 60th tubes.

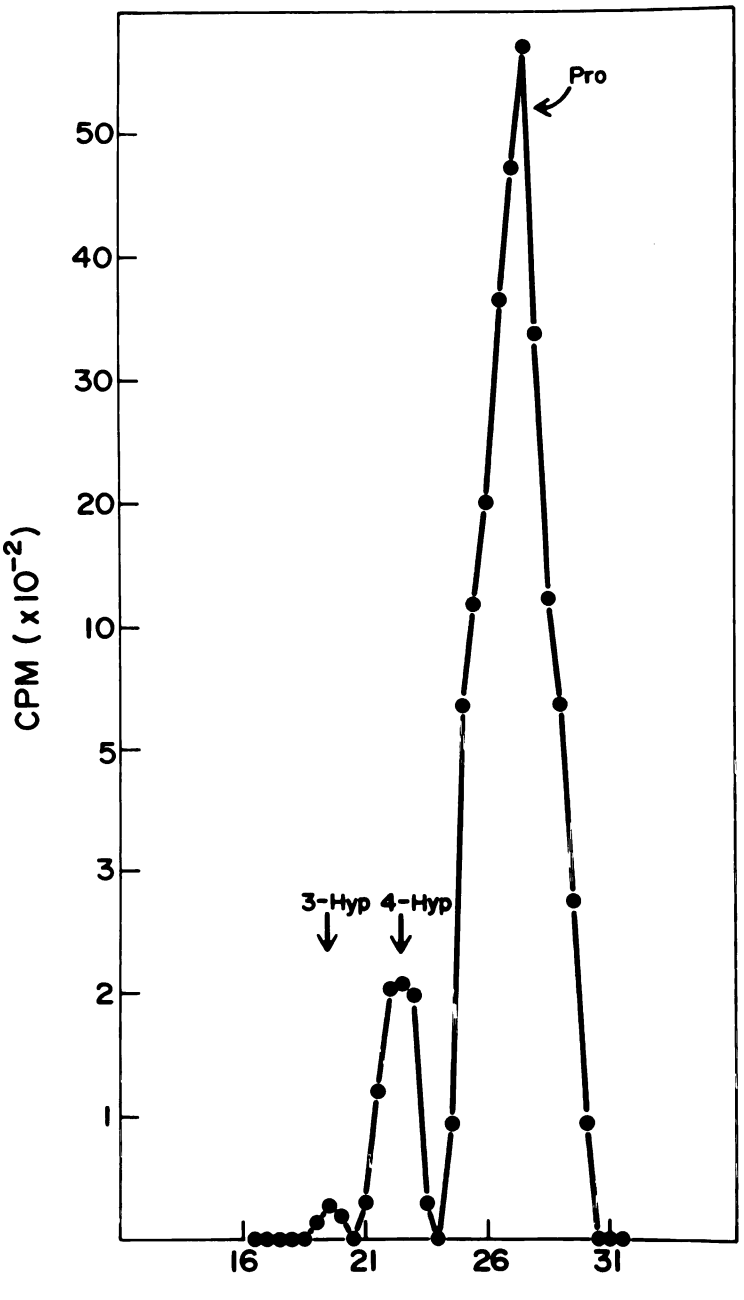


Figure 9:

Medium of adult bovine aortic endothelial cells labeled with [^{3}H] proline for 24 h. The hydrolysate was run as described under Methods. Strips of 0.5 cm width were cut out and extracted for counting. CPM was reported here without further correction. The ratio of total hydroxyproline to proline was 2.25%, close to that obtained by column chromatographic techqniue (Cutroneo et al., 1972) which is 2.17%. The ratio of 3-hydroxyproline to total hydroxyproline was 11.5% which again was close to the result of 10.2 ± 1.4% observed by Howard et al. (1976) using the amino acid analyzer. It demonstrated definitively that there was no different in recovery rates of these three imino acids, and thus the specific activity of each imino acid can be derived in the same manner.

6. DEAE-cellulose chromatography

To separate and quantitate procollagens which were secreted into the media, DEAE-cellulose chromatography was performed as described (Smith et al., 1972; Burke et al., 1977). After labeling for 24 h with [^{3}H] proline in the presence of ascorbic acid and BAPN as described above, media were dialyzed at 4°C, against 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5 containing 20 mM EDTA, 1mM PMSF, 1mM PCMB, and 1mM benzamidine, to remove the traces of free radiolabeled amino acid and to prevent conversion of procollagen to collagen. Saturated ammonium sulfate (100% w/v) was added to make the final concentration 30% (v/v). The resulting suspension was then stirred gently overnight at 4°C in the presence of 3 mg of unlabled carrier, lathyritic rat skin collagen. Precipitates were collected by centrifugation (7000 xg, 30 min, 4°C), dissolved in the initial buffer, 2 M urea, 50 mM Tris-HCl, pH 7.5, and dialyzed overnight (4°C) against the same solution. Prior to chromatography, any insoluble material was removed by filtering through cotton. Samples were applied to a column of DEAE-cellulose (1.5 x 4 cm) and washed with 25 ml of the initial buffer. Bound proteins were eluted using a linear gradient (0 to 0.2M) of NaCl (total volume of 160 ml, flow-rate 6.2 ml/h). Fractions of 2.45 ml were collected, of which a 0.3 ml aliquot was removed for counting. Three ml Aquasol was added and samples were counted in a Beckman Counter LS 8000, with a counting efficiency of 17.6% for the tritiated material.

7. Pepsin digestion and salt fractionation

The culture medium and the cell layer were treated with pepsin separately. Ammonium sulfate (30% v/v) was added to the media. Following centrifugation, the pellets were dissolved and dialyzed (24 h, 4°C)

against 0.5 N HAc and then lyophilized. The lyophilized samples were further dissolved in 0.5 N HAc containing pepsin A (100 ug/ml).

The cell layers together with the ECM were suspended in 0.5 N HAc and homogenized. Pepsin A (100 µg/ml) was then added. Both samples (media and cell layers) were digested separately for 24 h at 4°C and the reaction was then stopped by neutralization with NaOH to pH 8.5. Both solutions were dialyzed against 1 M NaCl, 50 mM Tris-HCl, pH 7.5, and collagens were precipitated stepwise by the slow addition of NaCl to a final concentration of 1.7, 2.6, and 4.5 M respectively (Chung and Miller, 1974) together with collagen standards as indicated. The developing precipitates were stirred overnight (4°C) and collected by centrifugation (7000 xg, 4°C, 30 min). Each fractionated precipitate was then dissolved in 0.5 N HAc dialyzed against 0.1 N HAc and lyophilized.

8. CM-cellulose chromatography

CM-cellulose column chromatography was performed as described previously (Epstein et al., 1971). Radioactive samples from each salt fractionation were dissolved in 40 mM sodium acetate, 2 M urea, pH 4.8, the initial buffer. Lathyritic rat skin collagen, containing primarily type I, was added (2-5 mg) as an internal standard and carrier. For certain samples, type III collagen prepared from fetal skin, or type V (A, B, C chains) from placenta was added. Prior to chrmatography, samples were heat-denatured at 42°C, for 30 min, and the insoluble material was removed by centrifugation (1,000 xg, 10 min) at room temperature. Aliquots were taken for determination of the total radioactivity in each salt fractionation. Samples were then applied to a 0.7 X 10 cm jacketed CM-cellulose column, pre-equilibrated with the dearated initial

buffer at 42°C, and washed with 25 ml of the initial buffer. Bound proteins were eluted using a linear gradient (0 to 0.1 M) of NaCl (total volume, 150 ml, flow-rate 37 ml/h). Fractions of 2.5 ml were collected. Absorbance at 230 nm was detected by a Gilford spectrophotometer, model 252-1, for the unlabeled carrier collagen. Aliquots of labeled material were counted by a Beckman LS 8000 scintillation counter with a counting efficiency of 28.8% for tritiated material. Conductivities were measured at room temperature. The recovery rate falls in the range of 65-75% for both radiolabeled and unlabeled samples.

9. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gel electrophoresis was performed using the method of Neville (1971), a discontinuous system with a 3% stacking gel and 7% separating gel.

10. DNA quantitation

DNA determinations were done as described by Burton (1956).

11. Cell counting

The cell number was obtained by trypsinizing triplicate cultures and counting cells in a Coulter counter.

III. RESULTS

A. Quantitative Study of Collagen Synthesis

Both bovine corneal endothelial subconfluent cell cultures (BCE) and bovine aortic endothelial subconfluent cell cultures (ABAE) were labeled with [^{3}H] proline for 24 h. As shown in Table 1, first column, in BCE culture, the amount of [^{3}H] proline incorporated into proteins present in the cell layer was nearly twice that secreted into the medium (data for BCE culture are presented in the upper portion of the bar). However, in ABAE culture, the [^{3}H] proline incorporation was about the same for both the medium and the cell layer (data for ABAE culture are presented in the lower portion of the bar). When the radioactivity recovered in the form of hydroxyproline was analayzed, the amount in the medium of BCE culture was 2.5 times (1.76×10^5 cpm per 10^6 cells) that recovered in the cell layer (6.8×10^4 cpm per 10^6 cells, Table 1, third column). This indicated more collagenous materials were elaborated into the medium than the cell layer. In ABAE culture, a smaller amount of hydroxyproline was recovered in both the medium and the cell layer compared to BCE culture. It was 6.1×10^5 cpm per 10^6 cells for the medium of ABAE culture, which was 5 times that of the cell layer, 1.2×10^5 cpm per 10^6 cells. This was attributed primarily to a higher ratio of hydroxyproline to total incorporation (Table 1, second column) indicating a higher amount of collagen made as a proportion of total protein synthesis by BCE culture compared to ABAE culture (Table 1, fourth column). By calculation (Green and Goldberg,1963), 2.17% and 0.47% of total protein synthesis was devoted to collagen in the medium and in the cell layer of BCE culture respectively, and about 1% (1.08%) of total newly-synthesized protein synthesis was devoted to collagen synthesis.

Table 1

$[^3H]$ PRO INCORPORATION AND COLLAGEN ACCUMULATION

BY SUBCONFLUENT BOVINE CORNEAL AND VASCULAR ENDOTHELIAL CELLS

BCE / ABAE	Total Incorporation $[^3H]$ cpm X 10^{-5} / 10^6 cells	Hydroxproline / Total Incorporation %	Hydroxyproline $[^3H]$ cpm X 10^{-4} / 10^6 cells	C/P[a] %	3-Hydroxyproline / Total Hydroxyproline %
Medium	27.3 / 28.0	6.45 / 2.17	17.6 / 6.1	2.17 / 0.75	6.7 / 11.5
Cell layer[c]	48.5 / 23.3	1.41 / 0.52	6.8 / 1.2	0.47 / 0.18	8.0 / 24.7
Total	75.8 / 51.3	3.20 / 1.42	24.4 / 7.3	1.08 / 0.48	7.1 / 13.7

NOTE: a) C/P indicates the ratio of collagen to total protein. It is calculated assuming that the hydroxyproline residue content of collagen is 12.2% and that the average proline content in total cellular is 4.1% (Green and Goldberg 1963).

b) The results presented here are the mean of four different measurements. Data for corneal endothelial cells (BCE) and vascular endothelial cells (ABAE) are presented as BCE/ABAE.

c) The cell layer denotes the cells and their ECM.

In ABAE culture the capacity was found to be 0.48%, one half of that of BCE culture. The media and cell layers were further analyzed for the distribution of the two hydroxyproline isomers, 3-and 4-hydroxyproline, markers for basement membrane collagen and interstitial collagen, respectively (Man and Adams, 1975). For both BCE and ABAE cultures, 3-hydroxyproline was found to be accumulated in greater amounts in the cell layer, reflecting the preferential association of basement membrane collagen with the cell layer (Table 1, fifth column).

B. Qualitative Study of Collagen Synthesis

1. DEAE-cellulose chromatography of medium proteins

The types of procollagens secreted into the culture media were analyzed. Media proteins precipitated by 30% ammonium sulfate were chromatographed on a DEAE-cellulose column. Chromatography of the BCE medium gave an elution profile (Fig. 10A) composed of two major peaks (denoted 1,3) and three minor peaks or regions (denoted 2,4 and 5). The first major peak, which was eluted in the initial wash, represented an unabsorbed fraction. The other major peak was eluted during the course of NaCl gradient between fractions 42-50 (peak 3). The hydroxyproline contents of these 5 peaks or regions, 23% for peak 1, 9% for peak 2, 16% for peak 3, 6% for region 4, and 3% for region 5, indicated that all contained collagenous materials. Further identification of each peak or region was made using human skin fibroblast procollagens as markers (Fig. 10B). By comparing elution profiles (Fig. 10 A & B), peak 2 was identified as procollagen type I and peak 3 as procollagen type III. Procollagen type IV is reported to elute from the DEAE-cellulose column in the initial wash (Crouch and Bornstein, 1978; Liotta et al., 1979) corresponding to peak 1. That peak 1 contained procollagen type IV was

also suggested by the higher content of 3-hydroxyproline. Regions 4 and 5 were tentatively identified as the precursors of type V collagen. The ratio between procollagen type III and procollagen type I was nearly 50:1. Thus, type III procollagen was the principal collagen secreted and accumulated into the media by BCE cells. When the same technique was applied to the medium protein of ABAE cultures, a similar elution profile was obtained (results are shown in the Fig. 1 of Chapter V). Procollagen type III was also the predominant collagen secreted into the medium by ABAE culture. There was no detectable amount of procollagen type I, whereas a higher peak corresponding to peak 4 or 5 of BCE culture was noted. All these results strongly suggested that type III collagen was the predominant type made by both corneal and vascular endothelial cells. These profiles were distinct from that obtained from fibroblasts, the major cell type of mesenchymal origin .

2. CM-cellulose chromatography of pepsin-digested proteins from the media and cell layers

To quantitate the collagen types produced by BCE cultures, samples from media and cell layers were analyzed after pepsin digestion under acidic conditions, followed by salt fractionation. NaCl was added stepwise to a final concentration of 1.7, 2.6, and 4.5 M under neutral conditions. Each salt concentration precipitates preferentially certain type-specific collagens (Sage and Bornstein, 1979; Epstein et al., 1971; Kresina and Miller, 1979). Each salt fractionate was analyzed by CM-cellulose chromatography. Acidic non-collagenous moieties are eluted in the initial wash, while different collagen chains are resolved by NaCl gradient elution under denaturing conditions. When the 1.7M NaCl fractions of both the media and the cell layers were analyzed, the

elution profiles were the same (Fig. 11A and B). Only type III collagen $[\alpha 1(III)]_3$ was observed. A prominent peak was observed which eluted prior to the elution position of α2 chain (Fig. 11A). This peak co-eluted with type III collagen. This carrier, prepared from the 1.7 M NaCl precipitate of human fetal skin collagen, contained 60% type III collagen as documented by both SDS-PAGE gel and CM-Cellulose chromatography (Fig.4). A minor peak co-eluting with the α1 position was also detected. When 2.6 M NaCl fractions were analyzed from both the media and the cell layers, α1(I) and α2 of type I collagen were detected and were observed to co-elute with the type I standard carrier. However, less α1 and α2 were observed in the media than in the cell layers (Fig. 12A & B). This reflected increased rates of processing of type I collagen, as described previously by Goldberg (1977).

The 4.5 M NaCl fractions of both samples were also analyzed, fractions containing primarily type IV and type V collagens (Sage and Bornstein, 1979; Kresina and Miller, 1979). Both cell layers and media yielded an elution profile with two major peaks which emerged between the elution positions of α1(I) and α2 chain of type I collagen (Fig. 13 A and B). This elution profile was similar to that of type V collagen, as shown when purified A, B, and C chains from a placental extract were cochromatographed with media samples (Fig. 13A). The first peak was identified as the A chain and the second peak as the B, and possibly C chains, which co-elute and cannot be resolved from each other by this method (Fig. 6). The shoulder of the A chain peak and the minor peak preceding the α1(I) elution position was tentatively identified as α1(IV), type IV collagen, compared to the elution profile of a type IV collagen standard (see Fig. 5 and Dehm and Kefalides, 1978). When the

4.5 M NaCl supernatant fraction was analyzed, no peaks could be detected, demonstrating that all collagenous materials had been precipitated by 4.5 M NaCl (results not shown).

The same technique was then employed in ABAE culture. The results are presented in Chapter V. In brief, type III collagen was found to be the major collagen made by ABAE culture and there was no detectable amount of type I collagen.

By integrating the peaks for the different types of collagen and combining the results of medium and cell layer, it was found that BCE cells synthesized collagen types I and III and basement membrane collagens at a ratio of 3:16:1. Nevertheless, ABAE cells synthesized negligible amount of type I collagen and the ratio of type III collagen to basement membrane collagens was 3:1 (Table 2, A and B).

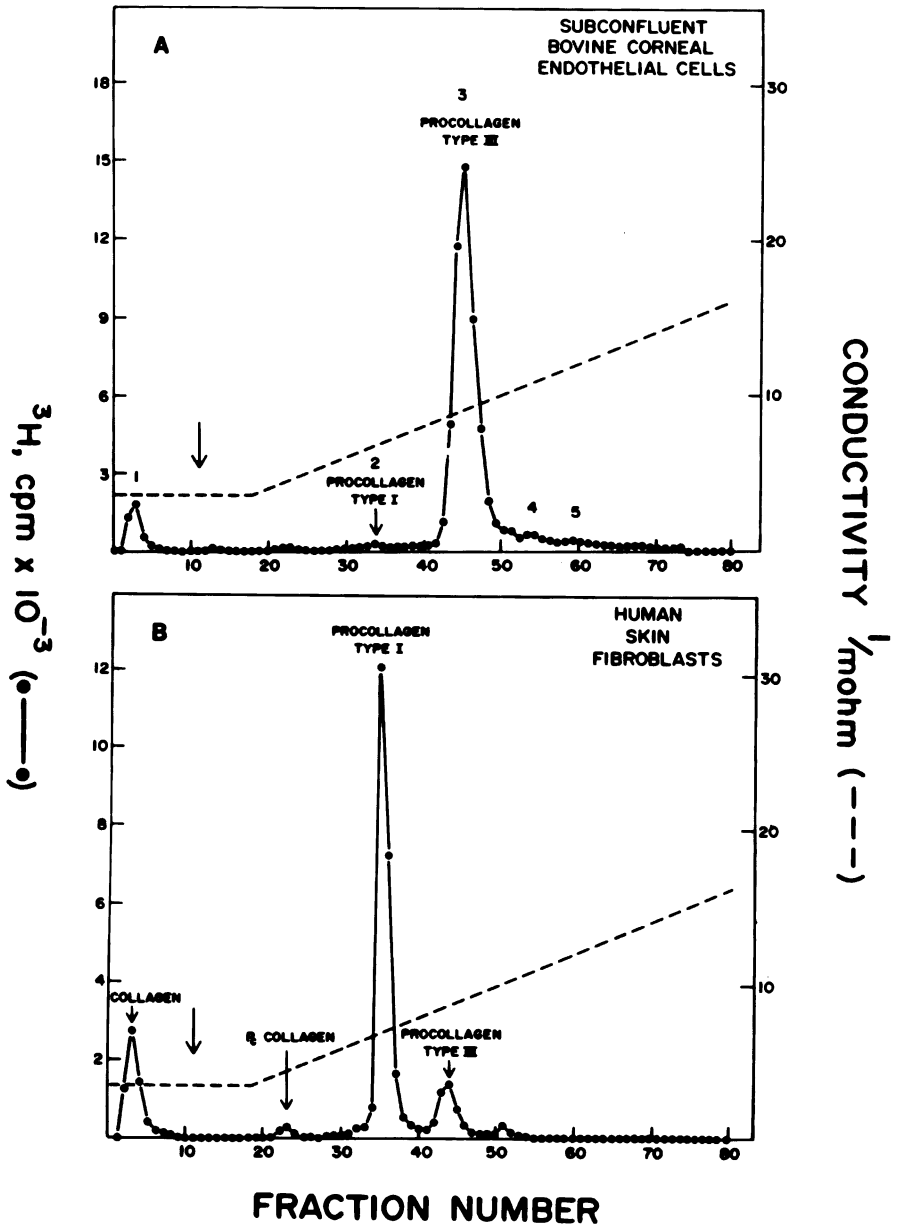


Figure 10:

DEAE-cellulose chromatograms of [^{3}H]proline-labeled medium proteins of (A) BCE cells and (B) human skin fibroblasts. Samples were processed and chromatographed as described under Materials and Methods. Arrows indicate start of NaCl gradient. Conductivities were measured at room temperature. Peak 1 was the unabsorbed fraction containing collagens and procollagen type IV. Peaks 2 and 3 corresponded to procollagen types I and III respectively. Peaks 4 and 5 were tentatively indentified as precursors for type V collagen.

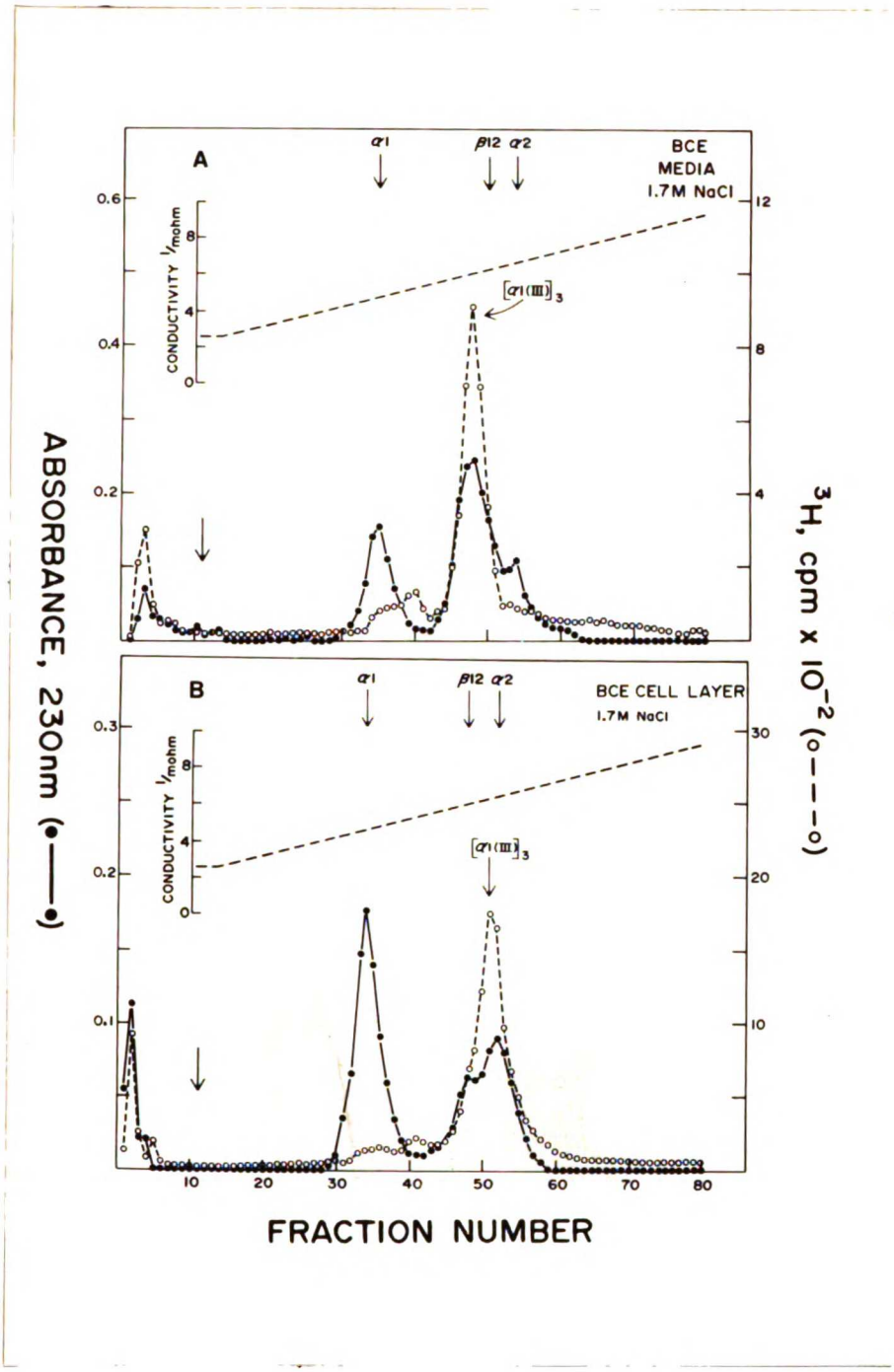


Figure 11:

CM-cellulose chromatograms of pepsin-digested [^{3}H]proline-labeled proteins in the media (A) and the cell layers (B) of BCE cells which were precipitated at 1.7 M NaCl under neutral conditions. The samples were processed and chromatographed as described under Materials and Methods. For the media (A), 1.7 M NaCl precipitate of human fetal skin, which contained 60% type III collagen, was cochromatographed as an internal standard and carrier. For the cell layers (B) lathyritic rat skin collagen, most type I, was employed. Both media and cell layers exhibited the same elution profile with a prominent peak of type III $[\alpha 1(III)]_3$ collagen. Arrows indicate the start of the NaCl gradient.

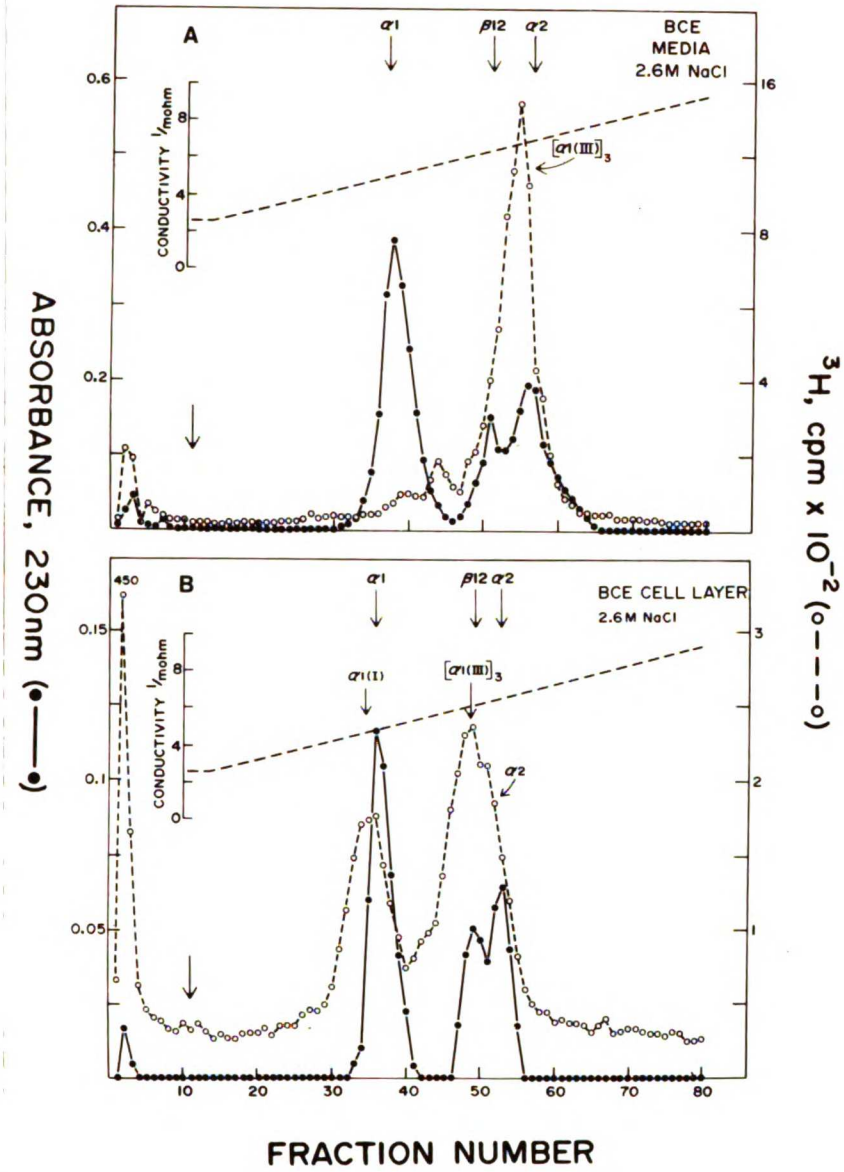


Figure 12:

CM-cellulose chromatograms of pepsin-digested [^{3}H]proline-labeled proteins in the media (A) and the cell layers (B) of BCE cells which were precipitated at 2.6 M NaCl under neutral conditions. Both samples were processed and chromatographed as described under Materials and Methods. Lathyritic rat skin collagen, mainly type I, was cochromatographed in both cases. A distinct peak coeluted with the α1(I) standard was readily observed in the profile of the cell layer (B). However, the media (A) contained primarily type III collagen $[\alpha 1(III)]_3$. Arrows indicate the start of the gradient.

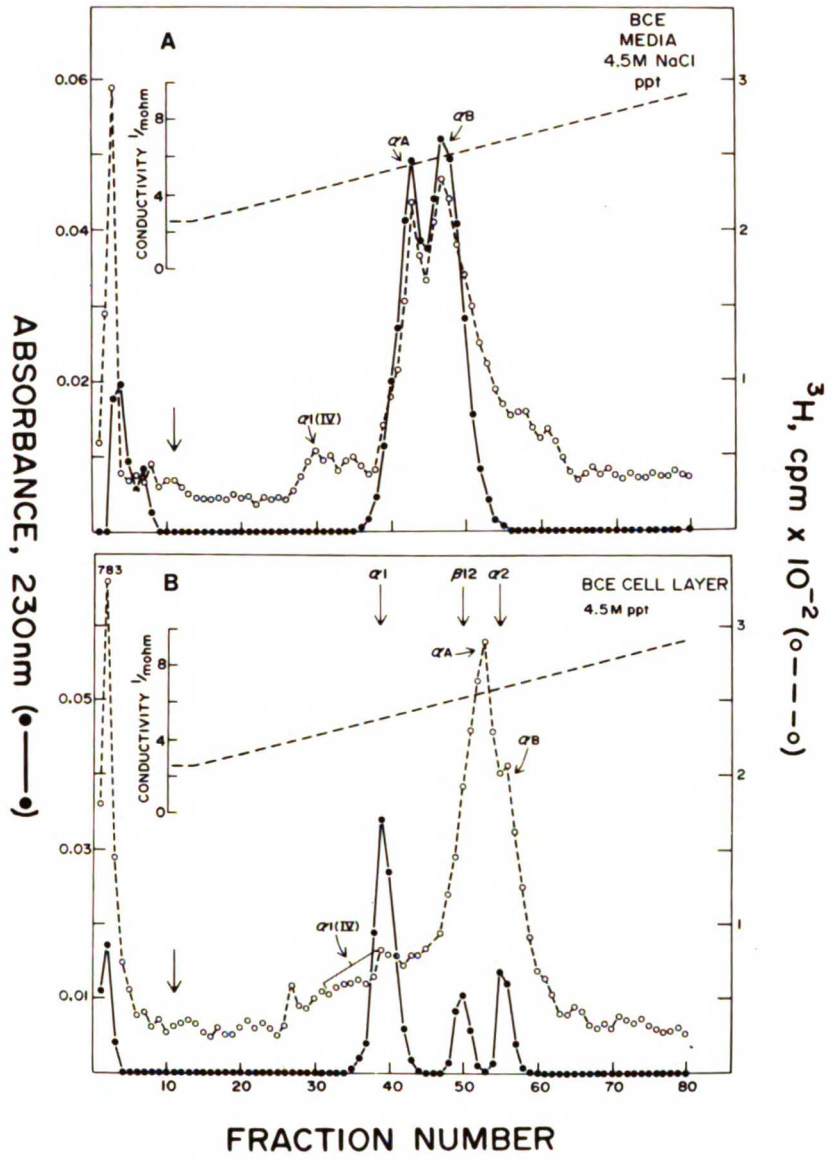


Figure 13:

CM-cellulose chromatograms of pepsin-digested [^{3}H]proline-labeled proteins in the media (A) and the cell layers (B) of BCE cells which were precipitated at 4.5 M NaCl under neutral conditions. The samples were processed and chromatographed as described under Methods. For the media (A), placenta extracts containing A, B, C, chains were cochromatographed as an internal standard and carrier. For the cell layers (B), however, lathyritic rat skin collagen, containing primarily type I, was cochromatographed. Both the media and the cell layers exhibited the same profile with two major peaks which eluted between α1(I) and α2 and represented the A and B chains respectivly. A minor peak eluting prior to α1(I) was identified as α1(IV). Arrows indicate the start of the NaCl gradient.

Table 2

RATIOS OF DIFFERENT COLLAGEN TYPES

SYNTHESIZED BY BOVINE CORNEAL ENDOTHELIAL

CELLS AND VASCULAR ENDOTHELIAL CELLS

	I	III	IV	V
(A) BCE CELLS				
Total radioactivity (cpm X 10^{-3})[a]	29.8	177.3	3.5	3.9
Ratio[b]	2.6	15.7	1	
(B) ABAE CELLS				
Total radioactivity (cpm) X 10^{-3})	0.4	261.9	23.1	80.9
Ratio	negligible	2.6	1	

NOTE:

a) Total radioactivity for each type of collagen was calculated from the results of CM-cellulose chromatograms, by integrating designated peaks from each salt fractionate and combining results of both media and cell layers. Type I collagen was calculated from the α1(I) peak by a correction factor of 3/2, based on its heteropolymer structure as $\alpha 1\ (I)_2 \alpha 2$. Data from the two cell cultures are presented after adjusting fro DNA levels.

b) Ratio was measured by combining the values for collagen types IV and V.

IV. DISCUSSION

In the eye, primary mesenchyme originating from the primary streak gives rise to both corneal endothelium as well as to the vascular endothelium lying between the retina and sclera (Lopashov and Stroeva, 1961; Sabin, 1920; Trelstad et al., 1967; Hay and Revel, 1968). Thus these two types of endothelial cells have a common embryological origin. Secondary mesenchyme which originates from the neural creast (Horstadius, 1950; Le Douarin et al., 1977) gives rise to the stroma proper of the cornea, composed entirely of fibroblasts. Thus, corneal and vascular endothelial cells, though of mesenchymal origin, are derived from an embryological source entirely different from that of fibroblasts.

An attempt was made to examine whether cells of a common embryological source maintain a similarity in the phenotypic expression of type-specific synthesis. The present study established that type III collagen is the major collagen synthesized by cultured endothelial cells. This clearly distinguishes endothelial cells from fibroblasts, which synthesize type I and type III at a ratio of approximately 3:1 (Smith et al., 1972). About the same time this work was completed, Sage et al. (1979b and 1981) reported that in their culture system, type III collagen is the major collagenous product of vascular endothelial cells, confirming the present results. The concept that collagen type synthesis can serve as a marker to identify cell types is examined in Table 3 which summarizes the collagen types synthesized by different cells in culture.

Table 3 TYPES OF COLLAGENS SYNTHESIZED BY VARIOUS CELLS IN CULTURE

CELL TYPES	COLLAGEN TYPES	KEY REFERENCES
A. Mesenchymal Cells	Interstitial Collagens I,II,III.	
1. Fibroblasts	I >>III $^{I}/_{III} = {}^{3}/_{1}$	Smith et al., 1972
2. Smooth muscle Cells	III > I $^{I}/_{III} = {}^{1}/_{2}$	Burke et al., 1977
3. Chondrocytes	II	Miller and Matukas, 1969
4. Osteoblasts	I	Stern et al., 1980
5. Endothelial Cells	III	Sage et al., 1979b, 1981 Tseng et al., 1981
B. Epithelial Cells	Basement membrane collagen	
1. Corneal	IV	Kefalides et al., 1976 Sunder et al., 1979
2. Amniotic		Crouch and Bornstein, 1979 Alitalo et al., 1980
3. Glomerular		Killen and Striker, 1979
4. Lens		Grant et al., 1972 Laurent et al., 1978

That type III is the predominant type of collagen synthesized by cultured endothelial cells distinguishes itself not only from other mesenchymal origin cell types such as fibroblasts, smooth muscle cells, chondrocytes, and osteoblasts, but also from epithelial cell types (Table 3).

A preponderance of type III collagen appears to be a characteristic of all endothelial cells, regardless of origin. However, small amounts of other collagens are also found among different kinds of endothelium. Type I collagen is made by corneal endothelium, while vascular endothelium does not produce it, even when both cell types are grown and maintained under the same optimal culture conditions. Thus, some degree of heterogeneity in the phenotypic expression of collagen synthesis occurs among similar types of cells, though the major collagen types remain the same. The phenomenon of microheterogeneity in making cell-specific products has been observed in many other cell systems. Different patterns of glycosaminoglycans are synthesized by fibroblast-like cells from chick cornea, heart, and skin (Conrad et al., 1977). Among smooth muscle cells from different blood vessels, a different pattern of intermediate-sized filaments is detected (Gabbiani et al., 1981). Epithelial cells from different tissue sources exhibit a different synthetic pattern of keratin (Doran et al., 1980). Herein another example in collagen type synthesis by endothelial cells is described. The microheterogeneity characterizes the uniqueness of a cell type in the course of terminal differentiation.

The collagenous composition of the intact bovine cornea has been analyzed by several investigators (Schmut, 1977; Freeman, 1978; Davidson et al., 1979; Praus, et al., 1979). However, such work has focused on

the cornea as a whole, without preliminary separation of the three component layers. The presence of type III collagen cited in these studies has been an inconsistent observation in the literature and remains controversial. An additional unresolved problem is whether type III collagen is present *in vivo* in the adult bovine corneal endothelium. To clarify this point, the collagenous composition of adult and fetal corneal endothelia was determined. Corneas from fetal and adult bovine species were initially separated into endothelial, stromal, and epithelial layers by crude dissection. Each layer was subsequently subjected to limited pepsin digestion and salt precipitation according to the procedure described under Methods. The salt precipitates at 0.9 M NaCl and 1.2 M NaCl under acidic condition represented the fraction of interstitial and basement membrane collagens respectively. Such precipitates were then identified for collagen types either by 7% SDS-PAGE directly or analysis of the CNBr-cleaved peptide mapping by 15% SDSPAGE. The results are presented in Fig. 14 and 15. Both type I and V were the major collagens of the stromal layer of the adult cornea. The difference between endothelial and stromal layers was that an additional protein band comigrating with one of the fragments of type IV collagen standard was noted in the endothelial layer. When the fetal cornea was analyzed and compared with the adult cornea, a dramatic difference was observed. Type III collagen became detectable and was present in a greater proportion in the stromal, endothelial, and epithelial layers of the fetal cornea. It was almost non-existent in the adult cornea. This provides additional evidence supporting the notion that type III collagen is prevalent in fetal or embryonic tissues. This has been observed previously in skin (Miller *et al.*, 1971; Epstein, 1974).

The preponderance of type III collagen made by cultured corneal endothelial cells suggests that the cell culture system of endothelial cells represents a reversion to an embryonic-like stage of cell growth or perhaps a type of dedifferentiation.

One of the major characteristics of corneal endothelium *in vivo* is that it is formed by a honeycomb-like monolayer of flattened, contact-inhibited, and highly polarized cells, which deposit an ECM exclusively underneath or on the basal cell surface. This ECM forms a distinct basement membrane structure termed Descemet's membrane. Previous results demonstrate that Descemet's membrane *in vivo* contains type IV and V (A and B chains) collagens (Kefalides and Denduchis, 1969; Kefalides *et al.*, 1976; Davison *et al.*, 1979). The present study has established that, in addition to interstitial collagen types III and I, basement membrane collagens types IV and V are also elaborated by corneal endothelial cells *in vitro* into their ECM. A greater ratio of basement membrane collagen was produced as the cells were grown for longer times, i.e. 2 weeks after confluence (results not shown). Thus the aforementioned characteristics were retained when cells were grown in long-term culture in the presence of FGF. These cells therefore offer a model for the study of the synthesis and asymmetrical deposition of ECM.

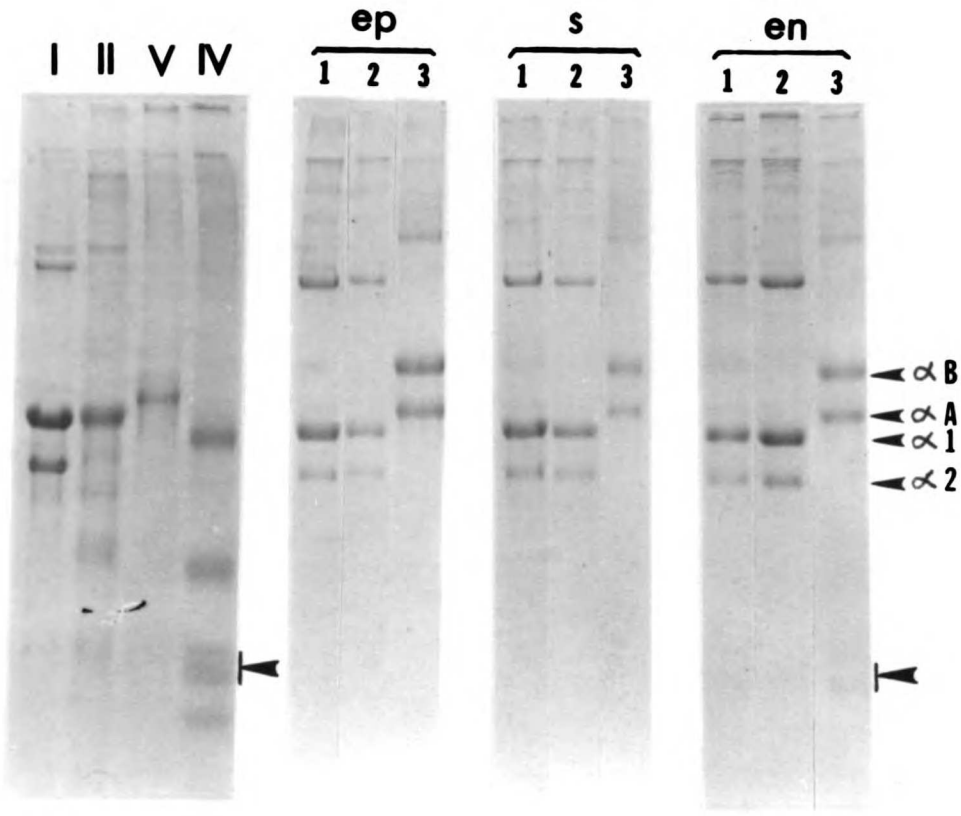


Figure 14:

Comparison of 7% SDS-PAGE pattern of the endothelial (En), stromal (S), and epithelial (Ep) layers of adult cornea. Each layer was subjected to limited pepsin digestion and salt fractionation at 0.9M and 1.2M NaCl respectively under acidic conditions as described in Methods. Different collagen type standards were coelectrophoresed and used to identify the collagenous bands of these unknowns. In the stromal layer, the major collagen was type I together with small amounts of type V, A and B chains. In the endothelial layer, a protein band comigrating with one of the fragments of type IV collagen was indicated by an arrow.

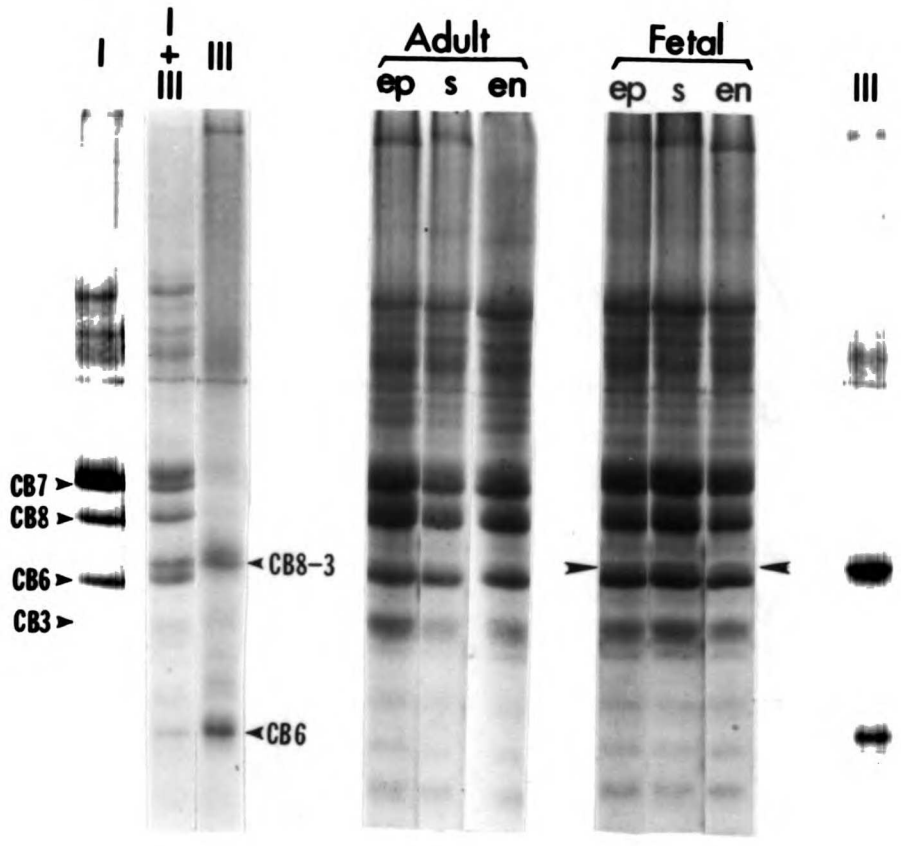


Figure 15:

Comparison of 15% SDS-PAGE pattern of CNBr-cleaved peptide mapping of the endothelial (En), Stromal (S), and epithelial (Ep) layers of adult corena and fetal cornea. The samples were obtained after the same treatment as described in the legend of Fig. 14 and Methods. In 0.9 M NaCl precipitate, type III was readily detected in fetal cornea as shown by the presence of CB8-3 (III) adjacent to CB6 (I), as indicated by the arrow. In contrast, such a CB fragment was not detected in adult cornea.

CHAPTER V MODULATION OF COLLAGEN SYNTHESIS BY FIBROBLAST GROWTH FACTOR

I. Introduction

As described in the experimental model system in Chapter II, a single peptide factor, fibroblast growth factor (FGF), is able to modulate cellular morphology in the culture of endothelial cells. When endothelial cells are grown in the presence of FGF and passaged at a low split ratio, they resemble vascular endothelium *in vivo* (Gospodarowicz *et al*. 1977; Birdwell *et al*. 1978; Vlodavsky *et al*. 1979). They form at confluence a monolayer of closely apposed and highly flattened cells which do not overgrow one another. The ECM is localized exclusively beneath the basal cell surface. In contrast, at a low split ratio and in the absence of FGF, cells become large, overgrow one another, and lose polarity. The ECM now is secreted and distributed uniformly over the entire cell surface.

Thus, by a simple manipulation, the addition or removal of FGF, cultured vascular endothelial cells are able to grow with two different phenotypes, one which maintains cell polarity and another in which this polarity is lost.

In this chapter, the synthesis of collagen is analyzed quantitatively and qualitatively under the two culture conditions described above. This is pursued in an attempt to test the working hypothesis stated in Chapter II, that one of the mechanisms of action of FGF is to modulate collagen synthesis specifically. The changes in collagen production will affect the organization and property of the ECM, which in turn might modify normal cell-matrix interactions.

II. EXPERIMENTAL PROCEDURE

A. Materials were described in the same section of Chapter IV.

B. Methods

1. Cell cultures

Bovine vascular endothelial cells (ABAE+F) were routinely cultured in DMEM (H-16) supplemented with 10% bovine calf serum, 2.5 ug/ml Fungizone, and 50 ug/ml Gentamycin. FGF (100 ng/ml) was added every other day until the cultures became confluent. Cell stocks were passaged weekly at a split ratio of 1:64.

Vascular endothelial cells maintained in the absence of FGF (ABAE-F) were passaged every 7 to 10 days at a split ratio of 1:8. Medium was renewed every three days. Within three passages, such cultures lose the morphological appearance of vascular endothelium. Cells become larger, lose their contact-inhibited properties, and begin growing in multiple layers without orientation (Vlodavsky et al., 1979).

2. The following procedures were the same as described in Chapter IV:

a. Metabolic labeling

b. Quantitative analysis of collagen synthesis by the determination of radiolabeled proline and hydroxyproline

c. Qualitative analysis of collagen synthesis by DEAE-cellulose and CM-cellulose chromatography

d. Cell counting and DNA quantitation

III. RESULTS

A. Quantitative Study of Collagen Synthesis

ABAE cells maintained in the presence (ABAE+F) or absence of FGF (ABAE-F) were labeled with [^{3}H] proline for 24 h. As shown in Table 1, under each of the growth conditions, the same amount of [^{3}H] proline was incorporated into proteins, both in the cell layer and secreted into the medium. The cell layer refers to both cells and their ECMs. The level of total protein synthesis and the proportion of total proteins secreted into the medium remained the same under both growth conditions. However, the radioactivity recovered in the form of hydroxyproline in ABAE-F cultures was twice (2.77%) that recovered in ABAE cultures (1.42%) (Table 1). There was twice the amount of collagen synthesis in endothelial cells in the absence of FGF. By calculation (Green and Goldburg, 1963), 0.5% versus 1.0% of the total proteins synthesized by ABAE cultures was devoted to collagen synthesis in the presence and absence of FGF respectively (Table 1).

The media and cell layers were further analyzed for the distribution of the hydroxyproline isomers, 3- and 4-hydroxyproline. In general, 3-hydroxyproline was preferentially accumulated in the cell layers, perhaps reflecting the preferential association of basement membrane with the cell layers (Man and Adams, 1975). However, a greater portion of hydroxyproline was recovered as 3-hydroxyproline in ABAE + F cultures (24.7% in the cell layer and 11.5% in the medium) than in ABAE-F cultures (9.1% in the cell layer and 8.3% in the medium) (Table 1). Thus, in the presence of FGF, endothelial cells seemed to synthesize more basement membrane components, although total collagen synthesis was decreased.

Table 1

[^{3}H] PROLINE INCORPORATION AND COLLAGEN ACCUMULATION

BY ADULT BOVINE AORTIC ENDOTHELIAL CELLS IN THE PRESENCE OR ABSENCE OF FGF

	+ FGF			- FGF		
	Medium	Cell[a] Layer	Total	Medium	Cell Layer	Total
1. Total incorporation cpm x 10^{-2}/ug of DNA[b]	300	250	550	342	246	588
2. Hyp/Total incorporation (%)	2.17	0.52	1.42	4.20	0.78	2.77
3. C/P (%) [c]	0.75	0.18	0.48	1.47	0.26	0.96
4. 3-Hyp/Total Hyp [d] (%)	11.5	24.7	13.7	8.3	9.1	8.4

NOTE:

a) The cell layer denotes both the cells and their ECMs.

b) DNA content for two 35 mm culture dishes of cells was 83 ug for ABAE + F, and 88 ug for ABAE-F cells.

c) C/P indicates the ratio of collagen to total protein. It is calculated assuming that collagen hydroxyrpoline residue content is 12.2% and the average content of proline residue in total cellular protein is 4.1% (Green and Goldberg, 1963).

d) Two isomers of hydroxyproline, 3- and 4-hydroxyproline were separated and measured by high voltage paper electrophoresis as described by Tseng et al. (1980). Ratios of 3-hydroxyproline to the sum of 3-hydroxyproline and 4-hydroxyproline are presented.

B. Qualitative Study of Collagen Synthesis

1. DEAE-cellulose chromatography of medium proteins

The types of procollagen secreted by cells into the media were analyzed under the two culture conditions. Media proteins precipitated by 30% ammonium sulfate were chromatographed on a DEAE-cellulose column. Chromatography of ABAE+F medium gave an elution profile composed of three major peaks (Fig. 1A). The first peak represented an unabsorbed fraction. Two other peaks were eluted during the course of NaCl gradient between fractions 44-62 and 69-72 respectively. The hydroxyproline contents of the three peaks, 16.8% for peak 1, 5.6% for peak 2, and 3.2% for peak 3, indicated that all contained collagenous materials. Further identification of each peak was made using human skin fibroblast markers (Fig. 1C). By comparing elution profiles (Fig. 1A & C), peak 1 was identified as unbound collagen and procollagen type IV, and peak 2 as procollagen type III. Peak 3 was tentatively identified as the precursor of type V collagen (unpublished observation). No detectable amount of procollagen type I was present in the media of vascular endothelial cell cultures.

DEAE-cellulose chromatography of the ABAE - F culture medium (Fig. 1B) yielded a distinctly different elution profile from that observed with ABAE + F medium. Compared to Fig. 1A, there were two additional peaks, (peaks 4 and 5), eluting between fractions 19-21 and 37-44, respectively. Peaks 2 and 3 had an elution profile identical to peaks 2 and 3 observed in that of ABAE + F culture medium (Fig. 1A). Peak 5 present in the media of ABAE - F cultures was identified as type I procollagen (Fig. 1B & C). Peak 4 was not identified. Its elution position was similar to that of EC, a unique collagen recently described by Sage *et al.* (1980) in aortic endothelial cell cultures.

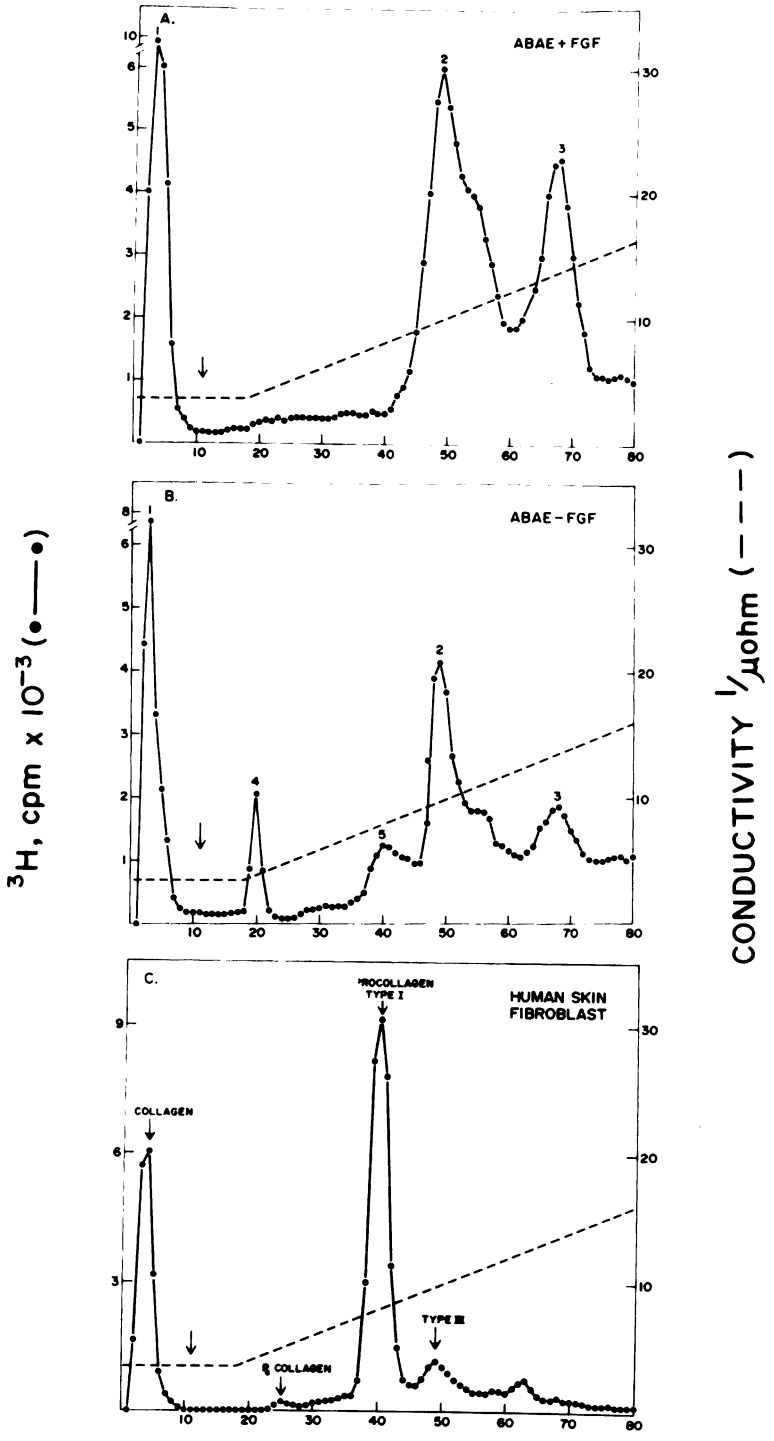


Figure 1:

DEAE-cellulose chromatograms of [^{3}H] proline-labeled mediums proteins of (A) ABAE + F cells, (B) ABAE - F cells, and (C) human skin fibroblasts. After labeling for 24 h with [^{3}H] radiolabeled proline in the presence of ascorbic acid and BAPN the 30% ammonium sulfate preciptate was chromatographed according to the procedure described in Methods of Chapter IV. Arrows indicate the start of the gradient. Conductivites were measured at room temperature. In both (A) and (B) peak 2 was procollagen type III and peak 3 was tentatively identified as procollagen type V. Peak 5 was procollagen type I. Peak I contained collagen type IV in both cases. Peak 4 was not identified but compatible to EC collagen described by Sage et al. (1980).

2. CM-cellulose chromatography of pepsin-digested proteins of both the medium and cell layers of ABAE + F and ABAE -F

To quantitate the types of collagens produced by ABAE + F and ABAE - F cultures, samples from media and cell layers of both types of cultures were further analyzed by CM-cellulose chromatography after pepsin digestion under acidic conditions and salt fractionation. NaCl was added stepwise to a final concentration of 1.7, 2.6, and 4.5 M under neutral conditions. Each salt fraction was then analyzed by CM-cellulose chromatography. Acidic non-collagenous moieties are eluted in the initial wash, while different collagen chains are resolved by NaCl gradient elution under denaturing conditions. Lathyritic rat skin collagens were used as internal standards. When the 1.7 M NaCl fraction of an extract of cell layers was subjected to chromatography, the elution profile was the same for cells maintained in either the presence or absence of FGF. Only type III collagen $[\alpha 1(III)]_3$ was observed (Fig. 2A & B). When the media were analyzed, the same type III collagen profiles were again obtained (results not shown). When the 2.6 M NaCl fractions from extracts of cell layers from cultures maintained in the presence or absence of FGF were analyzed, different elution profiles were observed. With ABAE + F cell cultures, only type III collagen was detected. In contrast, with ABAE - F cell cutlures, $\alpha 1(I)$ and $\alpha 2$ of type I collagen were clearly detected and co-eluted with the type I standard carrier (Fig. 3B). The same results were observed when media proteins of both cell cultures were analyzed (results not shown). Thus, in vascular endothelial cell cultures maintained in the presence of FGF, type III but not type I collagen was produced and could be detected in the media as well as in the cell layer. In the absence of FGF, synthesis of type I collagen became readily detectable.

The 4.5 M NaCl fractions of the cell layers from both cultures were analyzed. These fractions contain primarily type IV and type V collagens (Sage and Bornstein, 1979; Kresina and Miller, 1979). The cell layers of ABAE + F cultures yielded an elution profile with two major peaks which emerged between the elution positions of α1(I) and α2 chain of type I collagen (Fig. 4A). This elution profile was similar to that of type V collagen, as shown by comparison with the elution profile of purified A, B, and C chains from a human placental extract (Fig. 4C). The first peak of Fig. 4A eluted with the marker A chain and the second peak with the B, and possibly C chains which co-elute and cannot be resolved by this method (Sage and Bornstein, 1979, and Fig. 6, Chapter IV). The shoulder of the A chain-like peak and the minor peak preceding the α1(I) elution position were identified as α1(IV), type IV collagen, when compared to the elution profile of a type IV collagen standard (Fig. 4D). The profile of this collagen standard had major elution peaks which preceded α1(I). Each peak represents one component of the heterogeneous mixture, as shown by SDS-PAGE in Fig. 5, Chapter IV. In contrast, with the 4.5 M NaCl fraction of the cell layers of ABAE - F cultures, only the first peak, corresponding to the elution position of the placental A chain was detected by CM-cellulose chromatography (Fig. 4B). Two minor peaks eluted in the region of α1(I) were demonstrated and resembled α1(IV), type IV collagen. When the 4.5 M NaCl supernatant fraction was analyzed, no peaks could be detected (not shown), demonstrating that all collagenous materials had been precipitated by 4.5 M NaCl.

By integrating the peaks for the different types of collagen and combining the results of medium and cell layer, it was found that ABAE +

F cells synthesized collagen type III and basement membrane collagens at a ratio of 3:1. In contrast, ABAE-F cultures synthesized collagen type III and basement membrane at a ratio of 5:1, and types I and III at a ratio of 2:5 (Table 2). In the absence of FGF, the synthesis of types IV and V basement membrane collagen was 60% of that synthesized in the presence of FGF (Table 2).

In summary, under conditions which resemble the normal physiological situation, with the elaboration of the ECM only on the basal adherent surface, the vascular endothelial cells synthesized no type I collagen. Type III collagen was the major collagen species present under these culture conditions. In contrast, ABAE-F cultures synthesized type I collagen. Under conditions which resembled a pathological situation, in which ECM was elaborated over the entire cell surface, type I collagen constituted 25% of total collagen synthesis. Under these conditions, there was twice the amount of collagen synthesis, and the proportions of types IV and V collagen were decreased. Under both culture conditions, however, type III continued to be the major collagen species.

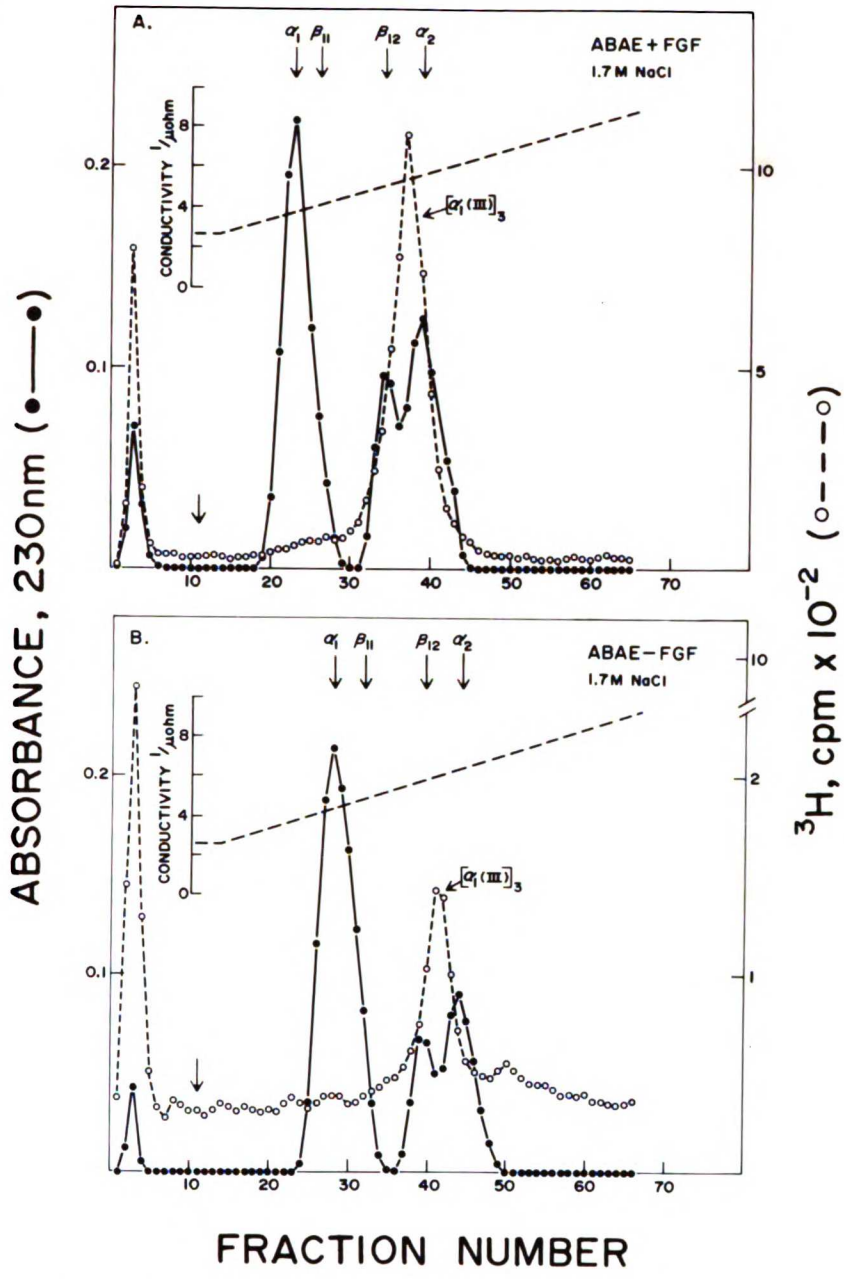


Figure 2:

CM-cellulose chromatograms of pepsin-digested [^{3}H] proline-labeled proteins in cell layer of (A) ABAE + F and (B) ABAE - F which were precipitated at 1.7 M NaCl under neutral conditions. The samples were processed and chromatographed as described under Method of Chapter IV. for both samples, lathyritic rat skin collagen, mostly type I, was cochromatographed as an internal standard and carrier. For unlabeled carrier collagen, absorbance at 230 nm was monitored (●——●) and elution peaks were identified as described in Fig. 2 of Chapter IV. For radiolabeled samples, aliquots were removed for liquid scintillation counting (O-----O). Arrow indicates the start of the gradient. Conductivities were measured at room temperature.

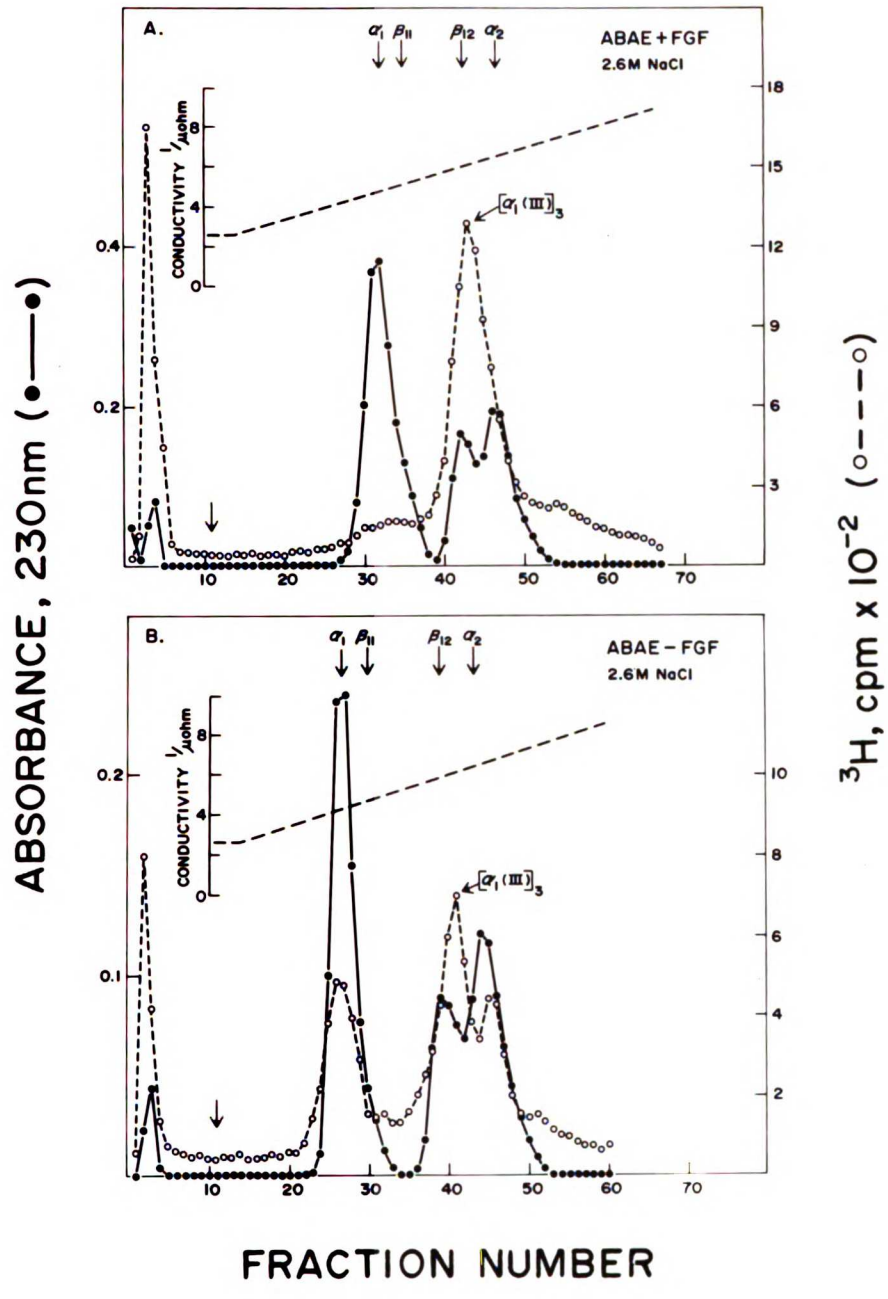


Figure 3:

CM-cellulose chromatograms of pepsin-digested [^{3}H] proline-labeled proteins in the cell layer of (A) ABAE + F and (B) ABAE - F, which were precipitated at 2.6 M NaCl under neutral conditions. Radioactive collagens were chromatographed in the presence of lathyritic rat skin collagen as described in the legend of Fig. 2 of this chapter.

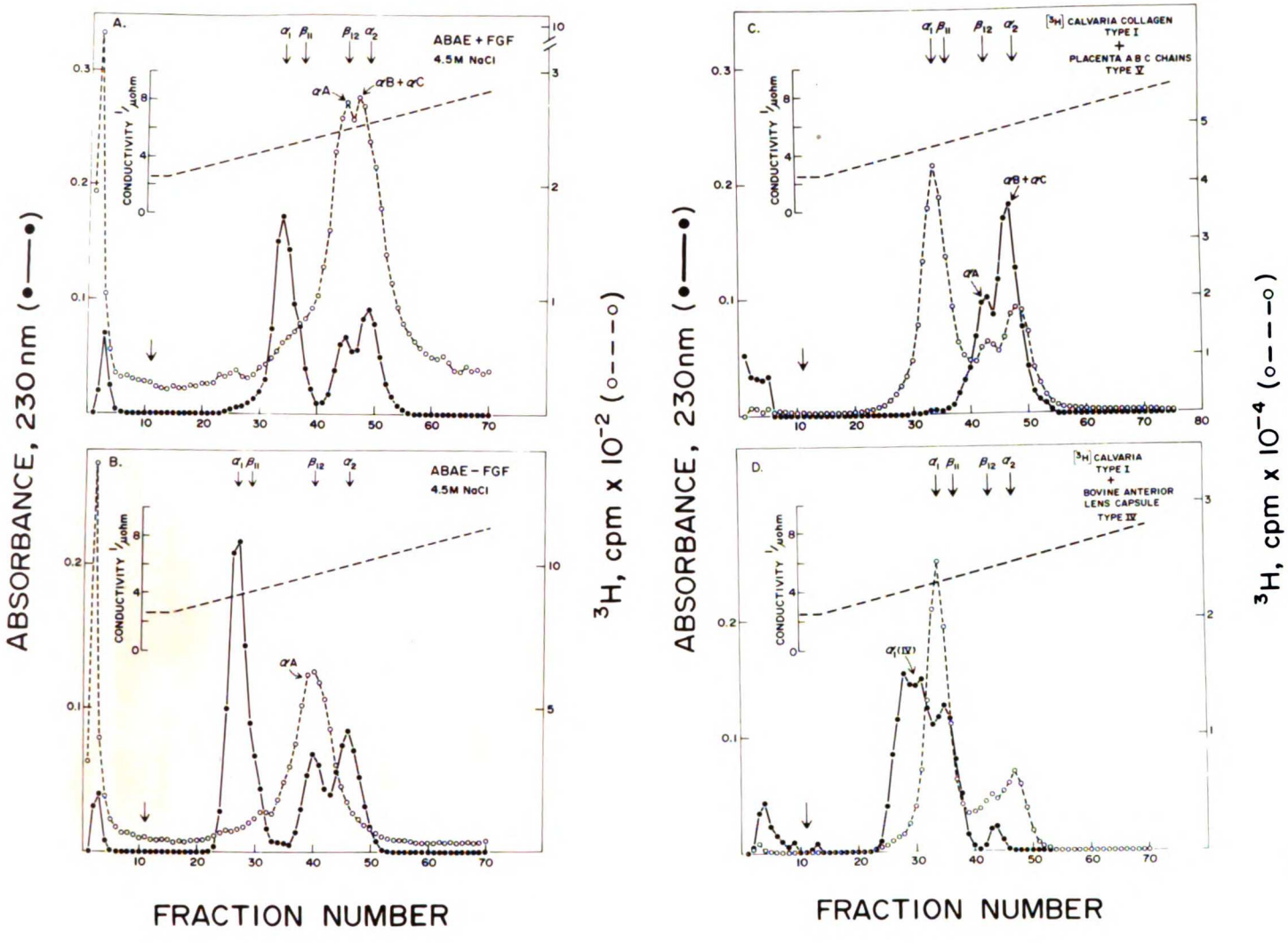


Figure 4:

CM-cellulose chromatograms of pepsin-digested [^{3}H] proline-labeled proteins in the cell layer of (A) ABAE + F and (B) ABAE - F which were precipitated at 4.5 M NaCl under neutral conditons. Radioactive collagens were chromatographed in the presence of lathyritic rat skin collagen carrier as described in the legend of Fig. 2. In (C), [^{3}H] proline-labeled chick calvaria, type I collagen, was cochromatographed with unlabeled placenta type V collagens, containing the A, B, C chains. In (D), type IV collagen from bovine anterior lens capsule was cochromatographed with calvaria collagen and gave a unique elution profile with major peaks preceding α1(I). It contained heterogeneous components as shown by the four major peaks which were further resolved by SDS-PAGE shown in Fig. 5, Chapter IV.

Table 2

PROPORTION OF DIFFERENT TYPES OF COLLAGENS ACCUMULATED IN ADULT BOVINE AORTIC ENDOTHELIAL CELLS IN THE PRESENCE OR ABSENCE OF FGF[a]

	I	III	IV	V
+ FGF				
Total Radioactivity[b] ($\times 10^{-3}$ cpm)	0.4	261.9	23.1	80.9
Ratio[c]	negligible	2.6	1	
-FGF				
Total Radioactivity ($\times 10^{-3}$ cpm)	150.2	340.8	4.3	64.3
Ratio	2.2	5.0	1	

NOTE:

a Numbers refers to counts in total cultures, media, cell layer plus ECM.

b Total radioactivity for each type of collagen is calculated from the results of CM-cellulose chromatograms by integrating designated peaks from each salt fractionate and combining results of both media and cell layers. Type I collagen is calculated from the $\alpha 1(I)$ peak by a correction factor of 3/2, based on its heteropolymer structure as $(\alpha 1)_2\alpha 2$. Data from the two cell cultures are presented after adjusting for DNA levels.

c Ratio is measured by combining the values for collagen types IV and V.

IV. DISCUSSION

Cultured endothelial cells (ABAE) passaged weekly at a low split ratio (1:64) and grown in the presence of FGF exhibit the differentiated properties of vascular endothelium *in vivo*. This correlates with the formation of an ECM localized exclusively on the basal cell surface. Cultures maintained in the presence of this mitogen exhibit a high mitotic index even when passaged weekly at a split ratio as low as 1:64 or 1:200 and survive longer than cultures maintained in the absence of FGF. Confluent ABAE cell cultures grown without FGF at a low split ratio lose their contact-inhibited properties after a few passages, as well as the polarity of secretion of ECM (Vlodavsky *et al.*, 1979).

The synthesis of collagen by cultured vascular endothelial cells has been examined previously at both the morphological and biochemical levels (Kefalides, 1971; Gay *et al.*, 1975; Jaffe *et al.*, 1976; Howard *et al.*, 1976; Levene and Heslop, 1977; Barnes *et al.*, 1978; Sage *et al.*, 1979). Because of the major changes in the pattern of matrix deposition in the presence of FGF, collagen synthesis in this system was re-examined.

The level of collagen synthesis was assessed by determining the content of hydroxyproline. Vascular endothelial cells cultured in the absence of FGF devoted twice their total protein synthetic capacity to collagen compared to cells grown in the presence of FGF. Type III was the major collagen synthesized by cultures maintained in the presence of FGF. This was confirmed by both DEAE and CM-cellulose chromatography and is consistent with the *in vivo* situation, in which the predominance of type III collagen located beneath the endothelial cell layer of arterial wall sections can be observed immunohistochemically (Gay *et al.*, 1975).

In contrast, in the absence of FGF, synthesis of type I collagen in addition to type III collagen emerged. When collagen type synthesis is correlated with the changes of endothelial cells in morphology and matrix deposition, it provides some meaningful insight. Type I collagen has been observed as a major interstitial collagen synthesized by endothelial cells in the culture system by Barnes et al. (1978). After several passages, their cells adopt a fibroblastic configuration (Levene and Heslop, 1977) with a ratio of hydroxyproline to proline similar to that observed in fibroblast cultures. Recently, Cotta-Pereira et al. (1980) further reported that sprout cells, a name used to described a focal growth of endothelial cells with a different morphology, secrete predominantly type I collagen. The present study together with these findings support a notion that the change in collagen types correlates with a cellular phenotypic change. The maintenance of type III collagen represents the normal stage of endothelial cell growth when a contact-inhibited monolayer with asymmetrical cell surface polarity is preserved in vitro. The emergence of type I collagen and increase in total collagen synthesis denotes a different stage of cell growth characterized by fibroblastic configuration. The present experiment confirmed this phenomenon and also demonstrated that a single peptide factor, FGF, was able to modulate these two forms of cell growth. Thus, FGF plays an important role not only in proliferation but also differentiation in the culture of endothelial cells. This experiment further disclosed that the maintenance of normal endothelial phenotype was achieved by the modulation of collagen synthesis which was under the apparent control of FGF.

The present data are also important for understanding a number of pathological processes. Normally, the outer media and adventitial layers of blood vessels are the domain of type I collagen (Ross and Glomset, 1973). Only in atherosclerosis is type I collagen found in association with the intimal layer (McCullagh and Balian, 1975). In this way the behavior of endothelial cells cultured in the absence of FGF resembles one aspect of the process of plaque formation. The appearance of an ECM on the apical cell surface correlates with a loss of its nonthrombogenic properties, as reflected by the cells' ability to bind platelets (Vlodavsky et al., 1979), and thus provides an important model for intravascular thrombosis.

The association of vascular endothelial cells with basement membranes has been demonstrated previously only by immunoflurescent studies in which antibodies prepared against lens capsule (Howard et al., 1976) and glomerular basement membrane (Jaffe et al., 1976) are used. Vascular endothelial cells synthesized collagen types IV and V under both culture conditions. The level was greater in the presence of FGF. With FGF, the profile of type V resembled that of the placental markers containing A chain and B, or possibly C chains. In the absence of FGF, only the peak which coeluted with the marker A chain was detected. Because of possible species differences in elution patterns, it was not possible to identify these peaks with absolute certainty. The biological significance of these biochemical changes in endothelial cells is not clear since the functional role for type V collagen remains unsolved. However these changes may correlate with changes in morphology and distribution of the ECM observed in immunofluorescent studies (Greenburg et al. 1980).

Extracellular matrix supports cell proliferation (Gospodarowicz et al., 1980), including the growth of vascular endothelial cells (Gospodarowicz and Ill, 1980), and obviates the requirements for this growth factor. Thus ECM appears to have a permissive effect on the proliferation of cultured cells. The present studies demonstrated the dramatic effect of FGF on the expression of collagen-type specific synthesis, not only in the distribution of that collagenous matrix, but also in the amounts and types of collagens synthesized. It is reasonable to believe that the property of ECM can be maintained in such a manner by the addition of FGF. This will in turn modulate the interactions between cells and their ECMs. The relationship of FGF, a soluble, diffusable peptide factor, and ECM, an insoluble matrix complex, in the action mechanism of controlling cell-matrix interactions will be further analyzed in the next chapter.

CHAPTER VI MODULATION OF COLLAGEN SYNTHESIS AND DEPOSITION BY EXTRACELLULAR MATRIX --- IMPLICATION OF A RELATIONSHIP BETWEEN FGF AND ECM

I. INTRODUCTION

As described in the Chapter II, Experimental Model System, the major characteristic of the endothelium *in vivo* is that it forms a well-organized monolayer of contact-inhibited, flattened cells with a cell surface polarity such that extracellular matrix (ECM) is elaborated exclusively under the subendothelial surface. This characteristic phenotype has been shown to be preserved by the addition of fibroblast growth factor (FGF) to the culture medium *in vitro* as plastic substrata are employed. When exposed to this mitogen, cells exhibit a high mitotic index and, upon reaching confluence, the same morphological properties as their *in vivo* counterparts. However, without such a growth factor cells grow poorly, lose orientation, and deposit ECM over the entire cell surface.

The ECM produced by cultured corneal endothelial cells is a major cell product at confluence. The natural substratum prepared from this ECM is capable of supporting a variety of cultured cells, including corneal endothelium. This substratum obviates the requirement for exogenous FGF. Thus, in cell culture either FGF or the natural substratum maintains the *in vivo* phenotypic characteristics of endothelium. One, if not all, of the mechanisms of action of FGF is by controlling ECM

production. In Chapter V, this working hypothesis has been tested and confirmed, that the amount and type of collagen synthesis is modulated by this mitogenic peptide.

In this chapter, a further exploration is made to elucidate the relationship between a diffusable factor, FGF, and an insoluble complex, ECM, in their ability to control cellular proliferation and to maintain differentiated properties. Since collagen is one of the major components in the ECM, synthesis and deposition of this macromolecule reflects cellular polarity in organizing ECM, which represents one of the key elements of the in vivo phenotype (see Chapter IV). To approach the aforementioned question, collagen synthesis and deposition was compared by growing the corneal endothelial cells under the following three different conditions:(A) on plastic plates without adding FGF, conditions under which the in vivo phenotype is lost, (B) on plastic plates with the addition of FGF, and (C) on natural substrata made from their own ECMs and in the absence of FGF, both the latter two conditions under which the in vivo phenotype is preserved. In order to investigate the initial effects of FGF and ECM, the analysis of collagen synthesis and deposition was performed through the entire course of the cell culture, that is, from sparse to confluence, and to post confluence.

II. EXPERIMENTAL PROCEDURES

A. MATERIALS The same as those described in Materials section of the Chapter IV.

B. METHODS

1. Preparation of plastic plates coated with natural substrata

Natural substrata were prepared as described (Gospodarowicz et al., 1980a). In brief, bovine corneal endothelial cells were plated at an initial density of 10^4 cells per 35-mm dish and were maintained in DMEM (H-16) supplemented with 10% (v/v) FCS, 5% (v/v) calf serum, 5% (w/v) dextran T-40 (Sigma), Gentamycin (50 µg/ml), and Fungisone (2.5 µg/ml). FGF (100µg/ml) was added every other day. Once the cultures became confluent, ordinarily within 6 days, the media were renewed and the cultures were further incubated for 6 days. The cultures were then washed with PBS and exposed for 30 min to 0.5% (v/v) Triton X-100 in the same buffer. Once the nuclei and the ECM became visible, the cultures were washed three times with PBS. After three washings, only a few cytoskeletal structures and nuclei were associated with the intact ECM.

2. Tissue culture of bovine corneal endothelial cells

Bovine corneal endothelial cell cultures were established from steer corneas. Stock cultures were maintained in DMEM (H-16) supplemented with 10% fetal calf serum and 5% calf serum. Endothelial cells were passaged weekly at a 1:64 split ratio and FGF (100 µg/ml) was added every other day until cultures were subconfluent.

Three different culture conditions were used in the experiment. In the first group (A), BCE cells were grown on plastic plates without the

addition of FGF. In the second group (B), the same plastic plates were used, but FGF was added in the same fashion as described above for stock cultures. In the third group (C), the cells were grown on natural substrata without adding any FGF. On day 0, three tissue cultures were initiated at a density of 1-2 x 10^4 cells per 35-mm dish. The rest of the cell culture conditions were the same for the three groups as described above. For the later two groups (B) and (C), cell cultures reached confluence on day 6. However, for group A, the cell density was lower than group B and C on each designated day (Gospodarowicz et al., 1981), despite the fact that cells were larger in size.

3. Metabolic labeling

Both sparse and subconfluent cultures of bovine corneal endothelial cells were preincubated for 24 h in glutamine-free DMEM (H-16) supplemented with 10% bovine calf serum and ascorbic acid (25 μg/ml). On day 0, 2, and 4, the cultures were exposed to ascorbic acid (25 μg/ml) and then L-[2,3,4,5,-^{3}H] proline (40 μCi/ml) on the next day, i.e. day 1, 3, and 5 for 24 h at 37°C. In one group of experiments, BAPN (80μg/ml) was added to compare the effect of BAPN exerted on the synthesis and distribution of collagen molecules, since BAPN is known to prevent collagen cross-linking. It was discovered that the effect of BAPN was negligible, thus BAPN was withdrawn from all the experiments in measuring the amount and distribution of radiolabeled hydroxyproline of these three culture conditions except in the experiment of quantitating different types of procollagens in the media by DEAE-cellulose chromatography. After 24 h, the media and cell layers were harvested and processed as described in the Methods section of the Chapter IV. In group B, additional measurements were made when cultures were 3 week-old, i.e., 2 weeks after confluence.

4. Assay of non-dialyzable radiolabeled proline and hydroxyproline

To measure the amount of newly-synthesized collagen in each cell culture condition, a method of quantitating radiolabeled proline and hydroxyproline was employed as described in the Methods section of Chapter IV. The ratios of [^{3}H]hydroxyproline/[^{3}H]proline + [^{3}H]-hydroxyproline were presented as such without further correction for the loss of radioactivity which occurs when proline residues become hydroxylated. Total hydroxyproline calculated from values of the total radioactive incorporation and the above ratios were regarded as newly-synthesized collagen. A correction factor for 10^5 cells in each culture condition was made. Triplicate samples were measured and the mean ±SD derived from these three measurements were presented in the results.

5. DEAE-cellulose chromatography

To quantitate the procollagens which were secreted into the media of the subconfluent cultures of three culture conditions, DEAE-cellulose chromatography was performed as described in the Methods section of the Chapter IV.

6. Assay of lysyl oxidase activity

To determine whether the extent of deposition of collagen was affected by the activity of lysyl oxidase, an extracellular enzyme responsible for cross-linking of collagen, the enzymatic activity was measured in both the media and cell layers of the three different cultures at the confluent stage, i.e. day 6. The procedure of preparation of the radiolabeled substrate is described in detail by Siegel et al. (1970) and Siegel (1974). The assay of lysyl oxidase activity was performed by a procedure described by Siegel and Fu (1976).

7. Cell counting

The cell number was obtained by trypsinizing parallel cultures and counting the cells in triplicate with a Coulter Counter.

III. RESULTS

A. Comparison of growth and morphology of BCE cells grown under different conditions

As observed previously by Gospodarowicz et al. (1980a), under these three different culture conditions described in Methods, BCE cells exhibited differences in morphology and in growth rates. BCE cells were initially plated at a density of $1\text{-}2\times10^4$ cells per 35 mm dish. Group A, in which cells were grown on plastic plates without the addition of FGF, had a cellular morphology different from that of either group B, whose cells were grown on plastic dishes to which FGF was added, or group C, in which natural substrata were substituted for plastic plates and to which no FGF was added. Cells of group A were larger than those in groups B and C and were, unlike cells in groups B and C, irregular in shape. The growth rate of group C was highest. Cells of group C reached confluence on day 5. The growth rate of group B was slightly slower than that of group C and reached confluence on day 6. The growth rate of group A was the slowest. These cells could not reach the same cell density even when cultured for longer periods of time at confluence were large and overlapping. On day 6, they reached a density half that of groups B and C.

B. Total incorporation of [^{3}H]proline in non-dialyzable proteins both secreted into the media and accumulated in the cell layers

After labeling for 24 h with [^{3}H]proline under the conditions described in Methods, the total incorporation of [^{3}H]proline into non-dialyzable proteins was analyzed first in the above three different culture conditions. These values reflected the general cellular ability to synthesize protein. When media and cell layers were measured together, the total incorporation of [^{3}H]proline into non-dialyzable proteins represented the cellular ability to produce newly-synthesized proteins. These abilities can be measured and compared, once these values have been corrected for number of cells in the three different culture conditions. As shown in the results of Table 1 and Fig. 1, the total incorporation of [^{3}H]proline in these three culture conditions was different, indicating that their abilities to make newlysynthesized proteins were different. Cellular activity in incorporating [^{3}H]proline increased in group A, in which cells were grown on plastic plates without the addition of FGF, but decreased in both group B, in which cells were grown on plastic plates with the addition of FGF, and group C, in which natural substrata were substituted for plastic plates and to which no FGF was added. In cultures of day 1, the value for group A was 2.3 times that of group B, and 1.8 times that of group C. When they reached subconfluence on day 5, it was 1.8 times and 3.3 times that of groups B and C respectively.

To examine the changes in the pattern of incorporation of [^{3}H]-proline when cell cultures were grown from sparse stage to the confluence and even later, the analysis was performed on each individual group at the progressive stages in the course of cell culture. The results showed a decreasing pattern of total incorporation, as cells reached confluence, with a noticeable exception in group B, whose

total incorporation increased slightly from sparse to subconfluent cultures, but decreased again in cultures two weeks post-confluence (three week-old culture). The decrease was greater for group C, whose value fell to 45% that of sparse cultures as cells reached confluence. However, for groups A and B the decreases were within the range of 10-20%.

The media and the cell layers were then analyzed separately to examine the distribution of incorporated proteins in these two compartments. The values presented here for the cell layers in each group included the cells and the surrounding ECM. The question was asked as to whether they could represent primarily the ECM. This was shown by the following experiment. When the cellular portion was denuded by the same method of preparing the natural substrata as described in Methods, less than 30% of total [^{3}H]proline stayed the remaining matrix. Yet more than 70% was observed in the recovery rate of [^{3}H]hydroxyproline. This indicated that [^{3}H]hydroxyproline, a better index for collagen synthesis, was primarily present in the ECM.

The measurements of total[^{3}H]proline in the media and the cell layers from each group gave a similar pattern, that more proteins were incorporated into the cell layers than were secreted into the media, regardless of the stage of culture. This was shown more clearly by the percentage of total incorporation present in cell layers (Table 1). When cultures of different stages i.e. day 1, 3, and 5 were compared in each individual group separately, an increase in this percentage was noted in groups A and B and a decrease in group C. In group A, the

percentage increased from 64% on day 1, to 66% on day 3, and finally 68% on day 5. The degree of incremental increase in percentage of group A was negligible and less than that of group B, where the percentage of total incorporation present in cell layers increased from 52% on day 1 to 59% on day 3, 66% on day 5, and finally 68% on 3 week-old cultures. It was primarily due to the increase in the total absolute amount of [^{3}H] proline incorporation present in the cell layer of group B (Table 1 and Fig. 1). Nevertheless, when the percentages were compared among the three different groups, the highest values were found in group C, where natural substrata were substituted for plastic plates, though a decrease in percentage was noted, 79% to 78% and then to 72%. These results indicated that the patterns in the ability and extent of incorporation of [^{3}H]proline into cell layers differed among these three groups. A unique pattern was present in Group B. Proportionally more [^{3}H] proline was incorporated into the cell layers as cultures approached confluence (Group B, Fig. 1).This suggested an active role of FGF in modulating the organization of cell layers. The highest value of group C, 79%, appeared at the initial sparse stage, i.e., day 1, indicating that the pre-existing ECM could markedly predispose the incorportion of proteins into the cell layers.

Table 1 [^{3}H] PROLINE INCORPORATION INTO NON-DIALYZABLE PROTEINS PRESENT IN BOTH THE MEDIA AND CELL LAYERS OF BOVINE CORNEAL ENDOTHELIAL CELLS AT DIFFERENT STAGES AND UNDER DIFFERENT CULTURE CONDITONS.

A. Plastic-FGF	Day 1	Day 3	Day 5	Week 3
MEDIA	6336 ± 132	5480 ± 212	4655 ± 340	--
CELL LAYERS	11404 ± 366	10526 ± 436	9980 ± 124	--
TOTAL	17740 ± 498	16006 ± 648	14635 ± 464	--
Cell Layer/Total (%)	64	66	68	--
B. Plastic + FGF				
MEDIA	3575 ± 222	3183 ± 110	2708 ± 34	2392 ± 94
CELL LAYERS	3947 ± 252	4492 ± 312	5351 ± 378	5082 ± 166
TOTAL	7522 ± 474	7675 ± 422	8059 ± 412	7474 ± 260
Cell Layer/Total (%)	52	59	66	68
C. ECM-FGF				
MEDIA	2068 ± 32	1566 ± 47	1228 ± 28	--
CELL LAYERS	7696 ± 234	5710 ± 210	3197 ± 122	--
TOTAL	9764 ± 266	7276 ± 257	4525 ± 150	--
Cell Layer/Total (%)	79	78	72	--

NOTE: 1. Mean value ± SD are reported here.

2. Each value represents cpm x $10^{-2}/10^5$ cells.

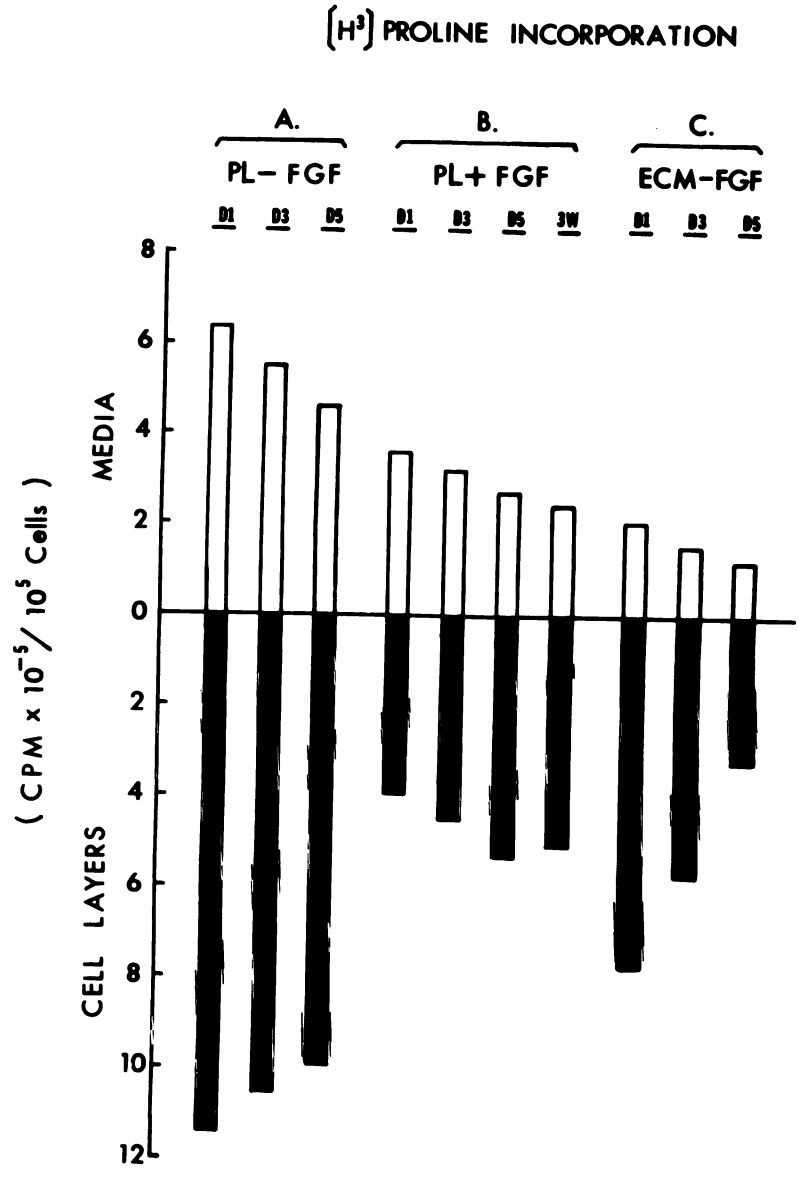


Figure 1:

Diagram of [^{3}H] proline incorporation into nondialyzable proteins which were present in both the media and cell layers of bovine corneal endothelial cells under three different culture conditions as described in Methods. Different stages, i.e., day 1, 3, and 5 (denoted as D1, D3, and D5) of each group and 3 week-old culture of group B (denoted as 3W) were analyzed and mean value of triplicates is drawn here. The detailed values and standard deviations are referred to Table 1.

C. Amount of [^{3}H]hydroxyproline recovered in non-dialyzable proteins both secreted into the media and accumulated in the cell layers

The collagenous contents of these three cultures were then analyzed. The proportion of collagen synthesized could be assessed when the [^{3}H]proline-labeled proteins were analyzed for the amount of [^{3}H]-hydroxyproline recovered after acid hydrolysis and the assay of [^{3}H]-hydroxyproline as described in Methods. The absolute amount of [^{3}H]-hydroxyproline represented the total collagenous content. The ratio of [^{3}H]hydroxyproline/total [^{3}H]proline was used as a parameter of the proportion of total protein synthesis devoted to collagen.

When media and cell layers were measured together, the amount of [^{3}H]hydroxyproline represented the total cellular level of collagen synthesis. As shown in the results of Table 2 and Fig. 2, the total amount of [^{3}H]hydroxyproline recovered in three culture conditions were markedly different, indicating that cells changed their capacity to make collagen when different culture conditions were employed. When cells were grown on plastic plates without adding any FGF (group A), the highest total amount of [^{3}H]hyroxyproline was recovered, 2.3 times that of group B. and 5.9 times that of group C on day 1. Similar contrasts were observed at subsequent stages: group A had a value 2.7 times and 4.4 times that of groups B and C on day 3, and 2.5 times and 5.0 times that of group B and C on day 5. These results demonstrated that, cells synthesized more collagen when they were grown on plastic alone without addition of FGF. When FGF was added exogeneously into the media, total synthesis of collagen per cell decreased to 40% of the original value (Table 2). This value decreased another 50-60% when the natural substrata were used (group C, Table 2). Noteworthy was the difference in

collagen synthesis between group A and C, between which the major change was the use of ECM (group C, Table 2 and Fig. 2). The decrease in collagen synthesis per cell was greater than that for total protein synthesis (group C, Table 1 and Fig. 1). The difference was primarily due to a lower ratio of [^{3}H]hydroxyproline/[^{3}H]proline (group C, Table 3). This further demonstrated the specific effect of a pre-existing ECM on collagen synthesis and deposition.

The entire course of cell culture, from sparse, day 1, to confluent day 5, for each group was compared next. A general pattern was revealed in which the total amount of [^{3}H]hydroxyproline described as cells reached confluence. This indicated that collagen production per cell decreased as cells reached a higher density. The percentage of decrease from the sparse to the confluent stage was in the range of 30% to 50% and was highest in group B and lowest in group C. The amount of decrease fell gradually in the course of culture. It was greater when cells went from the sparse to the confluent stage. Changes were not as pronounced when cells went from confluence to the post-confluence stage, as shown in group B (Table 2, Fig. 2). A decrease of 48% was observed before confluence, and 21% after confluence.

This suggested that in group B, to which FGF had been added, modulation of collagen synthesis and deposition was under the control of FGF and in a stepwise fashion. Whereas, in group C, in which the natural substrata were used, the control of ECM in collagen synthesis and deposition was prevalent at an early stage.

The media and cell layers of each group were then analyzed separately to examine the distribution of newly-synthesized collagen in these two compartments during the course of maturation of the ECM from

the sparse to confluence, and to even later stages. The pattern of distribution of newly-synthesized collagen in these two compartments directly reflected the polarity of secretion of this macromolecule. The asymmetrical cell surface deposition of ECM is one of the major characteristics of endothelial cells in vivo. This comparison among the three different culture conditions further demonstrated the degree and extent to which each culture achieved this property. This was pursued separately as follows.

In group A, a higher proportion of newly-synthesized collagen was elaborated into the media than was deposited into the cell layers. This result was observed in both sparse and confluent cultures. This was shown more clearly by examining the percentage of total [^{3}H] hydroxyproline present in the cell layers (group A, Table 2). The percentage rose from 21% on day 1, to 31% on day 3, and finally to 40% on day 5, suggesting that the collagen distribution changed as cells reached a higher density. More collagen was deposited into the cell layers than was elaborated into the media at the confluent stage compared to the sparse stage of growth. Despite the fact that the total amount of [^{3}H]hydroxyproline decreased as cells grew to a higher density, the amount present in the cell layers increased (group A, Table 2 and Fig. 2). Nevertheless, proportionally, more newly-synthesized collagen was still elaborated into the media even at confluence (40% on day 5). This indicated that endothelial cells grown on plastic plates without the addition of FGF failed to achieve asymmetric deposition of collagen into their cell layers. This failure occurred at the sparse as well as at confluent stages of cell growth.

Group B was examined next. A similar pattern in distribution of the collagen molecule between media and cell layers was observed. However, a gradual increase occurred in collagen deposited in the cell layers (group B, Table 2 and Fig. 2). The percentage of total [^{3}H] hydroxyproline present in cell layers increased from 13% on day 1, to 28% on day 3, and to 34% on day 5. A percentage of 56% was eventually reached when cultures were two weeks post-confluence. This suggested that when cells were grown on plastic plates in the presence of FGF, their normal phenotype was achieved in stepwise increments, as reflected by the polarity of secretion of ECM.

Group C was then analyzed. The pattern of distribution of newly-synthesized collagen in media and cell layers was entirely different from those of groups A and B. On day 1, a percentage of 52% was observed, suggesting the presence of an asymmetrical polarity in the deposition of collagen. This was achieved at an early stage of growth. This high percentage was maintained and even increased as cultures were grown to higher cell densities; 62% and 74% were observed for cultures on day 3 and 5 respectively. This was attributed primarily to the decrease in the amount of collagen elaborated into the media, since values for collagen in the cell layers remained almost constant (group C, Table 2 and Fig. 2). This further demonstrated that a pre-existing ECM caused the preferential deposition of newly-synthesized collagen into the cell layers. Such an effect was operative even at the sparse stage, since the natural substrata were present from the beginning.

In conclusion, the synthesis and deposition of collagen in cultured endothelial cells was modulated in a different mode by FGF and ECM. In

the presence of FGF, the cell surface polarity of secretion of newly-synthesized collagen was achieved in a stepwise fashion through the course of cell culture. Two weeks after confluence it finally reached a pattern of distribution of [^{3}H]hydroxyproline between media and cell layers similar to that seen in sparse cultures when ECM was present instead of FGF. Cell polarity for the elaboration of collagen occurred in the early cultures of group C, in which a pre-existing, ECM was present. This characterized the unique effect of ECM and may explain why FGF was no longer required for cell proliferation and differentiation under that culture condition.

Table 2 [^{3}H] HYDROXYPROLINE RECOVERED IN NON-DIALYZED PROTEINS PRESENT IN BOTH THE MEDIA AND CELL LAYERS OF BOVINE CORNEAL ENDOTHELIAL UNDER THREE CULTURE CONDITIONS.

A. Plastic-FGF	Day 1	Day 3	Day 5	Week 3
MEDIA	837 ± 38	425 ± 28	360 ± 36	--
CELL LAYERS	217 ± 18	223 ± 21	360 ± 36	--
TOTAL	1054 ± 56	648 ± 49	601 ± 50	--
Cell Layer/Total (%)	21	34	40	--
B. Plastic + FGF				
MEDIA	391 ± 22	174 ± 18	155 ± 21	81 ± 14
CELL LAYERS	60 ± 16	67 ± 12	81 ± 16	105 ± 17
TOTAL	451 ± 38	241 ± 30	236 ± 37	186 ± 31
Cell Layer/Total (%)	13	28	34	56
C. ECM-FGF				
MEDIA	86 ± 12	55 ± 7	31 ± 8	--
CELL LAYERS	93 ± 14	91 ± 10	92 ± 12	--
TOTAL	179 ± 26	146 ± 17	121 ± 20	--
Cell Layer/Total (%)	52	62	74	--

NOTE: 1. Mean value ± SD were reported.
2. Each value represented cpm x $10^{-2}/10^5$ cells

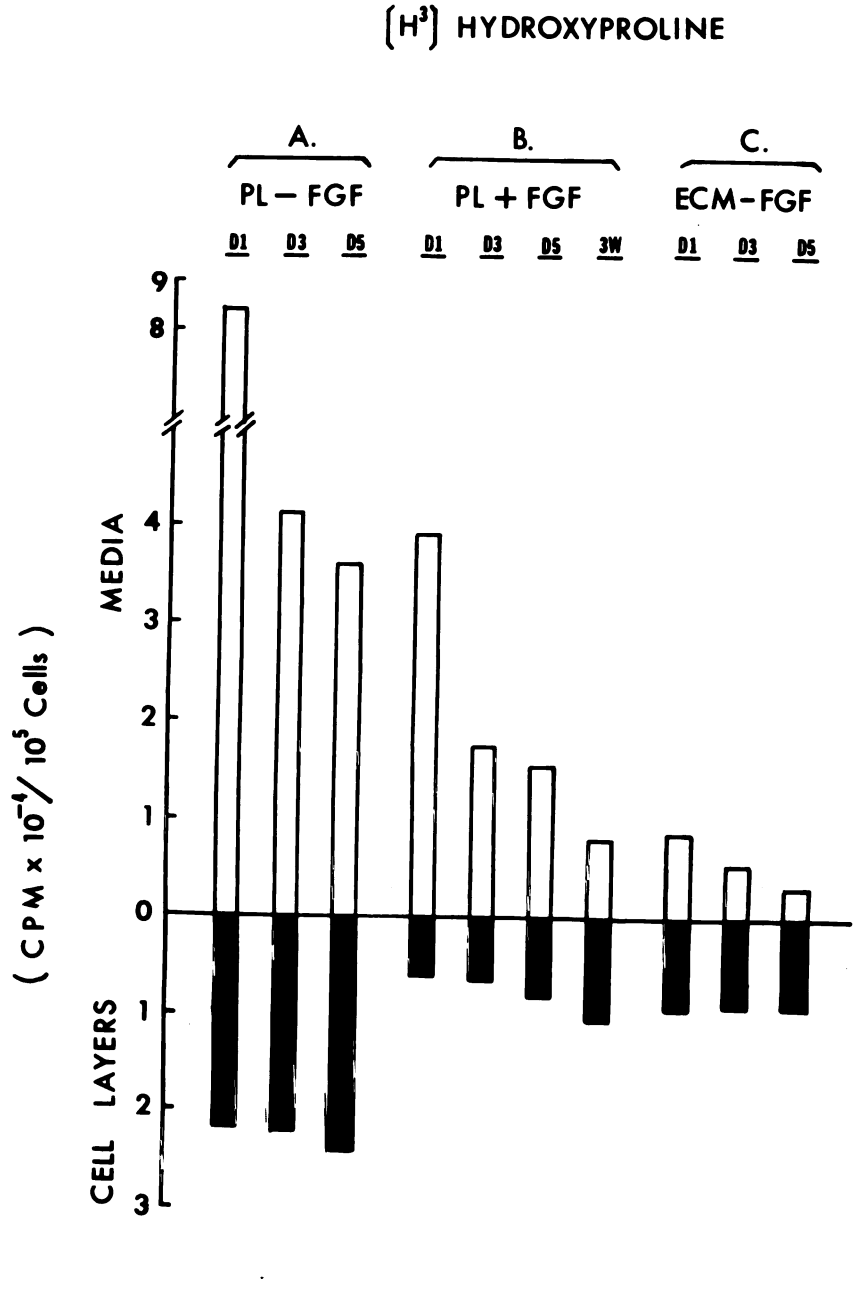


Figure 2:

Diagram of [^{3}H]hydroxyproline recovered from non-dialyzable proteins present in both the media and cell layers of bovine corneal endothelial cells under three different culture conditions as described in Methods. Different stages, i.e. day 1, 3, and 5 (denoted as D1, D3, and D5) of each group and 3 week-old culture of group B (denoted as 3W) were analyzed and mean values of triplicates are drawn here. The detailed values and standard deviations are referred to Table 2.

D. Comparison of the synthesis of collagen and non-collagenous proteins under the three culture conditions

The difference in the proportion of collagen and non-collagenous protein synthesis was examined in order to determine whether modulation of collagen synthesis and deposition by FGF_{Λ}^{or} ECM was specific. This comparison was made by measuring the ratio of [^{3}H]hydroxyproline/total [^{3}H]proline incorporation. These values can be further converted into the ratio between collagen and general protein synthesis by the same correction factor given by Green and Goldberg (1963). Table 3 summarized these ratios without such a correction. When the media and cell layers were analyzed together, group A and B had a ratio of 5.9% and 6% on day 1 respectively which were higher than that for group C, with a ratio of 1.8%. This indicated that on plastic plates, in both the presence and absence of FGF, collagen synthesis was higher than when a natural ECM was present. Pre-existing ECM thus enabled to suppress collagen synthesis specifically. As the cultures were grown to a higher cell density, the pattern of change in these ratios was different among these three groups. In both group A and group B, values were decreasing progressively on day 3, 5, and even later. The extent of decrease in group B was more dramatic than that in group A, in which a plateau was reached on days 3 and 5. Nevertheless, in group C, the ratio gradually increased from 1.8% on day 1 to 2.7% on day 5. The difference in the pattern of change of this ratio among these three groups suggested a different mechanism for the modulation of collagen synthesis by FGF and ECM. When the media and cell layers were analyzed separately in each group, a common pattern was shown that higher percentages of ratio were existing in the media compartment. This indicated that the media had a

higher fraction of elaborated collagenous proteins compared to the cell layers. That the ratio for the media was decreasing in all three cases as cultures reached to confluence explained the result presented in section C, where a similar pattern of decrease in the [^{3}H]hydroxproline content was noted in the media. Noteworthy was the exceptionally high ratio occurring in the media of group A. This suggested that the higher amount of collagen elaborated in the media of the sparse culture of group A was due to the higher elaboration of collagenous protein at that stage. When the cell layers were examined, the ratio was increasing in group A and C, and reached a steady state in group B. An increasing accumulation of [^{3}H]hydroxyproline in the cell layers of three groups was clearly demonstrated in the previous section. However, the mode by which the cells organized their collagenous matrices in the cell layers seemed to be different. For groups A and C, this proceeded with an ever increasing proportion of collagen incorporated into ECM. For group B, this was due to the increase of total [^{3}H]proline incorporation, since the ratio remained the same.

E. Lysyl oxidase activity under different culture conditions

In the above sections, different patterns of collagen synthesis and deposition between media and cell layers were observed. This led to the suggestion that the control mechanism in these three culture conditions was complex and different. As far as the level of control mechanism was concerned, it could occur at the level of intracellular biosynthesis as well as the level of extracellular deposition. In regard of the latter situation, a complex process has been postulated involving fibrillogenesis and cross-linking in both of which other components of the ECM can participate (Section I, Chapter I). To elucidate whether the effect

of FGF and ECM on collagen deposition into the cell layers could involved the cross-linking of collagen, the pattern of newly-synthesized collagen as measured by [^{3}H]hydroxyproline in media and cell layers was determined in the presence of BAPN (80μg/ml).This is a known lathyrogen and is able to prevent the cross-linking of collagen fibrils by inhibiting lysly oxidase (Table 1, Chapter I). The pattern was compatible with that shown in Fig. 2 (data not shown). It indicated that the pattern of collagen deposition was barely modulated by BAPN, and that the enzyme activity of lysyl oxidase did not play a major role in organizing collagenous matrices in the culture of endothelial cells. This was also confirmed by the comparatively low values of enzyme activity compared to that of fibroblasts, 5000 cpm/10^5 cells (Table 4). However, the activity changed along with the change of culture conditions in this experiment. This suggested that the action mechanism of FGF was possibly not restricted to the modulation of amounts and types of collagen synthesis (Chapter V). A wider range of action involving extracellular deposition was also suggested.

F. Qualitative study of collagen synthesis in the media proteins under three different culture conditions

The types of collagen synthesis was analyzed further by DEAE-cellulose chromatography. Media proteins of the three culture conditions were precipitated by 30% ammonium sulfate. The precipitates were then subjected to DEAE-cellulose chromatography as previously described under Methods of Chapter IV.

The elution profiles did not differ profoundly (Fig. 3). Procollagen type III remained the major collagen elaborated into the media under these three culture conditions (Fig. 3 A, B, and C). The major difference was characterized by the differences in proportional amounts present in the three major portions of the elution profile, the peak which was eluted before the gradient, by the gradient, and by the high salt. The fraction eluted by a salt concentration close to 0.2 M NaCl, represents glycoproteins. The fraction eluted at NaCl concentration higher than 0.2 M represents proteoglycans (Miller, 1971). These results suggested the possibility of modulation of other components of the ECM by FGF and by the pre-existing ECM. This was also supported by different ratios of collagen/total proteins which were presented in Table 3.

Table 3

RELATIVE RATIO OF COLLAGEN IN TOTAL PROTEIN SYNTHESIS IN BOTH MEDIA AND CELL LAYERS OF BOVINE CORNEAL ENDOTHELIAL CELLS UNDER DIFFERENT CULTURE CONDITIONS

A. Plastic-FGF	Day 1	Day 3	Day 5	Week 3
MEDIA	13.2	7.8	7.7	--
CELL LAYERS	1.9	2.1	2.4	--
TOTAL	5.9	4.1	4.1	--
B. Plastic + FGF				
MEDIA	8.9	5.5	5.7	3.4
CELL LAYERS	1.5	1.5	1.5	2.1
TOTAL	6.0	3.1	2.9	2.5
C. ECM-FGF				
MEDIA	4.1	3.5	2.5	--
CELL LAYERS	1.2	1.6	2.8	--
TOTAL	1.8	1.7	2.7	--

NOTE: 1. Ratios presented above were drived from dividing each mean value from Table 2 by each designated mean value from Table 1.

Table 4 LYSLY OXIDASE ACTIVITY UNDER DIFFERENT CULTURE CONDITIONS

	(A) Plastic-FGF	(B) Plastic + FGF	(C) ECM-FGF
MEDIA	80	118	158
CELL LAYERS	0	26	18
TOTAL	80	144	176

NOTE:
1. Activity was expressed as cpm of [^{3}H] release per 10^5 cells.
2. In each assay tube, 1 x 10^6 cpm of [6-^{3}H] lysine-labeled chick calvarial collagen was used as substrate.
3. The assay was performed according to the procedure described by Siegel and Fu (1976).
4. Cultures at the stage of day 6 were used. The results represented the mean of duplicates.

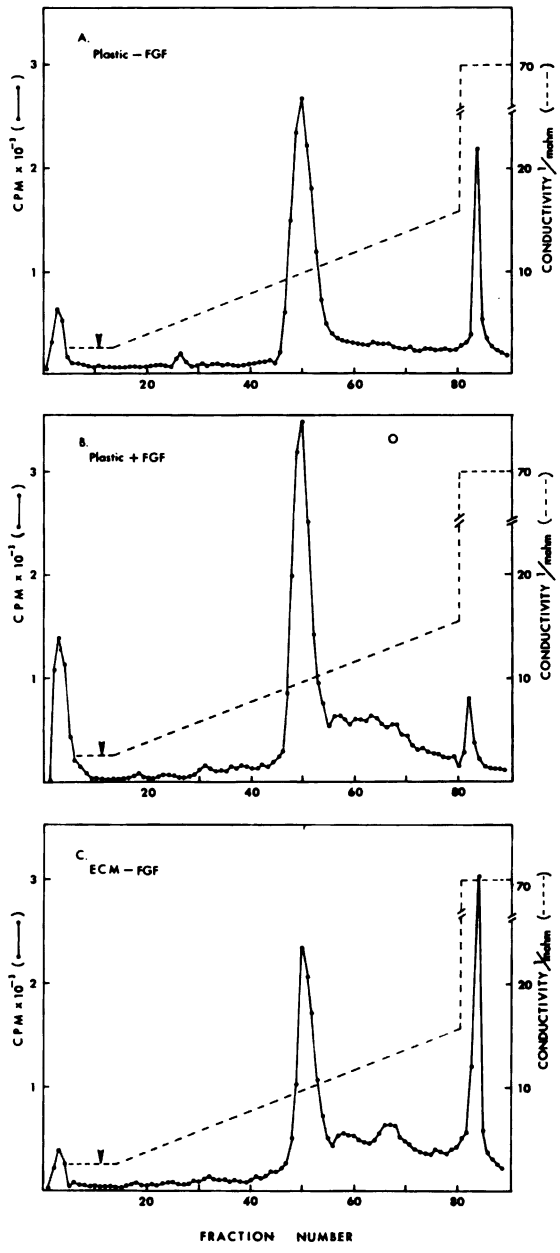


Figure 3:

DEAE-cellulose chromatograms of media proteins of three culture conditions, (A) group A--plastic-FGF, (B) group B--plastic + FGF and (C) group C--ECM-FGF. The major peak eluted by NaCl gradient was identified as procollagen type III. the peak eluted before gradient represented procollagen type IV. The minor peak eluted before the peak for procollagen type III was procollagen type I. Broad peak eluted after procollagen type III cotanined fibronectin and procollagen type V. The peak eluted after gradient by 1M NaCl wash represented proteoglycans and glycosaminoglycans.

IV. DISCUSSION

Cells are intimately associated with their surrounding ECMs onto which cells attach, migrate, proliferate, and differentiate. It has been postulated that such cellular activities are affected by their ECMs although the mechanism by which this occurs has not been established (Chapter I, Section IV).

In the endothelial cell culture system, the normal phenotype of cells grown on plastic plates is lost unless FGF is present (Chapter II, model system). However the normal phenotype is preserved if a natural ECM is used (Chapter II, model system). This suggests that an altered phenotype can result from an artificial extracellular environment or from the absence of a preformed ECM. Collagen, one of the major components of the ECM has been implicated as the primary molecule mediating cell-matrix interaction (Chapter I, Section II). Similarly, synthesis of collagen may itself be modulated by changes in the extracellular substrata. so that the ECM thus formed reflects the altered status of cells and can exert effects on cellular functions. In this chapter, evidence is provided to support this hypothesis, in an attempt to establish an association between the mechanism of action of FGF and that of ECM.

Most of the studies of collagen synthesis *in vitro* have been performed by culturing cells on plastic plates or at best, on collagen-coated plates, which is an artificial and possibly an inadequate environment. The influence of the natural substrata, which more closely resemble the situation *in vivo* has not been analyzed heretofore. Advantage can be taken of the asymmetric deposition of ECMs by corneal endothelial cells and the ease with which large amounts of ECM can be readily prepared.

This cell culture system is a good model for asking such a question as whether collagen synthesis can be modulated by physiological extracellular substrata. The relative amounts of collagen synthesis and deposition between media and cell layers were examined using conditions which compared a normal extracellular substratum with plastic plates. Results demonstrated that collagen synthesis and deposition were modulated significantly by ECM.

When bovine corneal endothelial cells were grown on their own ECMs, the total amount of collagen synthesis was decreased five to six fold and the percentage of collagen deposited into cell layers was proportionately higher compared to that grown on plastic plates (groups A and C of Table 2 and Fig. 2). This study is the first to demonstrate that collagen synthesis and deposition can be modulated by an ECM.

Analysis of collagen production on substrata other than plastic plates has so far been demonstrated in only one instance. In no studies has the synthesis of collagen by cells been measured using cells grown on their own preformed natural substrata. Meier and Hay (1974) demonstrate that killed lens capsule stimulates the synthesis and deposition of collagen by corneal epithelial cells. This system is similar, yet there is an important difference from the present study. The killed lens capsule is regarded as an adjacent tissue instead of the cells' own ECM. The assessment of collagen synthesis as measured by total radiolabeled proline incorporation is not as accurate as measuring radiolabeled hydroxyproline which was used in this report. Therefore, it is difficult to compare the present results with that study.

When an exogeneous ECM was provided, the total amount of collagen synthesis decreased because a certain amount of collagenous material was preexisting. In marked contrast, when cells were grown on plastic plates in which no ECM was provied, cells compensated for this shortage by producing more collagen. Thus a mechanism must exist by which cells sense and respond to the quality of their extracellular environment.

A feed-back regulatory mechanism described herein may have been operating at the the level of the extracellular space. This creates a new conceptual dimension in the regulatory mechanisms of collagen biosynthesis. This will be discussed in greater detail in Chapter VII, under Signifacance and Conclusion.

Not only the total amount of collagen synthesis was lowered, when cells were grown on an ECM but was there proportionately more collagen deposited into cell layers, indicating that an asymmetrical cell surface polarity of secretion was achieved, which mimicked the in vivo situation. In contrast, cells grown on plastic plates without a presupplemented ECM, large amounts of collagen were produced and a higher proportion was elaborated into the media. This was compatible with the altered phenotype, deposition of the ECM over all cell surface, as described in the model system of Chapter II. This indicated that the change in collagen synthesis and patterns of deposition correlated well with the alteration in cell phenotype in these cultured endothelial cells.

Synthesis and deposition of collagen were modulated in different modes as described above. This was resulted from different cellsubstrate interactions. A major difference was that the preformed substrata was provided at the beginning of cell culture. It is noteworthy

that a pattern of collagen deposition, with a higher proportion in cell layers, was present even at the early sparse stage of culture when cells were grown on ECM. This indicated that collagen synthesis accurately reflects the status of cell-matrix interactions. The recognition of the substrata environment is the first event in cell-matrix interactions, which influences subsequent cell functions, as will be mentioned below. It is also noteworthy that when cells are grown on plastic plates, a higher level of collagen synthesis was insufficient and unable to cause reversion of the phenotype back to normal. This was compatible with the study by Gospodarowicz et al. (1980) in which supplementation of collagen alone is shown insufficient for endothelial cells to achieve the degree of proliferation provided by the natural substrata.

Based on this observation, a third condition was evaluated under which the normal phenotype is maintained in plastic plates by adding FGF to the culture media. The synthesis and deposition of collagen was modulated so that cell surface polarity and asymmetric elaboration of collagen was finally achieved but only after confluence. The stepwise manner in the pattern of collagen synthesis and its deposition demonstrated an active role for FGF. At the early sparse stage, a contrasting picture was disclosed in the pattern of distribution of collagen between media and cell layers. Compared to their counterparts in the absence of FGF, a much smaller proportion of collagen was deposited into the cell layers, and the total amount decreased to one half (group A and B, Table 2 and Figure 2). At the later stage the amount of collagen synthesized and deposited in cell layers was gradually increased. This indicated different levels of collagen were needed for the organization of the ECMs during the entire course of cell culture. This also suggested

the participation of other components, such as fibronectin, laminin, glycosaminoglycans, and proteoglycans in organizaing ECM. This postulation was possible, since evidence suggest that several constituents of ECM can modulate the production of ECM. Nevo and Dorfman (1972) demonstrate that synthesis of chondromucoprotein by chondrocytes in suspension culture is enhanced by the addition of sulfated polysaccharides to the cultured medium. Kosher et al. (1973) find that chondromucoprotein enhances the synthesis of sulfated glycosaminoglycans by pre-chondrogenic somites. Meier and Hay (1974) demonstrate that glycosaminogylcans stimulates the synthesis of sulfated glycosaminoglycans but not collagen synthesis in developing cornea. David and Bernfield (1979) demonstrate that the pre-existence of collagen reduces the degradation of glycosaminoglycans by cultured mammary epithelial cells. All these studies suggest a generality, that mutual interactions occur between each of the components of the ECM (see reviews on glycosaminoglycans by Lindah and Hook, 1978; on fibronectin by Ruoslahti et al., 1981; and on collagen by Kleinman et al., 1981).

These results, and those described in Chapter V, suggest that modulation of collagen synthesis and its deposition and integration into an ECM is a complex, dynamic process. The data did not determine whether the action of FGF on collagen synthesis was primary or secondary? Nevertheless, it is clear that the role of FGF in modulation of collagen synthesis and deposition differed from that of ECM in the entire course of cell cultures (groups B and C, Table 2 and Fig. 2). A similar pattern achieved at a later stage (compare 3 week-old cultures of group B with group C) suggested that FGF played an active role of modulating the maturation of ECM and that the requisite ECM was important in maintaining a normal phenotype in vivo.

CHAPTER VII SIGNIFICANCE AND CONCLUSION

In this thesis study, experimental results have demonstrated that synthesis and deposition of collagen by cultured endothelial cells was significantly modulated by FGF in both quantity and quality. Evidence has been provided to support the working hypothesis proposed in the Chapter II, that one, if not all, of the mechanisms of action of FGF is to modulate collagen synthesis. Such a mechanism helps to explain how cellular functions can be affected by a soluble factor such as FGF. In the case of FGF, the effect can be regarded as modulating collagen synthesis, which in turn changes the property of ECM. Therefore, the relationship between a soluble factor, FGF, and an insoluble matrix complex, ECM, is clarified and summarized in Fig. 1. Based on this concept, several conclusions are drawn and are described as follows.

The model suggested by Grobstein and others that ECM does not function merely as a structural support, but contributes to the control of cellular function is further supported by the evidence provided in this thesis. Despite the fact that the exact nature or underlying mechanism of ECM in the control of cellular function is still unsolved, the present study together with several previous investigations in the same line emphasize the important functional role of ECM. The functional role of ECM has been demonstrated in studies of embryonic morphogensis. The present thesis further develop the concept that certain circulating soluble factors operate by modulating ECM production.

The model system states that in order to achieve normal differentiated phenotypic characteristics, endothelial cells derived from cornea and blood vessels require either an exogeneous supplement of ECM

prepared from precedent corneal endothelial cell cultures or the introduction of FGF into the culture media. This is compatible with the preceding formulation. The concept described so far is useful in the understanding of the extracellular environmental factors which are necessary and important for maintaining cellular growth and differentiaton in culture.

As far as the underlying mechanism of ECM in mediating all these cell-matrix interactions is concerned, future studies are required to solve the question, what is the molecular basis for this event to occur. As shown in the introductory Section IV of Chapter 1, compositonal components of ECM are multiple. When temporal organization among these components is considered, the complexity of ECM is even more complicated than one could have predicted. Collagen is known as a major component of ECM. Modulation of collagen synthesis quantatively and qualitatively is believed to change the property of ECM. However from this study, it is still not known whether collagen is the molecule sine qua non mediating all these interactions between cells and their ECMs. Cellular functions are dynamically modulated by ECMs and differentiated phenotypic characteristics are achieved at the final stage. The cell growth in culture resembles to some extent that of the embryonic stage. The indispensibility of ECM for cell growth in culture can be invoked similarly. In culture, cells are plated after being dissociated from tissue or a previous culture. Under such conditions, the surrounding ECM is usually lost. Therefore, unless cells have a capacity to replenish ECM in culture, an abnormal cell-matrix interaction ensues due to the presence of a artificial plastic substratum. As a result, the cellular functions are altered. Therefore, in culture cellular ability to maintain cell growth and differentiation is dependent upon their capacity to make their own ECMs in an appropriate quantity and quality. This capacity

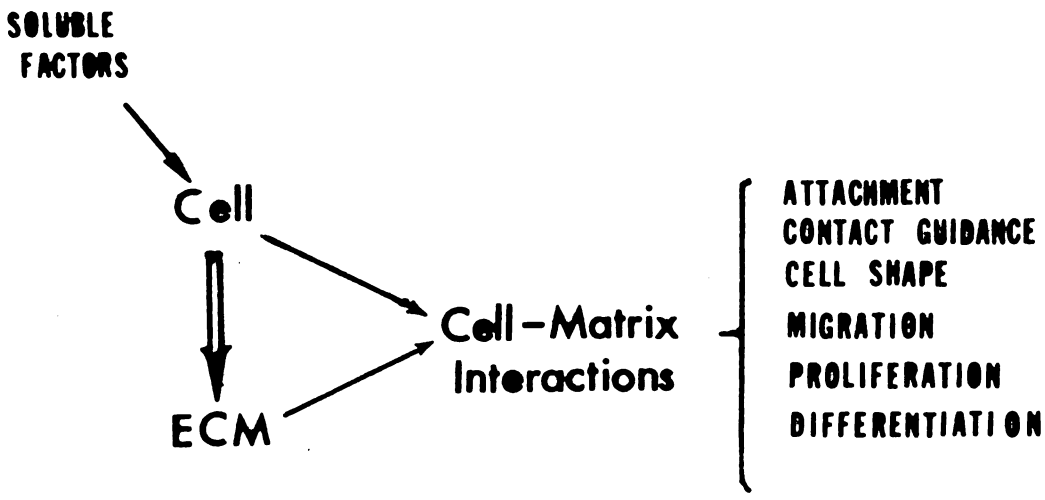


Figure 1

Schematic summary of the relationship between certain soluble factors and extracellular matrix which can exert effects of controlling cell-matrix interactions.

varies among different cell types, relatively limited in epithelial cells, but largely preserved in mesenchymal cells (see Table 6, Chapter I). Based on this concept, the normal cell-matrix interaction can be maintained only when an appropriate ECM is present in the early stages of cell growth in culture systems. This can be achieved either by exogeneously providing the ECM or by facilitating the cell's capacity to make their own ECMs.

Quantitative and qualitative synthesis of collagen is modulated by several soluble factors and processes (Tables 4 and 5, Chapter I). Aberrations of these may correlate with a number of pathologic situations. This study serves as another example to demonstrate the dramatic difference in cell growth and phenotypic expression induced by different culture conditions. In this model system, the major phenotypic expression for abnormal cell growth is characterized by the anomalous deposition and oversynthesis of collagen in cultured endothelial cells. As a result, the property of non-thrombogenicity of the apical cell surface is lost. This process simulates the pathogenesis of a number of vascular diseases which are often times initiated by thrombosis due to abnormal platelet aggregation. The basic mechanism of such an abnormal process resides in the alteration of the regulatory system of collagen synthesis and deposition. This thesis study suggests that a soluble factor such as FGF can control the regulatory system and directs the cells to synthesize and deposit collagen in an appropriate manner. More importantly, the results also demonstrate that anomalous deposition and oversynthesis of collagen resulted from an initial abnormal cell-matrix interaction. This abnormal cell-matrix interaction can result from changes in cellular components or more interestingly, as shown in

the study, it can also be brought forth by the loss of an appropriate ECM. Very obviously, this study creates a new understanding of the regulatory mechanism of collagen synthesis and deposition. As shown in Fig. 2, collagen synthesis is subjected to the influence of the extracellular environment upon which cells rest. This creates a new dimension in understanding the pathogenesis of pathological fibrosis. More importantly, this scheme provides a number of new previously untried testable hypotheses for the pathogenesis of that fibrosis.

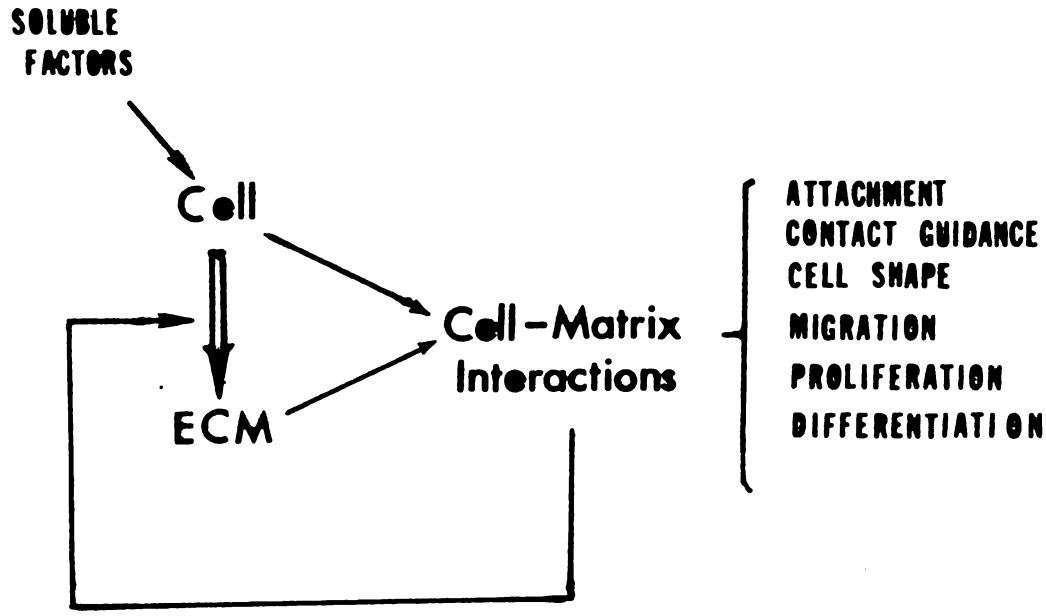


FIGURE 2:

Schematic drawing of the possible mechanism for pathologic fibrosis. Note that the feed-back fashion of control in the regulatory system of synthesizing and depositing matrix components.

REFERENCES

1. Aalto M, Kulonen E (1974) FEBS Lett. 49:70-72.
2. Aalto M, Potila M, Kulonen E (1976) Exp Cell Res. 97:193-202.
3. Abe S, Steinmann BV, Wall LM, Martin GR (1979) Nature 279:442-444.
4. Adams M, Vitasek R, Deyl Z, Felsch G, Musilova J, Olsovska Z (1976) Clin. Chim. Acta 70:61-69.
5. Adams SL, Sobel ME, Howard BL, Olden K, Yamada KM, de Crombrugghe B, Pastan I (1977) Proc. Natl. Acad. Sci. USA 74:3399-3403.
6. Adams SL, Alwine JC, de Crombrugghe B, Pastan I (1979) J. Biol Chem. 254:4935-4938.
7. Alitalo K, Kurkinen M, Vaheri A (1980) Cell 19:1053-1062.
8. Atfield GN, Morris CJOR (1961) Biochem. J. 81: 606-614.
9. Bailey AJ, Bazin S, Sims TJ, Le Lous M, Nicoletis C, Delaunary A (1975) Biochim. Biophys. Acta 405:412-421.
10. Bailey AJ, Sims TJ, Gabbiani G, Bazin S, Le Lous M (1977) Clin. Sci. Mol. Med. 53:499-502.
11. Bailey AJ, Sims TJ, Duance VC, Light ND (1979a) FEBS Lett. 99:361-366.
12. Bailey AJ, Shellswell GB, Duance VC (1979b) Nature 278:67-69.
13. Barnes MJ, Morton LF, Bennet RC, Bailey AJ, Sims TJ (1976) Biochem. J. 157:263-266.
14. Barnes MJ, Morton LF, Levene CI (1978) Biochem. Biophys. Res. Commun. 84:646-653.
15. Baum BJ, Moss J, Breul SD, Crystal RG (1978) J. Biol. Chem. 253:3391-3394.
16. Baum BJ, Moss J, Breul SD, Berg RA, Crystal RG (1980) J. Biol. Chem. 255:2843-2847.

17. Bazin S, Le Lous M, Delaunary A, Bzowski A, Bailey AJ, d'Andiran G, Gabbiani G (1977) C. R. Acad. Sci. D. 285: 439-441.

18. Becker U, Nowack H, Gay S, Timpl R (1976) Immunology 31: 57-65.

19. Bentz H, Bachinger HP, Glanville R, Kuhn K (1978) Eur. J. Biochem. 92: 563-567.

20. Berg RA, Schwartz ML, Crystal RG (1978) Fed. Proc. 38: 801.

21. Bernfield MR, Banerjee SD, Cohn RH (1972) J. Cell Biol. 52: 674-689.

22. Benya PD, Padilla SR, Nimni ME (1977) Biochemistry 16: 865-872.

23. Benya PD, Padilla SR, Nimni ME (1978) Cell 15: 1313-1321.

24. Bhatnagar RS, Prockop DJ (1966) Biochim. Biophys. Acta 130: 383-392.

25. Bhatnagar RS, Hussain MZ, Streifel JA, Tolentino M, Enriquez B (1978) Biochem. Biophys. Res. Commun. 83: 392-397.

26. Bienkowski RS, Baum BJ, Crystal RG (1978a) Nature 276: 413-416.

27. Bienkowski RS, Cowan MJ, McDonald JA, Crystal RG (1978b) J. Biol. Chem. 253: 4353-4363.

28. Birdwell CR, Gospodarowicz D, Nicolson G (1978) Proc. Natl. Acad. Sci. USA 75: 3273-3277.

29. Blumenkrantz N, Sondergaard J (1972) Nature (New Biol.) 239:246.

30. Bornstein P, Piez KA (1966) Biochemistry 5: 3460-3473.

31. Bornstein P, Traub W (1979) in "The Proteins" ed. Neurath H and Hill RL 4: 411-632 (New York, Academic. 3rd ed).

32. Bornstein P, Byers PH (1980) in "Metabolic Disease and Control Metabolism" ed. PK Bondy, LE Rosenberg, pp. 1089-1153 (Philadelphia,Saunders, 8th ed.).

33. Bornstein P, Sage H. (1980) Ann. Rev. Biochem. 49: 957-1003.

34. Bransome ED, Grower MF (1970) Anal. Biochem. 38: 401-408.

35. Brown RA, Shuttleworth CA, Weiss JB (1978) Biochem. Biophys. Res. Commun. 80: 866-872.

36. Bunge RP, Bunge MB (1978) J. Cell. Biol. 78: 943-950.

37. Burgeson RE, Adli EL, Kaitila II, Hollister DW (1976)
Proc. Natl. Acad. Sci. USA 73: 2579-2583.

38. Burgeson RE, Hollister DW (1979)
Biochem Biophys. Res. Commun. 87: 1124-1131.

39. Burke JM, Balian G, Ross R, Bornstein P (1977)
Biochemistry 16:3243-3249.

40. Butler WT, Finch JE Jr., Miller EJ (1977)
J. Biol. Chem. 252:639-643.

41. Byers PH, McKenney KH, Lichtenstein JR, Martin GR (1974)
Biochemistry 13: 5243-5248.

42. Castor CW (1975) Arthritis Rheum. 18:451-460.

43. Cheung HS, Harvey W, Benya PD, Nimni ME (1976)
Biochem. Biophys.Res. Commun. 68:1371-1378.

44. Chlapowski FJ, Kelly LA, Butcher RW (1975)
Adv. Cyclic Nucleotide Res. 6: 245-338.

45. Chung E, Miller EJ (1974) Science 183: 1200-1201.

46. Chung E, Rhodes RK, Miller EJ (1976)
Biochem. Biophys. Res Commun. 71: 1167-1174.

47. Church RL, Tanzer ML, Lapiere CM (1973)
Nature (New Biol.) 244: 188-190.

48. Chvapil M, Madden JW, Charlson EC, Peacock EE (1974)
Exp. Mol. Path. 20: 363-373.

49. Clark CC, Veis A (1972) Biochemistry 11: 494-502.

50. Conrad GW, Hamilton C, Haynes E (1977)
J. Biol. Chem. 252: 6861-6870.

51. Cotta-Pereira G, Sage H, Bornstein P, Ross R, Schwartz S (1980)
J. Cell Physiol. 102: 183-191.

52. Crouch E, Bornstein P (1978) Biochemistry 17: 5499-5509.

53. Crouch E, Bornstein P (1979) J. Biol. Chem. 254: 4197-4204.

54. Crouch E, Sage H, Bornstein P (1980)
Proc. Natl. Acad. Sci. USA 77:745-749.

55. Curtis ASG, Varde M (1964) J. Natl. Cancer. Invest. 33: 15.

56. Cutroneo KR, Costello D, Fuller GC (1971)
Biochem. Pharm. 20:2797-2804.

57. Cutroneo KR, Guzman NA, Liebelt AG (1972)
Cancer Res. 32: 2828-2833.

58. Cutroneo KR, Counts DF (1975) Mol. Pharmacol. 11: 632-639.

59. Daniels JR, Chu GH (1978) J. Biol. Chem. 250: 3531-3537.

60. David G, Bernfield MR (1979)
Proc. Natl. Acad. Sci. USA 76: 786-790.

61. Davison PF, Hong B-S, Cannon DJ (1979) Exp. Eye Res. 29: 97-107.

62. Dehm P, Kefalides NA (1978) J. Biol. Chem. 252: 6680-6686.

63. Deschmukh K, Kline WG (1976a) Eur. J. Biochem. 69: 117-123.

64. Deschmukh K, Kline WG, Sawyer BD (1976b) FEBS Lett. 67: 48-51.

65. Deschmukh K, Kline WG, Sawyer BD (1977a)
Biochim. Biophys. Acta 499: 28-35.

66. Deschmukh K, Sawyer BD (1977b)
Proc. Natl. Acad. Sci. USA 74: 3864-3866.

67. Deschmukh K, Sawyer BD (1978) FEBS Lett. 89: 230-232.

68. Dixit SN, (1978) FEBS Lett. 85: 153-157.

69. Dixit SN, Kang AH (1979) Biochemistry 18: 5686-5692.

70. Dixit SN, Kang AH (1980) Biochemistry 19: 2692-2696.

71. Dodson JW (1963) Exp. Cell Res. 31: 233.

72. Dodson JW, Hay ED (1971) Exp. Cell Res. 65: 215-220.

73. Doran TI, Vidrich A, Sun T-T (1980) Cell 22: 17-25.

74. Duance VC, Restall DJ, Beard H, Bourne FJ, Bailey AJ (1977) FEBS Lett. 79: 248-252.

75. Dunn GA, Ebendal T (1978) Exp. Cell Res. 111: 475-479.

76. Ebendale T (1974) Zoon. 2: 99.

77. Ebendale T (1976) Exp. Cell Res. 98: 159-169.

78. Ehrmann RL, Gey GD (1956) J. Natl. Cancer Inst. 16: 1375-1400.

79. Elsdale T, Bard J (1972) J. Cell Biol. 54: 626-637.

80. Emerman JT, Enami J, Pitelka DR, Nandi S (1977a) Proc. Natl. Acad. Sci. USA 74: 4466-4470.

81. Emerman JT, Pitelka DR (1977b) In Vitro 13: 316-328.

82. Engvall E, Ruoslahti E (1977) Int. J. Cancer 20: 1-5.

83. Engvall E, Ruoslahti E, Miller EJ (1978) J. Exp. Med. 147: 1584-1595.

84. Epstein EH Jr., Scott RD, Miller EJ, Piez KA (1971) J. Biol. Chem. 246: 1718-1724.

85. Epstein EH Jr., (1974) J. Biol. Chem. 249: 3225-3231.

86. Evans CA, Peterkofsky B (1976) J. Cell Physiol. 89: 355-368.

87. Eyre DR (1979) Int. Rev. Connect. Tissue Res. 8: 227.

88. Fessler JH, Fellser LI (1978) Ann. Rev. Biochem. 47: 129-162.

89. Fielding PE, Vlodavsky I, Gospodarowicz D, Fielding CL (1979) J. Biol. Chem. 254: 749-755.

90. Fietzek PP, Kuhn K (1976) Int. Rev. Connect. Tissue Res. 7: 1-60.

91. Fisher M, Solursh M (1979) Exp. Cell Res. 123: 1-14.

92. Fujii T, Kuhn K (1975) Hoppe-Seyler's Z. Physiol. Chem. 356: 1793.

93. Furcht LT, Mosher DF, Wendelschafer-Crabb G, Woodbridge PA, Foidart JM (1979) Nature 277: 393-395.

94. Fracis G, Butler WT, Finch JE Jr. (1978) Biochem. J. 175: 921-930.

95. Freeman IL (1978) Invest. Ophthalmol. Vis. Sci. 17: 171-177.

96. Gabbiani G, Schmid E, Winter S, Chaponnier C, De Chastonay C, Vanderkerckhove J, Weber K, Franke WW (1981) Proc. Natl. Acad. Sci. USA 78: 298-302.

97. Gay S, Balleisen L, Remberger K, Fietzek PP, Adelmann BC, Kuhn K (1975) Klin Wschr. 53: 899-902.

98. Gay S, Martin GR, Muller PK, Timpl R, Kuhn K (1976) Proc. Natl. Acad. Sci. USA 73: 4037-4040.

99. Gay S (1978) in "Collagen-Platelet Interaction" ed. Gastpar H, Kuhn K, Mark R pp 171-179 (New York, Schattauer).

100. Gay S, Miller J (1978) in "Collagen in the Physiology and Pathology of Connective Tissue" (Nw York, Fisher).

101. Gay S, Rhodes RK, Gay RE, Miller EJ (1981) Coll. Res. 1: 53-58.

102. Gey GO, Svotelis M, Foard M, Bang FB (1974) Exp. Cell Res. 84: 63-71.

103. Glanville RW, Rauter A, Fietzek PP (1979) Eur. J. Biochem. 95: 383-389.

104. Goldberg B (1977) Proc. Natl. Acad. Sci. USA 74: 3322-3325.

105. Gospodarowicz D, Moran J, Braun D (1977a) J. Cell Physiol. 91: 377-385.

106. Gospodarowicz D, Mescher AL, Birdwell CR (1977b) Exp. Eye Res. 25: 75-89.

107. Gospodarwicz D, Mescher AL (1978) Natl. Cancer Inst. Monogr. 48: 109-130.

108. Godpodarowicz D, Greenburg G, Birdwell CR (1978a) Cancer Res. 38: 4155-4171.

109. Gospodarowicz D, Bialecki H, Greenburg G (1978b) J. Biol. Chem. 253: 3736-3743.

110. Gospodarowicz D, Greenburg G, Vlodavsky I, Alvarado J, Johnson LK (1979a) Exp. Eye Res. 29: 485-509.

111. Gospodarowicz D, Vlodavsky I, Greenburg G, Alvarado J, Johnson LK, Moran J (1979b) Recent Prog. in Hormone Res. 35: 375-448.

112. Gospodarowicz D, Delgado D, Vlodavsky I (1980a) Proc. Natl. Acad. Sci. USA 77: 4094-4098.

113. Gospodarowicz D, Ill C (1980) J. Clin. Invest. 65: 1351-1364.

114. Godpowarowicz D, Vlodavsky I, Savion N (1981) Vision Res. 21: 87-103.

115. Grant ME, Kefalides NA, Prockop DJ (1972) J. Biol. Chem. 247: 3539-3544.

116. Grant ME, Jackson DS (1976) Essays in Biochemistry 12: 77-113.

117. Green H, Goldberg B (1963) Nature 250: 1097-1098.

118. Green H, Goldberg B (1965) Proc. Natl. Acad. Sci. USA 53: 1360-1365.

119. Green H, Todaro GJ, Goldberg B (1966a) Nature 209: 916-191.

120. Green H, Goldberg G, Todaro GJ (1966b) Nature 212: 631.-633.

121. Grinnell F, Martin D (1978) Proc. Natl. Acad. Sci. USA 75: 4408-4412.

122. Grobstein C (1953) J. Exp. Zool. 124: 383-414.

123. Grobstein C, Cohen J (1965) Science 150: 626-628.

124. Grobstein C (1967) Natl. Cancer Inst. Monogr. 26: 279-299.

125. Grobstein C (1975) in "Extracellular Matrix Influences on Gene Expression" ed. Slavkin HC & Grenlich RC pp 9-16; 804-814 (New York, Academic).

126. Hall DA, Jackson DS (1976) Int. Rev. Connect. Tissue Res. 7: 1-60.

127. Hance AJ, Crystal RG (1977) Nature 268: 152-154.

128. Hance AJ, Crystal RG, (1975) Am. Rev. Resp. Dis. 112: 657-671.

129. Hassell JR, Orkin RW (1976) Dev. Biol. 49: 80-88.

130. Hata R, Peterkofsky B (1977)
Proc. Natl. Acad. Sci. USA 74: 2933-2937.

131. Hauschka SD, Koningsberg IR (1966)
Proc. Natl. Acad. Sci. USA 55: 119-126.

132. Hauschka SD (1974) Dev. Biol. 37: 329-344.

133. Hay ED, Revel JP (1968) in "Epithelial-Mesenchymal Interactions"
ed. Fleischmajer R; Billingham RE, pp 31-55
(Baltimore, Williams & Wilkins Co.).

134. Hay ED (1973) Amer. Zool. 13: 1085-1107.

135. Hay ED (1977) in "Cell and Tissue Interaction" ed. Lash JW, Burger MM
pp. 115-137 (New York, Raven Press).

136. Hay ED (1980) Int. Rev. Cytol. 63: 263-322.

137. Hayakawa T, Hino M, Fuyamada H, Nagatsu T, Aoyama H, Izawa Y (1977)
Clin. Chim. Acta 75: 137-142.

138. Herbage D, Bouillett J, Bernengo J-C (1977)
Biochem. J. 161: 303-312.

139. Hewitt AT, Kleinman HK, Pennypacker JP, Martin GR (1980)
Proc. Natl. Acad. Sci. USA 77: 385-388.

140. Hotlzer H, Abbott J (1968) in "Stability of the Differentiated State"
ed. H. Ursprung, pp. 1-17 (Berline, Springer-Verlag).

141. Hong B-S, Davison PF, Cannon DJ (1979) Biochemistry 18: 4278-4282.

142. Horstadius S (1950) (ed.) The Neural Crest (London, Oxford Univ. Press).

143. Howard BV, Macarak EJ, Gunson D, Kefalides NA (1976) Proc. Natl.
Acad. Sci. USA 73: 2361-2364.

144. Hoard BH, Adams SL, Sobel ME, Pastan I, de Crombrugghe B (1978)
J. Biol. Chem. 253: 5869-5874.

145. Jaffe EA, Minick CR, Adelman B, Becker CG, Nachman R (1976)
J. Exp. Med. 144: 209-225.

146. Jimenez SA, Rosenbloom J (1974)
Arch. Biochem. Biophys. 163: 459-465.

147. Jimenez SA, Bashey RI, Benditt M, Yankowski R (1977)
Biochem Biophys. Res. Commun. 78: 1354-1361.

148. Jimenez SA, Yankowski R, Bashey RI (1978)
Biochem. Biophys. Res. Commun. 81: 1298-1306.

149. Johnson RC, Manasek FJ, Vinson WC, Seyer JM (1974)
Dev. Biol. 36: 252-271.

150. Kao W W-Y, Berg RA (1979) Biochim. Biophys. Acta 586: 528-536.

151. Kefalides NA, Denduchis B (1969)
Biochim. Biophys. Acta 400: 121-131.

152. Kefalides NA (1971) Biochem. Biophys. Res. Commun. 45: 226-234.

153. Kefalides NA (1973) Int. Rev. Connect. Tissue Res. 6: 63-104.

154. Kefalides NA, Cameron JD, Tomichek EA, Yanoff M (1976)
J. Biol. Chem. 251: 730-733.

155. Kemshead JT, Askonas BA (1978) Immunology 34: 1071-1076.

156. Kent G, Gay S, Inouye T, Bahn R, Minick OT, Popper H (1976)
Proc. Natl. Acad. Sci. USA 73: 3719-3722.

157. Killen PD, Striker GE (1979)
Proc. Natl. Acad. Sci. USA 76: 3518-3522.

158. Kivirikko KI (1970) International Rev. of Connective Tissue
5: 93-163.

159. Klebe RJ (1975) J. Cell Physiol. 86: 231-236.

160. Kleinman HK, McGoodwin EB, Martin GR, Klebe RJ, Fietzek PP, Woolley
DE (1978a) J. Biol. Chem. 253: 5642-5646.

161. Kleinman HK, Murray JC, McGoodwin EB, Martin GR (1978b)
J. Invest. Derm. 41: 9-11.

162. Kleinman HK, McGoodwin EB, Rennard SI, Martin GR (1979) Anal. Biochem. 94: 308-312.

163. Kleinman HK, Klebe RJ, Martin GR (1981) J. Cell Biol. 88: 473-485.

164. Konigsberg IR, Hauschka SD (1965) in "Reproduction-Molecular Submolecular and Cellular" ed. Locke, M. pp 243-290 (New York, Academic Press).

165. Kosher RA, Church RL (1975) Nature 258: 327-329.

166. Kosher RA (1976) Dev. Biol. 53: 265-276.

167. Krause NJ, Rowe DW, Fujimoto WY, Bornstein P (1978) Biochim. Biophys. Acta 540: 101-116.

168. Kresina TF, Miller EJ (1979) Biochemistry 18: 3089-3097.

169. Krieg T, Horlein D, Wiestner M, Muller PK (1978) Arch. Dermatol. Forsch. 263: 171-180.

170. Kosher RA, Lash JW, Minor RR (1973) Dev. Biol. 35: 210-220.

171. Kuloneu E, Potila M (1975) Biochem. Pharm. 24: 1671-1678.

172. Kruze D, Wierzchowski P (1967) Anal. Biochem. 19: 226-233.

173. Langness U, Udenfriend S (1974) Proc. Natl. Acad. Sci. USA 71: 50-51.

174. Lash JW, Vasan NS (1977) in "Cell and Tissue Interactions" ed. Lash JW, Burger MM. pp 101-113 (New York, Raven Press).

175. Laurent M, Lonchampt M-O, Regnault F, Tassin J, Courtois Y (1978) Exp. Cell Res. 115: 127-142.

176. Le Douarin NM, Teillet MA, Le Lievre C (1977) in "Cell and Tissue Interactions" ed. Lash JW, Burger MM. pp. 11-26 (New York, Raven Press).

177. Lembach KJ, Branson RE, Hewgley PB, Cunningham LW (1977) Eur. J. Biochem. 72: 379-383.

178. Lesot H, von der Mark K, Ruch J (1978)
C.R. Acad. Sci. Ser. D. 286: 765-768.

179. Levene CI, Heslop J (1977) J. Mol. Med. 2: 145-151.

180. Levinson W, Bhatnager RS, Liu T-Z (1975)
J. Natl. Cancer Invest. 55: 807-810.

181. Lichtenstein JR, Martin GR, Kohn LD, Byers PH, McKusick VA (1973)
Science 182: 298-300.

182. Lin RC, Snodgrass PJ (1975) Biochem. Biophys. Res. Commun. 64: 725-734.

183. Lindahl U, Hook M (1978) Ann. Rev. Biochem. 47: 385-477.

184. Lindstedt S, Prockop DJ (1961) J. Biol. Chem. 236: 1399-1403.

185. Linsenmayer TF, Toole BP, Trelstad RL (1973) Dev. Biol. 35: 232-239.

186. Linsemayer TF, Gibney E, Toole BP, Gross J. (1978)
Exp. Cell Res. 116: 470-474.

187. Liotta LA, Vembu D, Kleinman HK, Martin GR, Boone C (1978)
Nature 272: 622-624.

188. Liotta LA, Wicha MS, Foidart JM, Rennard SI, Garbisa S, Kidwell WR
(1979) Lab. Invest. 41: 511-518.

189. Liu S, Karasek M (1978) J. Invest. Dermat. 70: 288-293.

190. Long E, Kohler V, Kelly MJ (1976) in "Liquid Scintillation Science
and Technology" ed. Noujaim AA, Ediss C, Wiebe LI,
pp 47-68 (New York, Academic Press).

191. Lopoashov GV, Stroeva OG (1961) Adv. Morphogenesis 1: 331-337.

192. MacPherson I, Montagnier L (1964) Virology 23: 291-294.

193. Madri JA, Furthmayer H (1979) Am. J. Pathol. 94: 323-332.

194. Man M, Adams E (1975) Biochem. Biophys. Res. Commun. 66: 9-16.

195. Mayne R, Vail MS, Miller EJ (1975)
Proc. Natl. Acad. Sci. USA 72: 4511-4515.

196. Mayne R, Vail MS, Mayne PM, Miller EJ (1976a) Proc. Natl. Acad. Sci. USA 73: 1674-1678.

197. Mayne R, Vail MS, Miller EJ (1976b) Dev. Biol. 54: 230-240.

198. Mayne R, Vail MS, Miller EJ (1978) Biochemistry 17: 446-451.

199. Maxwell GD (1976) Cell Tissue Res. 172: 325-330.

200. McCullagh KA, Balian G (1975) Nature 258: 73-75.

201. McGee J O'D, O'Hare RP, Patrick RS (1973) Nature (New Biol.) 243: 121-123.

202. Meier S, Hay ED (1974a) Dev. Biol. 38: 249-270.

203. Meier S, Hay ED (1974b) Proc. Natl. Acad. Sci. USA 71: 2310-2313.

204. Michalopoulos G, Pitot HD (1975) Exp. Cell Res. 94: 70-78.

205. Miller EJ, Matukao VJ (1969) Proc. Natl. Acad. Sci. USA 64: 1264-1268.

206. Miller EJ (1971) Biochemistry 10: 1652-1658.

207. Miller EJ, Epstein EH Jr., Piez KA (1971) Biochem. Biophys. Res. Commun. 42: 1024-1029.

208. Miller EJ, Mathews (1974) Biochem. Biophys. Res. Commun. 60: 424-430.

209. Miller EJ (1976) Mol. Cell Biochem. 13: 165-192.

210. Muir HM (1977) in "Cell and Tissue Interactions" ed. Lash JW, and Burger MM, pp. 32-42 (New York, Raven Press).

211. Muller PK, Lemmen C, Gauss V, Kuhn K (1977) Exp. Cell Res. 108: 47-55.

212. Murray JC, Stingle G, Kleinman HK, Martin GR, Katz SI (1979) J. Cell Biol. 80: 197-202.

213. Myllyla R, Kuutti-Savolainen E-R, Kivirikko KI (1978) Biochem. Biophys. Res. Commun. 83: 441-448.

214. Nakagawa H, Bentley JP (1970) J. Pharm. Pharmacol. 23: 399-406.

215. Newsome DA (1976) Dev. Biol. 49: 496-507.

216. Narayanan AS, Page RC (1977) FEBS Lett. 80: 221-224.

217. Neville DM Jr. (1971) J. Biol. Chem. 246: 6328-6334.

218. Nevo A, Dorfman A (1972) Proc. Natl. Acad. Sci. USA 69: 2069-2072.

219. Nigra TP, Martin GR, Eagle H (1973) Biochem. Biophys. Res. Commun. 53: 272-281.

220. Nimni NE (1980) Biorheology 17: 51-82.

221. Norby DP, Malemud CG, Sokoloff L (1977) Arthritis Rheum. 20: 709-716.

222. Ochoa S, de Hara C (1979) Ann. Rev. Biochem. 48: 549-580.

223. Ohara PT, Buck RC (1979) Exp. Cell Res. 121: 235-249.

224. Oohira A, Kimata K, Suzuki S, Takata K, Suzuki I, Hoshino M (1974) J. Biol. Chem. 249: 1637-1645.

225. Paglia L, Wilczek J, de Leon LD, Martin GR, Horlein D, Muller PK (1979) Biochemistry 18: 5030-5034.

226. Parry G, Soo W-J, Bissell MJ (1979) J. Biol. Chem. 254: 11763-11766.

227. Pearlstein (1978) Int. J. Cancer 22: 32-35.

228. Peng CT (ed.) (1977) in "Sample Preparation in Liquid Scintillation Counting", pp. 42-55, (Arlington Heights, Amersham Co.).

229. Perez-Tamayo R (1978) Am. J. Path. 92: 508-537.

230. Peterkofsky B, Prockop DJ (1962) Anal. Biochem. 4: 400-406.

231. Peterkofsky B (1972) Biochem. Biophys. Res. Commun. 49: 1343-1350.

232. Peterkofsky B, Prather WB (1974) Cell 3: 291-299.

233. Pope FM, Martin GR, Lichtenstein JR, Penttinen R, Gerson B, Rowe DW, McKusick VA (1975) Proc. Natl. Acad. Sci. USA 72: 1314-1316.

234. Pouyssegur J, Pastan I (1979) Exp. Cell Res. 121: 373-382.

235. Praus R, Brettschneider I, Adam M (1979)
Exp. Eye Res. 29: 469-477.

236. Prockop, DJ, Berg RA, Kivirikko KI, Uitto J (1976) in "Biochemistry of Collagen", ed. Ramachandran GN, Reddi AH, pp 163-273.

237. Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA (1979)
N. Engl J. Med. 301: 13-23, 75-85.

238. Rath NC, Reddi AH (1979) Nature 278: 855-857.

239. Reddi AH, Anderson WA (1976) J. Cell Biol. 69: 557-572.

240. Reddi AH, Gay R, Gay S, Miller EJ (1977)
Proc. Natl. Acad. Sci. USA 74: 5589-5592.

241. Reid LM, Rojkind M (1979) Methods in Enzymology 58: 263-278.

242. Rhodes RK, Miller EJ (1978) Biochemistry 17: 3442-3448.

243. Risteli J, Tryggvason K, Kivirikko KI (1977)
Eur. J. Biochem. 73: 485-492.

244. Rojkind M, Gonzalez E (1974) Anal. Biochem. 57: 1-7.

245. Rojkind M, Kershenobick D (1975) in "Collagen Metabolism in the liver" ed. Popper H, Becker K., pp. 129-138 (New York, Stratton).

246. Ronnemaa T, Juva K, Kulonen E (1975) Atherosclerosis 21: 315-324.

247. Rosenbloom J, Prockop DJ (1971) J. Biol. Chem. 246: 1549-1555.

248. Ross R, Glomset JA (1973) Science 180: 1332-1339.

249. Rovensky YA, Slavnaya IL, Vasiliev JM (1971) Exp. Cell Res. 65: 193-201.

250. Rowe DW, Moen RC, Davison JM, Byers PH, Bornstein P, Palmiter RD (1978) Biochemistry 17: 1581-1590.

251. Ruoslahti E, Vuenta M, Engvall E (1978)
Biochim. Biophys. Acta 534: 210-218.

252. Ruoslahti E, Engvall E, Hayman EG (1981) Coll. Res. 1: 95-128.

253. Sabin FR (1920) Contrib. Embryol. Carneg. Inst. 9: 213-262.

255. Sandmeyer S, Bornstein P (1979) J. Biol. Chem. 254: 4950-4953.

255. Sage H, Bornstein P (1979) Biochemistry 18: 3815-3822.

256. Sage H, Woodbury RG, and Bornstein P (1979a) J. Biol. Chem. 254: 9893-9900.

257. Sage H, Crouch E, and Bornstein P (1979b) Biochemistry 18: 433-442.

258. Sage H, Pritzl P, Bornstein P (1981) Biochemistry 20: 436-442.

259. Schmut O (1977) Exp. Eye Res. 25: 505-509.

260. Schor SL, Court J (1979) J. Cell Sci. 38: 267-281.

261. Schwartz S (1978) In Vitro 14: 966-980.

262. Siegel RC, Pinnell SR, Martin GR (1970) Biochemistry 9: 4486-4492.

263. Siegel RC (1974) Proc. Natl. Acad. Sci. USA 71: 4826-4830.

264. Siegel RC, FU, JCC (1976) J. Biol. Chem. 251: 5779-5785.

265. Siegel RC (1977) J. Biol. Chem. 252: 254-259.

266. Sirica AE, Richards W, Tsukada Y, Sattler CA, Pitot HE (1978) Proc. Natl. Acad. Sci. USA 76: 283-287.

267. Slavkin HC, Trump GN, Brownell A, Sorcente N (1977) in " Cell and Tissue Interactions" ed. Lash JW and Berger MM, pp. 29-46 (New York, Raven).

268. Smith BD, Byers PH, Martin GR (1972) Proc. Natl. Acad. Sci. USA 69: 3260-3262.

269. Smith BD, Martin GR, Miller EJ, Dorfman A, Swarm R (1975) Arch Biochem. Biophys. 166: 181-186.

270. Stenman S, Vaheri A (1978) J. Exp. Med. 147: 1054-1063.

271. Stenn KS, Madri JA, Roll FJ (1979) Nature 277: 229-232.

272. Stern R, Wilczek J, Thorpe WP, Rosenberg SA, and Cannon G (1980) Cancer Res. 40: 325-328.

273. Stoker M, O'Neill C, Berryman S, Waxman V (1968)
Int. J. Cancer 3: 683-693.

274. Sundar Raj CV, Freeman IL, Church RL, Brown SI (1979)
Invest. Ophthal. Vis. Sci. 18: 75-84.

275. Swann DA, Constable E, Harper E (1972)
Invest. Ophthalmol. 11: 735-738.

276. Switzer BR, Summer CK (1971) Anal. Biochem. 39: 487-491.

277. Szmanovicz G, Mercier O, Bandoux A, and Borel JP (1979)
Biochim. 60: 499-503.

278. Takeuchi T, Prockop DJ (1969) Biochim. Biophys. Acta 175: 142-155.

279. Takeuchi T, Rosenbloom J, Prockop DJ (1969)
Biochim. Biophys. Acta 175: 156-164.

280. Thesleff I, Stenman S, Vaheri A, Timpl R (1979)
Dev. Biol. 70: 116-126.

281. Thompson EB, Lippman ME (1974) Metabolism 23: 159-202.

282. Timpl R, Martin GR, Bruckner P, Wick G, Wiedemann (1978)
Eur. J. Biochem. 84: 43-52.

283. Timpl R, Bruckner P, Fietzek PP (1979a) Eur. J. Biochem. 95: 255-263.

284. Timpl R, Rhode H, Robey PG, Rennard SI, Foidart J-M, Martin GR (1979b)
J. Biol. Chem. 254: 9933-9937.

285. Toole BP, Jackson G, Gross J (1972)
Proc. Natl. Acad. Sci. USA 69: 1384-1386.

286. Trelstad RL, Hay ED, Revel JP (1967) Devel. Biol. 16: 78-106.

287. Tryggvason K (1977) Eur. J. Biochem. 7: 177-180.

288. Tryggvason K, Kivirikko KI (1978) Nephron 21: 230-235.

289. Tseng SCG, Stern R, Nitecki D (1980) Anal. Biochem. 102: 291-299.

290. Tseng SCG, Savion N, Gospodarowicz D, Stern R (1981) J. Biol. Chem. 256: 3361-3365.

291. Uitto J, Prockop DJ (1974) Biochim. Biophys. Acta 336: 234-251.

292. Vater CA, Harris ED Jr., Siegel RC (1979) Biochem. J. 181: 639-645.

293. Victor PT, Rohrback DH, Martin GR (1980) Cell 22: 719-726.

294. Vlodavsky I, Fielding PE, Fielding CJ, Gospodarowicz D (1978) Proc. Natl. Acad. Sci. USA 75: 356-360.

295. Vlodavsky I, Johnson LK, Greenburg G, Gospodarowicz D (1979) J. Cell Biol. 83: 468-486.

296. von der Mark H, von der Mark K, Gay S (1976a) Dev. Biol. 53: 153-170.

297. von der Mark H, von der Mark K, Gay S (1976b) Dev. Biol. 53: 237-249.

298. von der Mark K, von der Mark H, Timpl R, Trelstad RL (1977a) Dev. Biol. 59: 75-85.

299. von der Mark K, von der Mark H (1977b) J. Bone Jt. Surg. Br. 53: 205-208.

300. von der Mark K, Gauss V, von der Mark H, Muller P (1977) Nature 267: 531-532.

301. von der Mark H, von der Mark K (1979) FEBS Lett. 99: 101-105.

302. Weber L, Meigel WN, Spier W (1978) Arch. Dermatol. Res. 261: 63-71.

303. Weiss P (1959) Rev. Modern Phys. 31: 11-20.

304. Weiss JB, Shuttleworth CA, Brown R, Sedowfia K, Baildam A, Hunter JA (1975) Biochem. Biophys. Res. Commun. 65: 907-912.

305. Werum LN, Gordon HT, Thornburg W (1960) J. Chromat. 3: 125-145.

306. Wessells NK (1964) Proc. Natl. Acad. Sci. USA 52: 252-259.

307. Wessells NK, Cohen JH (1968) Dev. Biol. 18: 294-309.

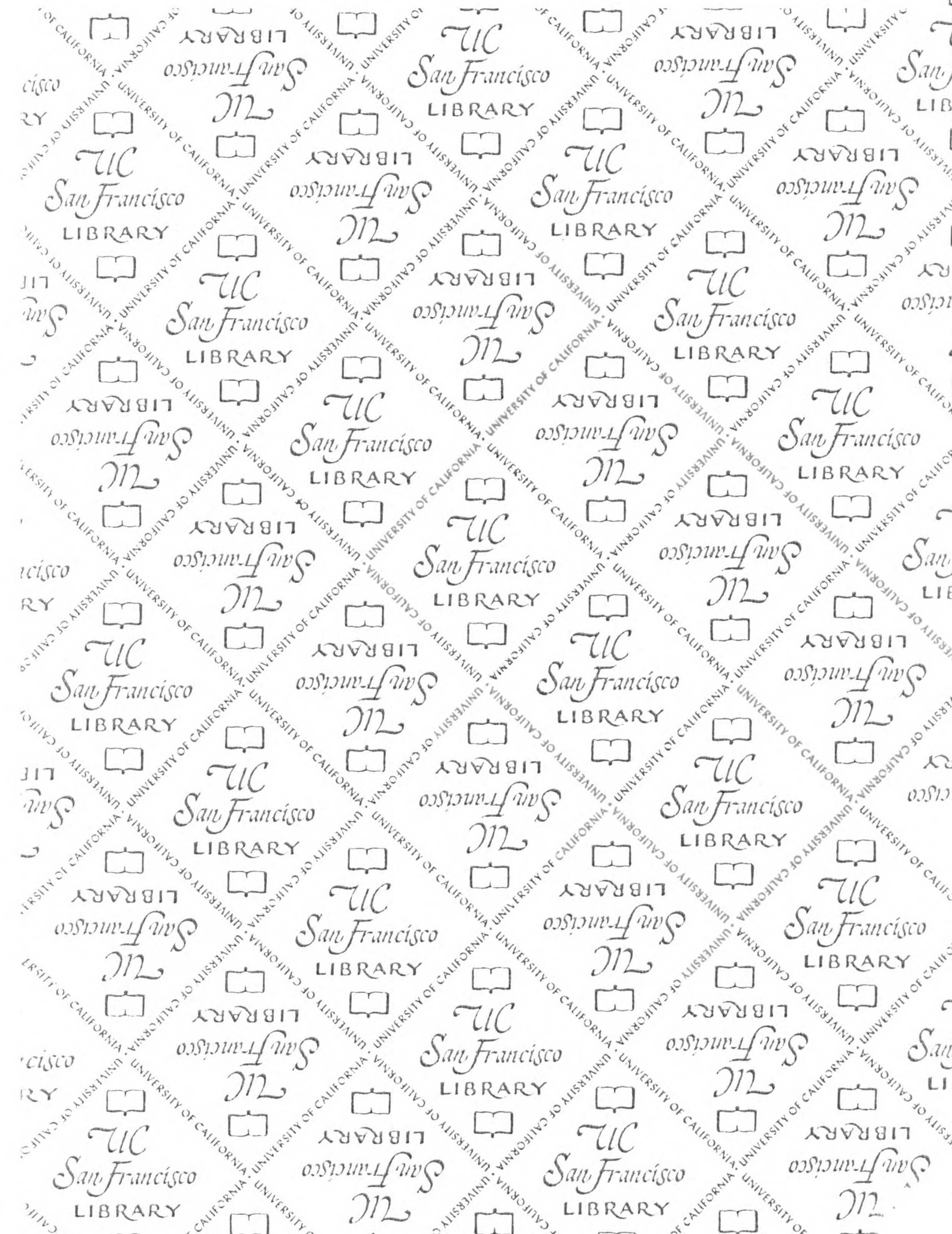
308. West CM, Lauza R, Rosenbloom J, Lowe M, Holtzer H (1979) Cell 17: 491-501.

309. Wiestner M, Krieg T, Horlein D, Glanville RW, Fietzek PP, Muller PK (1979) J. Biol. Chem. 254: 7016-7023.

310. Whitehouse MW, Bostrom H (1965) Biochem. Pharm. 14: 1173-1184.

311. Yang J, Richard J, Bowman P, Cuzman R, Enami J, McCormick K, Hamamoto S, Pitelka D, Nandi S (1979) Proc. Natl. Acad. Sci. USA 76: 3401-3405.

312. Yen EHK, Sodek J, Melcher AH (1979) Biochem. J. 178: 605-612.


UC
San Francisco
LIBRARY
UNIVERSITY OF CALIFORNIA · UNIVERSITY OF CALIFORNIA