1983

# COLLAGENASE AND ELASTASE ACTIVITIES IN HUMAN AND MURINE CANCER CELLS AND THEIR MODULATION BY DIMETHYLFORMAMIDE

David Ray Olsen
*University of Rhode Island*

# COLLAGENASE AND ELASTASE ACTIVITIES IN HUMAN AND MURINE CANCER CELLS AND THEIR MODULATION BY DIMETHYLFORMAMIDE

BY

DAVID RAY OLSEN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

1983

MASTER OF SCIENCE THESIS

OF

DAVID RAY OLSEN

APPROVED:

Thesis Committee

Major Professor _____

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A. A. Michel

Dean of the Graduate School

UNIVERSITY OF RHODE ISLAND

1983

## ABSTRACT

Olsen, David R.; M.S., University of Rhode Island, 1983. Collagenase and Elastase Activities in Human and Murine Cancer Cells and Their Modulation by Dimethylformamide. Major Professor: Dr. Clinton O. Chichester.

The transformation from carcinoma *in situ* to invasive carcinoma occurs when tumor cells traverse extracellular matracies allowing them to move into parenchymal tissues. Tumor invasion may be aided by the secretion of collagen and elastin degrading proteases from tumor and tumor-associated cells. In this study the production of Type I and Type V collagen degrading activities and elastolytic activities by DLD-1 human colon carcinoma cells, B16-F10 murine melanoma cells, and normal human dermal fibroblasts was examined. DLD-1 cells and normal fibroblasts produced similarly high levels of collagenolytic activity. DLD-1 cells also produced high levels of elastinolytic activity; this activity was found exclusively in the extracellular medium. DLD-1 cells and normal fibroblasts produced more collagen and elastin degrading activity than did B16-F10 melanoma cells, a cell line characterized as highly metastatic. The Type I and Type V collagenolytic

activities from DLD-1 cells were separated and characterized using DEAE cellulose chromatography and gel filtration chromatography. The Type I collagenolytic activity appeared to be an anionic protein at pH 8.3, and two forms of the enzyme were detected, one with a molecular weight of 60,000 and the other with a molecular weight of 35,000 daltons. The Type V collageno-ytic activity was a cationic protein at pH 8.3 and two forms of this activity were detected, one with a molecular weight of 80,000 and the second with a molecular weight of 54,000 daltons.

The effect of the polar solvent, dimethylformamide (DMF) on the production of collagen and elastin degrading activities was studied using cultured DLD-1 cells. DMF treated cells produced significantly higher levels of Type I collagenolytic, Type V collagenolytic and cell-associated elastase activities than did control cells. DMF treatment had no significant effect on media elastase levels. Treatment of DLD-1 cells with DMF elevated the cellular levels of protein and RNA while these same cells had less DNA per cell. DMF treated cells were also able to synthesize protein at a significantly faster rate than control cells. This increased rate of protein synthesis may account for part of the increased production of connective tissue degrading enzymes seen in DMF treated cells.

However, the effect of DMF on enzyme production was still present when the cells were treated with cycloheximide. The failure of cycloheximide to prevent the DMF induced increase in enzyme activity suggests that the effect of DMF is not entirely dependent on protein synthesis. These data suggest that DLD-1 human colon carcinoma cells produce at least two different collagen degrading enzymes and that DMF treatment may facilitate the invasive movement of cancer cells during the matastatic process by increasing the secretion of connective tissue degrading enzymes.

## ACKNOWLEDGEMENTS

The author wishes to thank Dr. Clinton Chichester for his guidance, encouragement, and dedicated support throughout the research and writing of this thesis.

I would like to thank my parents for their endless encouragement, advice, and support. Lastly, I wish to extend a special thank you to Ellen Austin.

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# INTRODUCTION

The metastatic growth of secondary neoplasms is a major problem facing the oncologist because the pharmacological agents and medical interventions available cannot sufficiently control this spread. The development of multiple metastatic foci leads to the loss of the normal function of the invaded tissues or organs and ultimately results in death. The metastatic process begins when tumor cells or tumor emboli disseminate from the primary tumor mass and invade the vasculature entering the circulation. The transported tumor cells arrest and invade the parenchymal tissue of adjacent organs, proliferate and develop into secondary metastases. During the invasion phase of metastasis, connective tissue matracies are traversed. Electron micrographs of the invaded regions reveal the local dissolution of basement membranes (Birbeck and Wheatly, 1965). The mechanism of basement membrane destruction is uncertain.

The extracellular matrix (ECM) is composed of several macromolecules including elastin, collagen, and proteoglycans. Elastin and collagen are resistant to degradation by most proteases. The only enzymes which can degrade collagen are collagenases. Enzymes which specifi-

cally degrade elastin are termed elastases.

Elastase activity is associated with several tumor associated cells and tumor cell lines (Mainardi et al, 1980a; Kao et al, 1982). Collagenases are also synthesized by several tumor and tumor associated cells (Horwitz et al, 1977; Mainardi et al, 1980a,b; O'Grady et al, 1982) and recently collagenases specific for the different types of collagen have been identified in the medium of certain tumor cell lines (Liotta et al, 1981a,b; Salo et al, 1983). During invasion, elastases and collagenases may be involved in the local destruction of basement membranes and stromal tissues.

In this study Types I and V collagen degrading activities and elastase activities were measured in several tumor cell lines. The collagenolytic activities which were secreted by cultured DLD-1 human colon carcinoma cells were analyzed by ion exchange chromatography and gel filtration chromatography to determine if different enzymes were responsible for the degradation of Types I and V collagen. The results of these studies may indicate that destruction of ECM components by invading tumor cells results from the action of more than one specific degradative enzyme.

A novel attempt to control the spread of cancer has been the use of biological inducers as chemotherapeutic agents. Biological inducers are compounds which induce malignant cancer cells to differentiate into benign cells (Sachs, 1981). One such compound, dimethylformamide (DMF) has induced a human colon carcinama cell line, DLD-1, to differentiate *in vitro* and these treated cells have a reduced tumorigenic capacity *in vivo* (Dexter *et al*, 1979). This study will also examine the effects of DMF treatment on the levels of connective tissue degrading proteases in the DLD-1 cell line. The reduction in tumorigenicity seen with DMF may be paralleled by a decrease in the cell's ability to invade host tissues.

# LITERATURE SURVEY

## Metastasis

Metastasis is a multistep process in which cells derived from a primary tumor detach and are transported to a distant site where they form a secondary tumor (Poste and Fidler, 1980). The metastatic process is initiated when cells or clumps of cells dissociate from the primary tumor and enter blood or lymph vessels. The tumor cells interact with host cells as they are transported by the circulation or lymph. During this process some of the tumor cells may be destroyed by host defense mechanisms (Old et al, 1961). The circulation of tumor cells stops when they encounter a vessel which is too small to allow passage (Ziedman, 1961) or when they attach to vascular endothelium due to cell-cell interactions (Winkelhade and Nicolson, 1976). The cells may exit the vessel at this point, invade surrounding tissue, proliferate, and develop into a secondary tumor. Alternatively the arrested cells may be engulfed and destroyed by host macrophages and lymphocytes (Vose, 1980; Hibbs et al, 1977).

The intravenous injection of mice with syngeneic tumor cells is an experimental model of metastasis. This model is employed to study various aspects of the metastatic process including the organ distribution of blood borne tumor cells (Fidler, 1973). The growth of tumors in specific organs following intravenous injection of cells has been demonstrated with several cell lines (Kinsey, 1960; Fidler and Nicoloson, 1976; Nicoloson and Winkelhade, 1975; Brunson et al, 1978). Cell types which metastasize to specific organs and variants of the same parent line which have different capacities to form tumor nodules following intravenous injection exhibit difference in their cell surface components (Dobrassy et al, 1981; Brunson et al, 1978; Raz et al, 1980). Cell lines which are characterized as "highly metastatic" following intravenous injection have greater quantities of cell surface sialic acid, increased levels of sialyltransferase and other cellular glycosidase activities (Dobrassy et al, 1981). Sialic acid is a component of cell surface glycoproteins involved in cell-cell and cell-substratum adhesion. Others have used the intravenous injection model with different tumor cell lines and noted random growth of metastases (Proctor, 1976). Tumor nodules arose in organs where capillary beds were first encountered.

Models of metastasis which employ intravenously injected cells are inadequate because they do not allow the examination of the entire metastatic process. They bypass the first step of metastasis when cells break off the primary tumor and invade the vasculature. In the future better models will further our understanding of the pathogenesis of metastasis.

## Invasion

During invasion tumor cells traverse stromal tissue and blood vessels to infiltrate the parenchyma of host organs. The invading cells then proliferate to eventually form secondary tumors. The mechanisms involved in the invasive movement of tumor cells is not completely understood. Invasion may occur as the result of mechanical pressures exerted by rapidly proliferating tumor cells (Eaves, 1973). The fast growing tumors create regions of increased pressure and invasion occurs at points where the host tissues were weakest. Infiltration of host tissues may also result from tumor cell products acting on stromal tissues and blood vessels (Fidler et al, 1978). Tumor cells synthesize and secrete proteases capable of degrading the host ECM (Dresden et al, 1972; Hashimoto et al, 1973). Areas where the matrix

is weakened will then be invaded by proliferating tumor cells. Invasion of target organs by malignant tumor cells *in vitro* can be inhibited when the incubation medium contains several types of protease inhibitors (Latner *et al*, 1973).

Stromal tissues and blood vessels are partially composed of the structural proteins collagen and elastin, these proteins are degraded by specific enzymes called collagenases and elastases. Analysis of the media from cultured tumor cells has confirmed the presence of collagenase and elastase activities (Liotta *et al*, 1979; Kao *et al*, 1982; O'Grady *et al*, 1982). These enzymes may aid invading tumor cells but this has not been proven. Invasion most likely results from a combination of mechanical forces exerted by proliferating tumor cells and degradative enzymes secreted by tumor and tumor associated cells.

## Modulation of Tumor Cell Growth

Tumor cells established *in vitro* are less differentiated than the normal cell-type from which they originated (Market, 1968). The decreased extent of differentiation characteristic of malignant cells is

thought to result from an imbalance between the number of genes which express and suppress normal growth and differentiation (Rabinowitz and Sachs, 1970). In vitro induction of differentiation of tumor cells has been achieved using several compounds. Dimethylsulfoxide has induced erythroleukemia cells to differentiate resulting in their synthesis of hemoglobin (Friend et al, 1971). Cyclic adenosine 3′, 5′ monophosphate and dibutyryl cyclic adenosine monophosphate stimulated cellular differentiation leading to increased melanin synthesis, cellular enlargement and increased dendrite formation in B16 melanoma cells (Kreider et al, 1973). Hexamethylene bisacetamide induced morphological changes (cell elongation) and maturational changes in a malignant mesenchymal cell line (Rabson et al, 1977). The agent caused a shift from the synthesis of Type III collagen to the synthesis of Type I collagen. Dimethylformamide (DMF) has induced morphological changes and reduced the tumorigenicity of rhabdomyosarcoma cells (Dexter, 1977). DMF has also induced the loss of tumorigenicity and clonogenicity of the human colon carcinoma cell line DLD-1 (Dexter et al, 1979). DLD-1 cells treated in vitro with DMF have exhibited a decreased expression of tumor associated antigens and an increase in the amount of normal colonic surface antigen (Hager et al, 1980). DMF alters several in vitro growth

characteristics of DLD-1 cells causing their doubling time to increase and their saturation density to decrease (Dexter et al, 1979). These data suggest that DMF has induced a malignant cell type to differentiate and mature into a cell-type with benign phenotypes. The effect of biological inducers such as DMF on the invasive capacity of tumor cells has not been studied, these experiments may provide information on the modulation of the metastatic potential of tumor cells.

## Collagen Heterogenity

Collagens are a group of triple-helical, inflexible, rod-shaped proteins found in mammalian connective tissues. Collagens have a 20% imino acid (proline and hydroxyproline) content, a 30% glycine content and are glycosylated (Burgeson, 1982). The precursor subunits of collagen, procollagen alpha (pro$\alpha$) chains undergo several post-translational modifications. The first modification is the enzymatic hydroxylation of specific prolyl and lysyl residues (Prockop and Juva, 1965). Carbohydrate moieties are then added to some of the hydroxylysyl residues by galactosyl and glucosyltransferases (Spiro and Spiro, 1971). These events are followed by intra- and interchain disulfide bond fomration and packaging of pro$\alpha$ chains into

a triple helix (Uitto and Prockop, 1973). Triple helical procollagen molecules are packaged into secretory granules in the Golgi complex and are ready for export (Weinstock and Leblond, 1974). Extracellularly procollagen molecules are modified by procollagen peptidases (Lapiere et al, 1971). These enzymes cleave off non-helical peptides from the $NH_2$ and COOH terminal ends of procollagen to form collagen.

In the extracellular matrix collagen fibers aggregate to form orderly fibrils via parallel lengthwise interactions (Prockop et al, 1979). The fibrils are stabilized by intra- and intermolecular covalent crosslinks formed following oxidative deamination of lysine and hydroxylysine residues by the enzyme lysyl oxidase (Pinell and Martin, 1968). Crosslinks are formed when enzymatically produced aldehydes react with lysine or hydroxylysine residues (Siegel and Martin, 1970). Crosslink formation increases the collagen fibrils resistance to degradation by collagenase (Vater et al, 1979).

Presently five major collagen types and several minor collagenous proteins have been identified (Burgeson, 1982). The major collagens, designated Types I, II, III, IV, and V, differ in subunit composition, carbohydrate content,

tissue distribution and arrangement in the extracellular matrix (Bornstein and Sage, 1980). The minor collagenous proteins which have been partially characterized include the E and F chains of human hyaline cartilage (Burgeson and Hollister, 1979), the HMW and LMW collagenous fragments of chick hyaline cartilage (Reese et al, 1979), EC collagen synthesized by cultured endothelial cells (Sage et al, 1980) and a collagenous fragment isolated from placental tissue (Furuto and Miller, 1980).

Types I, II, and III collagen, collectively called the interstitial collagens, are the major structural components of connective tissues. The interstitial collagens are composed of three subunit polypeptide chains called alpha ($\alpha$) chains. Alpha chains from Types I, II, and III collagen differ in amino acid composition and size, additionally the three $\alpha$ chains of Type I collagen are not similar. Type I collagen is composed of two different subunits designated $\alpha 1(I)_2$ $\alpha 2(I)$. Type II collagen is composed of three idential $\alpha$ chains represented by the formula $\alpha 1(II)_3$. Type III collagen has a molecular configuration of $\alpha 1(III)_3$ (Bornstein and Sage, 1980).

The basement membrane collagens Types IV and V make up a minor portion of the total collagenous fraction of the extracellular matrix. Type IV collagen has been localized to the basement membranes of most tissues (Bornstein and Sage, 1980). Type IV collagen is composed of three $\alpha$ chains which are biochemically distinct from those of the interstitial collagens. Two different $\alpha$ chains have been identified and are designated $\alpha$ 1(IV) and $\alpha$ 2(IV) (Sage and Bornstein, 1979). Type IV collagen $\alpha$ chains contain more 4-hydroxyproline than 3-hydroxyproline which is present in interstitial collagens. They contain more carbohydrate residues than interstitial collagens and these chains are stabilized by disulfide bonds (Sage et al, 1979). The IV collagen $\alpha$ chains are wound into a triple helix and form fibers which are different from other collagenous fibers. Type IV collagen fibers are composed of four distinct regions; at one end there is a non-collagenous globular peptide called NC1, the second region is the major triple helical domain which contains several regions where the triple helix is interrupted. The third region is another non-collagenous domain called NC2, and the other end of the fiber is a collagenous peptide called the 7S domain (Timpl et al, 1981; Schuppan et al, 1980; Glanville and Rauter, 1981). Type IV collagen fibers do not appear to be processed by procollagen peptidases

(Dehm and Kefalides, 1978). These fibers exhibit a specific orientation in basement membranes. The globular heads (NC1) of two fibers are connected in a non-covalent manner while the 7S domains of four fibers from aggregates (Timpl et al, 1981). The association of four 7S domains yields a structure termed 7S collagen (Risteli et al, 1980). 7S collagen is more stable than other collagenous proteins due to extensive disulfide bonding between subunits and it is partially resistant to digestion by bacterial collagenase.

Type V collagen, discovered in 1976 by two groups of investigators (Chung et al, 1976; Burgeson et al, 1976) represents approximately 5% of the total collagenous fraction of tissues. Immunofluorenscence has localized Type V collagen in basement membranes and on the pericellular surface of cells (Madri and Furthmayr, 1980; Gay et al, 1981). This type of collagen has been isolated from several tissues including vasculature, skin, synovium, gingiva, liver, tendon, placenta, bone, lung, and muscle (Bornstein and Sage, 1980). The exact function of Type V collagen is not known, it may be involved in cell migration (Stenn et al, 1979) and may play a role in stabilizing cytoskeletal architecture.

The physical properties of Type V collagen are similar to the other collegens. Four different Type V collagen $\alpha$ chains have been isolated and are designated $\alpha$ 1(V), $\alpha$ 2(V), $\alpha$3(V), and $\alpha$4(V) (Sage and Bornstein, 1979; Fessler et al, 1983). Presently, the exact molecular configuration of Type V collagen is not established. Most preparations are composed of two $\alpha$ 1(V) chains and one $\alpha$ 2(V) chain, the $\alpha$3(V) or $\alpha$4(V) chains may be present depending on the tissue source (Rhodes and Miller, 1978; Bentz et al, 1978; Sage and Bornstein, 1979; Fessler et al, 1983). Type V collagen is glycosylated like the other collagens, it's carbohydrate content is intermediate between the interstitial and basement membrane collagens (Burgeson et al, 1976). It is secreted into the extracellular matrix in a procollagen form and undergoes limited proteolysis by procollagen peptidases (Kumamoto and Fessler, 1981). The globular extension peptides which remain intact are larger than those of Types I, II, and III collagen but smaller than those of Type IV collagen. The triple helical region of Type V collagen is also larger than the triple helicies of Types I, II, and III collagen (Burgeson et al, 1976). The configuration of Type V collagen fibers in the extracellular matrix is not firmly established. The fibers appear to interact forming parallel longitudinal arrays of fibrils similar to those

formed by interstital collagens (Bentz et al, 1978).

The thermal stability of the Type V collagen triple helix appears to be similar to the helicies of other collagens, all have mid-point melting temperatures ($T_M$) of 37°C (Rhodes and Miller, 1978). The Type V collagen melting curve shows a slight inflection at 33°C-35°C which suggests the existence of two distinct molecules, one being less heat stable than other collagenous molecules. This unstable species may allow catabolism of the molecule by proteases which do not degrade triple helical collagen but can degrade denatured collagen. Type V collagen is degraded by thrombin, trypsin, chymotrypsin and elastase at temperatures above 34°C (Sage et al, 1981). At these temperatures the triple helix of Type V collagen begins to denature making the molecule more susceptible to degradation.

## Mammaliam Collagenase

The catabolism of collagen is initiated by the enzyme collagenase. Collagenases are a family of enzymes which play an important role in tissue remodeling (Mainardi et al, 1980a) and certain pathological states such as rheumatoid arthritis (Evanson et al, 1968). They are

the only enzymes capable of initiating the breakdown of mature triple helical collagen (Gross et al, 1974). Collagenases act by making one break in all three $\alpha$ chains in the triple helical region of the molecule. The cleavage site in Type I collagen has been localized to glycine residue 778 and isoleucine residue 779 which occur three quarters of the distance down the length of the $\alpha$ chains from the amino terminal end (Gross et al, 1974). Two digestion fragments are formed as a result of enzymatic cleavage, the $TC_A$ and $TC_B$ fragments. The $TC_A$ fragment represents 75% of the $\alpha$ chain and the $TC_B$ fragment is 25%. The production of specific reaction products at neutral pH and temperature which supports a triple helical conformation of the substrate is one method of identifying a protease as a true collagenase (Harris and Vater, 1982). These specific reaction products were first identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following incubation of Type I collagen with tadpole tailfin collagenase (Gross and Nagai, 1965).

Following the initial cleavage by collagenase the collagen triple helix denatures under physiological conditions, the $TC_A$ and $TC_B$ fragments are not thermally stable at temperatures above 34°C (Sakai and Gross, 1967). The denatured molecule is now susceptible to degradation

by non-specific proteinases (Harper, 1980). Denatured collagen fragments are degraded into smaller peptides by enzymes called gelatinases (Vaes et al, 1978). Collagenase isolated from fibroblasts can degrade denatured as well as native collagen; however, the rate of proteolysis of native substrate is faster (Welgus et al, 1982).

The study of collagen catabolism has led to the discovery of substrate specific collagenases. Collagen Types I, II, and III are degraded by classic or interstitial collagenase (Harper, 1980). This enzyme does not degrade Types IV and V collagen (Sage et al, 1979; Liotta et al, 1981a; Woolley et al, 1978). Interstitial collagenases have been isolated from several cell types including fibroblasts (Bauer et al, 1975), macrophages (Mainardi et al, 1980a), polymorphonuclear leukocytes (PMN) (Horwitz, 1977), eosinophils (Hibbs et al, 1982), bone cells (Puzas and Brand, 1979), rheumatoid synovial cells (Woolley et al 1975a) and several tumor cell lines (Wolf et al, 1982; O'Grady et al, 1982; Liotta et al, 1979). All collagenases studied share several properties including: (1) a pH optimum in the range of neutrality; (2) require $Ca^{2+}$ for maximal activity; (3) are inhibited by ethylenediamine tetracetic acid (EDTA), a metal ion

chelator and thus are characterized as metalloproteinases; (4) produce $TC_A$ and $TC_B$ reaction products under non-denaturing conditions; and (5) they are synthesized in a latent form requiring activation for maximal activity.

Collagenases which degrade interstitial collagen have been purified from several sources using a variety of techniques. A range of molecular weights from 30,000 to 150,000 daltons have been reported for the different purified collagenases (Nagai, 1973). Collagenase in tissue culture medium or tumor homogenates is usually purified by ammonium sulfate fractionation followed by ion exchange chromatography and/or affinity chromatography. These procedures are followed by gel filtration chromatography and molecular weight determination on SDS-PAGE.

Collagenase isolated from the medium of cultured human skin fibroblasts is the best characterized form of the enzyme. Fibroblast collagenase appears to have both cationic and anionic regions, it binds to anionic resins (Woolley et al, 1973), and cationic resins (Stricklin et al, 1977) at similar pH and salt concentrations. Fibroblasts secrete collagenase in a latent procollagenase form (Bauer et al, 1975), two different procollagenases with molecular weights of 60,000 and 55,000 daltons have been

identified (Stricklin et al, 1977). Procollagenase does not bind to collagen substrates, upon activation the enzyme binds tightly to its substrate (Stricklin et al, 1978). The two forms of procollagenase can be activated by limited tryptic digestion to give two active species of enzyme with molecular weights of 50,000 and 45,000 daltons (Stricklin et al, 1977). Fibroblast procollagenase can also be activated by organomercurial compounds, this reaction proceeds without an initial decrease in enzyme molecular weight (Stricklin et al, 1983). Lower molecular weight species are formed, however, their production is not coincident with the increase in enzyme activity (Stricklin et al, 1983). Autocatalytic cleavage is responsible for the eventual decrease in enzyme molecular weight (Stricklin et al, 1977).

Collagenases which degrade interstitial collagen have been purified to homogeneity from several sources. Collagenase from rabbit $V_2$ ascites cell carcinomas and Walker 256 carcinoma cells have been purified to homogeneity and molecular weights (MW) of 34,000 and 42,000 daltons, respectively, were reported (McCroskery et al, 1975; Wolf and Wirl, 1982). Other collagenases which have been isolated in pure form include human rheumatoid collagenese MW 33,000 daltons (Woolley et al, 1975a), porcine

synovial collagenase MW 44,000 daltons (Cawston and Tyler, 1979), neutrophil collagenase MW 70,000 daltons (Christner et al, 1982), and guinea pig skin collagenases MW 40,000 and 130,000 daltons (Huang and Abramson, 1975). Fiedler-Nagy et al (1977) have isolated collagenase from human fibroblast cultures. They reported a series of peaks of enzymatic activity following gel filtration chromatography which corresponded to MW of 40,000 to 150,000 daltons. They found that the enzyme was present in a tightly bound complex with a hydroxyproline containing peptide, most likely a fragment of collagen. Complexes of collagenase and different sized collagenous fragments were responsible for the numerous peaks of enzyme activity. These types of complexes may also account for the differences in MW values reported for different purified collagenases.

Collagenases which specifically degrade Type IV collagen have been isolated from human leukocytes (Uitto et al, 1980) and from the medium of a cultured metastatic murine sarcoma cell line (Liotta et al, 1979). Type IV collagenase from sarcoma cells was later purified (Salo et al, 1983). The isolated activity had a MW of 160,000 daltons, if Triton X-100 was added to the preparation and rechromatographed its MW decreased to 70,000 daltons.

This indicated the enzyme has hydrophobic properties and the large molecular weight form may be an aggregate of enzyme molecules. SDS-PAGE further separated the activity into two distinct components with MW of 68,000 and 62,000 daltons. The Type IV collagenase was secreted in a latent form, required $Ca^{2+}$ for activity and produced specific reaction products.

The degradation of Type IV collagen by PMN elastase has been reported (Mainardi et al, 1980b). PMN's also contain a collagenase which degrades interstitial collagens (Horwitz et al, 1977). The PMN Type IV collagenase described by Uitto et al (1980) is different from the interstitial collagenase, however, it may be an elastase-like protease similar to that reported by Mainardi et al (1980b). It has several properties which are characteristic of elastases including inhibition by phenylmethylsulfonyl floride (PMSF), an inhibitor specific for serine proteases (Fahrney and Gold, 1963).

Type V collagen specific degrading activities have been detected in the medium of cultured normal and malignant macrophages (Mainardi et al, 1980a; Liotta et al, 1981b). Mainardi separated normal macrophage Type I and Type V collagenases using DEAE cellulose chromatography.

The Type V collagenase was characterized as a neutral protease inhibited by EDTA but not PMSF. Liotta et al (1981b) characterized a Type V collagen degrading activity synthesized by malignant macrophages. This activity was secreted in a latent form, was inhibited by EDTA but not PMSF, produced specific reaction products and had a MW of 80,000 daltons as determined by gel filtration chromatography.

## Regulation of Collagenase Activity

Collagenases are secreted by most cells in a latent form, requiring activation for maximal activity (Bauer et al, 1975). The exact nature of latent collagenases has not been established. The enzyme may be secreted as an enzyme-inhibitor complex (Sellers et al, 1977) or in zymogen form (Stricklin et al, 1977). Activation of latent collagenases usually proceeds with a decrease in molecular weight of 10,000 to 20,000 daltons (Stricklin et al, 1977; Sellers et al, 1977; Wolf and Wirl, 1982). Limited proteolysis of latent collagenase results in increased enzymatic activity. In vitro several proteases catalyze this activation process, included are trypsin (Bauer et al, 1975), plasmin (Vaes et al, 1975), mast cell proteinase (Birkedal-Hansen et al, 1976), endogenous serine protease (Woessner, 1977), tadpole proteinase

(Harper et al, 1971), lysozomal protease (Eeckhout and Vaes, 1977) and metalloproteinases (Horwitz et al, 1976).

Collagenase can be activated in vitro by several non-proteolytic mechanisms. Incubation of latent enzyme with chaotropic ions such as $I^-$ and $SCN^-$, organomercurial compounds (Stricklin et al, 1983) and disulfide containing compounds (McCartney et al, 1980) all produce a more active species of collagenase. These compounds act by dissociating enzyme-inhibitor complexes or by disrupting the configuration of the native zymogen enzyme (Stricklin et al, 1983). These processes are followed by autocatalytic intramolecular activation, and the active enzyme formed is of lower molecular weight (Bauer et al, 1975; Stricklin et al, 1977).

The production of collagenases is regulated by the capacity of the cell to synthesize the protein (Valle and Bauer, 1977) and by the degree to which the cell is stimulated by surrounding cells. Johnson-Muller et al (1978) showed that normal epithelial cells stimulate the release of collagenase from stromal cells via the action of a soluble factor. Macrophages and T-lymphocytes also secrete a soluble factor which stimulates release of collagenase from synovial cells (Dayer et al, 1979).

Secretion of collagenases by fibroblasts is increased when the fibroblasts are cocultured with B-16 melanoma and A-10 adenocarcinoma cells, two epithelial-like tumor cell lines (Biswas, 1982). Rat mammary adenocarcinoma cells, also an epitelial-like tumor cell secrete plasminogen activator (PA). PA catalyzes the formation of plasmin, this protease can stimulate collagenase activity *in vivo* by activating latent collagenases present in stromal tissues (O'Grady *et al*, 1980). Cell-cell interactions may be important during invasion, such interactions can result in increased levels of collagen degrading enzymes in the extracellular matrix. These enzymes may facilitate the breakdown of connective tissue barriers encountered by invading tumor cells.

## Collagenase Inhibitors

Collagenases are inhibited by both tissue specific inhibitors and by several components of serum. Normal serum has been fractionated by isoelectric focusing and three inhibitory zones have been identified according to their electrophoretic mobility. They are the $\alpha$, $\beta$, and $\gamma$ zones (Broth *et al*, 1981). The major inhibitory component of serum, found in the $\alpha$-zone is $\alpha_2$ macroglobulin. $\alpha_2$ macroglobulin is a non-specific inhibitor of most endopeptidases (Seifter *et al*, 1970). Also present in this fraction are antithrombim III and $\alpha_1$ antitrypsin, the latter has only minor collagenase inhibitory

capacity (Woolley et al, 1975b). The second fraction of serum, the $\beta$-zone contains a low molecular weight protein named $\beta_1$- anticollagenase which specifically inhibits collagenase but not other proteases (Woolley et al, 1976). The $\gamma$ electrophoretic zone of serum contains a cationic protein which inhibits collagenase. This protein may be the C1q component of complement which has a collagen-like structure. It inhibits collagenase by acting as a competitive substrate (Nagai et al, 1978).

Several tissue specific collagenase inhibitors have been identified. Keuttner et al (1976) characterized a low molecular weight (11,000 daltons) cationic protein from bovine aorta and cartilage which inhibited collagenases under physiological conditions. Two groups have isolated and characterized a 28,000 dalton protein from amniotic fluid which inhibits collagenolytic activity (Murphy et al, 1981a; Aggeler et al, 1981). Additional tissue specific collagenase inhibitors have been isolated from human synovium (Murphy et al, 1981b), rabbit bone cells in culture (Cawston et al, 1981), cultured human skin fibroblasts (Welgus et al, 1979), and porcine gingival explants (Pettigrew et al, 1981).

## Elastin

Elastin is a structural protein found in the extracellular matracies of several tissues including lung, skin, blood vessels, cartilage, and breast (Werb et al, 1982). Elastin provides these tissues with tensile strength and elasticity (Anwar et al, 1977). Alterations in elastin metabolism are associated with several disease states (Sandberg et al, 1981). Desmoplasia, the excessive deposition of stromal connective tissue is often associated with the growth of invasive breast carcinomas (Barsky et al, 1982). Accumulation of newly synthesized elastin is a common feature associated with the desmoplastic response (Lundmark, 1972).

The primary structure of elastin is unique in that it contains large quantities of non-polar amino acids (Keeley et al, 1974). Tropoelastin, the subunit of mature elastin contains 11 repeating sequences of the pentapeptide val-pro-gly-val-gly, a second hexapeptide pro-gly-val-gly-val-ala found in a trypsin-sensitive region of tropoelastin repeats itself 6 times (Foster et al, 1973). Tropoelastin also has a high lysine content, these residues are present in pairs separated by 1 to 3 alanine residues and preceded by 1 to 8

alanine residues (Sandberg et al, 1972). Tropoelastin subunits are crosslinked to form elastin following the oxidative deamination of specific lysine residues by the enzyme lysyl oxidase (Pinell and Martin, 1968). The enzymatically formed reactive aldehyde groups of adjacent subunits spontaneously react to form covalent bonds. The crosslinking residues desmosine and isodesmosine are formed when three deaminated lysine residues and one epsilon amino group of a fourth lysine from tropoelastin monomers are joined (Lent et al, 1969). Crosslinks stabilize the protein and make it resistant to proteolysis (Stone et al, 1982). Elastin fibers in the extracellular matrix consist of two components: (1) a microfibrillar component which is very susceptible to degradation and (2) an amorphous component which is resistant to proteolysis (Werb et al, 1982).

## Elastase

Mature elastin is degraded by several enzymes known as elastases. Elastolytic activity has been detected in the β-cells of the pancreas (Marshall et al, 1969), PMN's (Ohlsson and Olsson, 1974), macrophages (Banda and Werb, 1981), platelets (Hornebeck et al, 1980), smooth muscle cells and fibroblasts (Boudillon et al, 1980),

human breast carcinoma cells (Kao et al, 1982) and metastatic human tumor cells (Jones and DeClerck, 1980). These elastolytic activities cleave the elastin molecule in different sites, are inhibited by different compounds, and have different cellular locations. Elastin has multiple cleavage sites and is degraded to several low molecular weight peptides by elastases (Werb et al, 1982). The cleavage site for pancreatic, macrophage, and PMN elastases have been identified. Pancreatic elastase cleaves peptide bonds in elastin which are carboxyl to glycine, valine and alanine residues (Barrett et al, 1980). This elastase is inhibited by PMSF and classified as a serine protease (Fahrney and Gold, 1963). Macrophage elastase only cleaves peptide bonds on the amino side of leucine residues (Banda and Werb, 1981). Macrophage elastase is characterized as a metal-dependent proteinase, its activity is blocked by EDTA (White et al, 1980). Elastase from PMN's cleaves elastin at sites which are carboxyl to alanine and valine residues (Barrett et al, 1980). This enzyme is inhibited by PMSF and is a serine protease. Fibroblast elastase has been characterized as a metal-dependent protease (Bourdillon et al, 1980). The elastolytic activity associated with cultured human breast carcinoma cells is inhibited by both EDTA and PMSF, these

cells may contain more than one protease which degrades elastin substrates (Kao et al, 1982).

Pancreatic elastase is secreted from the β-cells in an inactive form and is activated by trypsin in the intestine (Barrett et al, 1980). PMN elastase has been localized inside the cell in azurophil granules (Werb et al, 1982). Elastase from macrophages and human breast carcinoma cells is found in the medium when these cells are maintained in culture (Banda and Werb, 1981; Kao et al, 1982). The elastolytic activity of metastatic human tumor cells was not detected in the medium or inside the cells and was present only when the cells were attached to a suitable substrate (Jones and DeClerk, 1980). Smooth muscle cell and fibroblast elastases are not secreted into the growth medium but these activities can be extracted from cell sonicates using Triton X-100, suggesting they are membrane bound (Hornebeck et al, 1980).

## Elastase Inhibitors

The activity of elastases are limited by the presence of several natural inhibitors (Werb et al, 1982). The two major elastase inhibitors are found in the serum, they are

$\alpha_1$ antitrypsin and $\alpha_2$ macroglobulin (Ohlsson and Olsson, 1974). Pancreatic and PMN elastases are more susceptible to inhibition by $\alpha_1$ antitrypsin than $\alpha_2$ macroglobulin. Macrophage elastase is primarily inhibited by $\alpha_2$ macroglobulin. Macrophage elastase also inactivates $\alpha_1$ antitrypsin by degrading it, freeing other bound inactivated elastases (Banda et al, 1980). Imbalances in the ratio of elastases to inhibitors where elastases are in excess are thought to be involved in disease states such as chronic obstructive pulmonary disease (Eriksson, 1979), rheumatoid arthritis (Cox and Huber, 1976), and emphysema (Stone et al, 1982).

# EXPERIMENTAL

## Materials

All reagents used in this study were of analytical grade. Reagents used included [$^{3}$H]-sodium borohydride, specific activity 100 mCi/mmol, [2-$^{3}$H]-glycine, specific activity 44 Ci/mmol, [1-$^{14}$C]-acetic anhydride, specific activity 10 mCi/mmol and Atomlight liquid scintilation fluid, purchased from New England Nuclear Corporation. Trypsin, 2X crystallized was purchased from Worthington Biochemical Corporation. Soybean trypsin inhibitor type II-S, pepsin, 2X crystallized and bacterial collagenase Type VII were from Sigma Chemical Company. Acrylamide, N´, N´-methylene bisacrylamide, sodium dodecyl sulfate, N, N, N, N tetramethylenediamine, ammonium preoxysulfate and Coomassie Brilliant Blue were obtained from Eastman Kodak. Ultrogel AcA44 was purchased from LKB and diethylaminoethyl cellulose (DE-52) was from Whatman. All tissue culture reagents were obtained from Grand Island Biological Company.

## Cell Culture

The cell lines used in this study were obtained from the Roger Williams General Hospital Cancer Center and

included: DLD-1 a human colon carcinoma (Dexter et al, 1979), B16-F10 a murine melanoma (Fidler, 1973), and normal human dermal fibroblasts. DLD-1 cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 100 U penicillin/ml, 100 µg streptomycin/ml, 2.5 µg Fungizone/ml, 20 µg gentamycin/ml, 60 µg Tylocine/ml and buffered with 0.075% $NaHCO_3$, 10 µM Hepes and 10 µM Tricine. Fibroblasts were grown in Dulbecco's Modified Eagle (DME) medium supplemented with 10% FCS, 3.5 mg glucose/ml, 100 U pencillin/ml and 100 µg streptomycin/ml. B16-F10 cells were grown in Minimum Essential Medium supplemented with 10% FCS, 100 U pencillin/ml, 100 µg streptomycin/ml, 2.5 µg Fungizone/ml, 200 µg gentamycin/ml, 20 mM gultamine, 1 mM non-essential amino acids, 1 mM sodium pyruvate and buttered with 0.1% $NaHCO_3$ and 2 µM Hepes.

Cells were maintained in a humidified environment at 37°C containing 5% $CO_2$ and 95% air in 100 x 20 mm tissue culture dishes (Falcon 3003). Confluent monolayers were subcultured by removing the medium by suction, washing the cell layer with isotonic phosphate buffered saline (PBS) pH 7.0 and incubating the cultures in PBS which contained 0.25% trypsin, 0.2 mg glucose/ml and 0.2 mg EDTA/ml. Detached cells were centrifuged for 5 minutes at 500 Xg and

resuspended in fresh medium. Each dish of cells was replated into four 100 x 20 mm tissue culture dishes.

## Preparation of Culture Media for Enzyme Assays

Media from cell cultures were assayed for Types I and V collagen degrading activities and elastase activity. The secreted enzymes were precipitated and concentrated by ammonium sulfate fractionation using the method of Liotta et al (1981). The medium of late log phase cultures was decanted and the cell layers were washed three times with PBS. Fresh serum-free media (10 mls/100 x 20 mm plate) was added and cultures were incubated for 24 hours. The medium from three 100 x 20 mm plates was pooled and centrifuged for 5 minutes at 900 Xg to remove cells and debris. Medium proteins were precipiated by addition of crystalline ammonium sulfate to 60% of saturation and centrigured for 60 minutes at 27,000 Xg at 4°C. Precipitates were resuspended in 2 ml of 0.05M Tris-HCl pH 7.5 containing 0.01M $CaCl_2$ and dialyzed against 10 liters of this buffer for at least 16 hours at 4°C. Aliquots of the dialysate were activated by incubation with trypsin (10 µg/ml) at a 4:1 dialysate to trypsin ratio for 5 minutes at 37°C. The reaction was halted by addition of a five molar excess of soybean trypsin inhibitor, samples were cooled to 4°C, and then assayed for enzymatic activity.

## Preparation of Cells for Elastase, Protein and DNA Assays

Cell layers were harvested by incubation with PBS containing 1 mM EDTA at 37°C. Detached cells were collected and washed three times with 0.05M Tris-HCl pH 7.5 containing 0.9% NaCl and 0.01 M $CaCl_2$. The cells were resuspended in 1 ml of idalysis buffer which contained 0.1% Triton X-100 and sonicated three times for 10 seconds at 50 watts. Aliquots of the sonicate were used for elastase assays and determination of protein and DNA content.

## Dimethylformamide (DMF) Treatment

DMF was added to RPMI culture medium to give a final concentration of 0.8% (V/V). DLD-1 colon carcinama cells were grown in this media and only cells which had been subcultured 3-5 times were used. This dose of DMF is not toxic to this cell line and the cells displayed the morphologic changes described by Dexter *et al* (1979) after 3 passages.

## Measurement of Protein Synthesis

The effect of DMF treatment on the capacity of DLD-1 colon carcinoma cells to synthesize and secrete protein was

studied by measuring the incorporation of $^{3}$H-glycine into trichloracetic acid (TCA) precipitable cellular and media protein. Control and DMF treated DLD-1 cells were plated into 100 x 20 mm culture dishes at a density of 1 x $10^{6}$ cells/plate in 10 ml of media. The experiments were performed when the cultures were in the late log phase of growth. The medium was removed and replaced with 10 ml of fresh medium supplemented with 1% FCS and 5 µCi/ml 2-$^{3}$H-glycine. Incorporation of $^{3}$H-glycine into protein was measured after 3 and 6 hours of incubation. At these times the media were collected and the cells were immediately washed 3 times with PBS containing 5 µg cycloheximide/ml. The cells were detached with 0.25% trypsin-PBS, counted and sonicated in PBS containing 5 µg cycloheximide/ml 3 times for 10 seconds at 50 watts. Medium and cell proteins were precipiated by addition of ice cold TCA to a final concentration of 10%. Protein precipitates were washed three times with 5 ml of ice cold 10% TCA then hydrolyzed in 1 ml of Digestol (Yorktown Research) for 3 hours at 60°C. The hydrosylates were mixed with 10 ml of Econofluor liquid scintilation fluid and the radioactivity determined. Aliquots of the cell sonicates were removed prior to precipitation and used for protein and DNA determinations.

## Protein Determination

All cell and medium enzyme values in this study were normalized using total intracellular protein values. Protein content was determined by the method of Lowry et al (1951); bovine serum albumin was used as a standard. An aliquot of 0.01 ml of the cell sonicate was used in each determination.

## DNA Determination

The DNA content of cell momoayers was determined using the fluorometric assay of Prasad et al (1972). Bovine thymus DNA was used as a standard. In a typical assay 0.1 ml of cell sonicate was diluted with 0.4 mls of PBS. Ethidium bromide (10 µg) was added to each assay, this compound intercalates nucleic acids making them fluorscent. Any RNA present was digested by addition of 200 µg of ribonuclease. Fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 590 nm. The fluorescence observed before addition of ribonuclease minus that observed after its addition was used to calculate the amount of DNA present. The decrease in fluorescence following ribonuclease treatment represents the RNA content.

## Purification of Collagens

Type I collagen previously purified in this laboratory by the method of Fuji and Kuhn (1975) was labeled with $^{14}C$-acetic anhydride and used as a Type I collagenase substrate. Type V collagen was purified by the method of Rhodes and Miller (1978). Human placentas were collected and stored forzen at -20°C until sufficient quantities were accumulated. Placentas were thawed, the amnionic membranes were removed and used in subsequent steps. All of the following procedures were carried out at 4°C. The membranes were extracted for 2 days in 0.9% NaCl and for 2 days in 0.5 M acetic acid, both extractions were performed in the presence of protease inhibitors. The purification procedure is outlined in Figure 1. Amnions were minced and centrfuged and the wet weight was determined. The minced tissues were suspended in 0.5 M acetic acid at a ratio of 20 grams wet tissue/100 ml of acetic acid. Pepsin, 1500 units/ml, was added to the suspension, stirred for 24 hours then centrifuged for 45 minutes at 30,000 Xg. The supernatant fluid was collected and crystalline NaCl was added to a final concentration of 0.8 M. The solution was stirred for 24 hours, then centrifuged for 45 minutes at 30,000 Xg, and the pellet was discarded. The supernatant NaCl concentration was increased to 1.2 M by the addition of crystalline NaCl, and the solution was

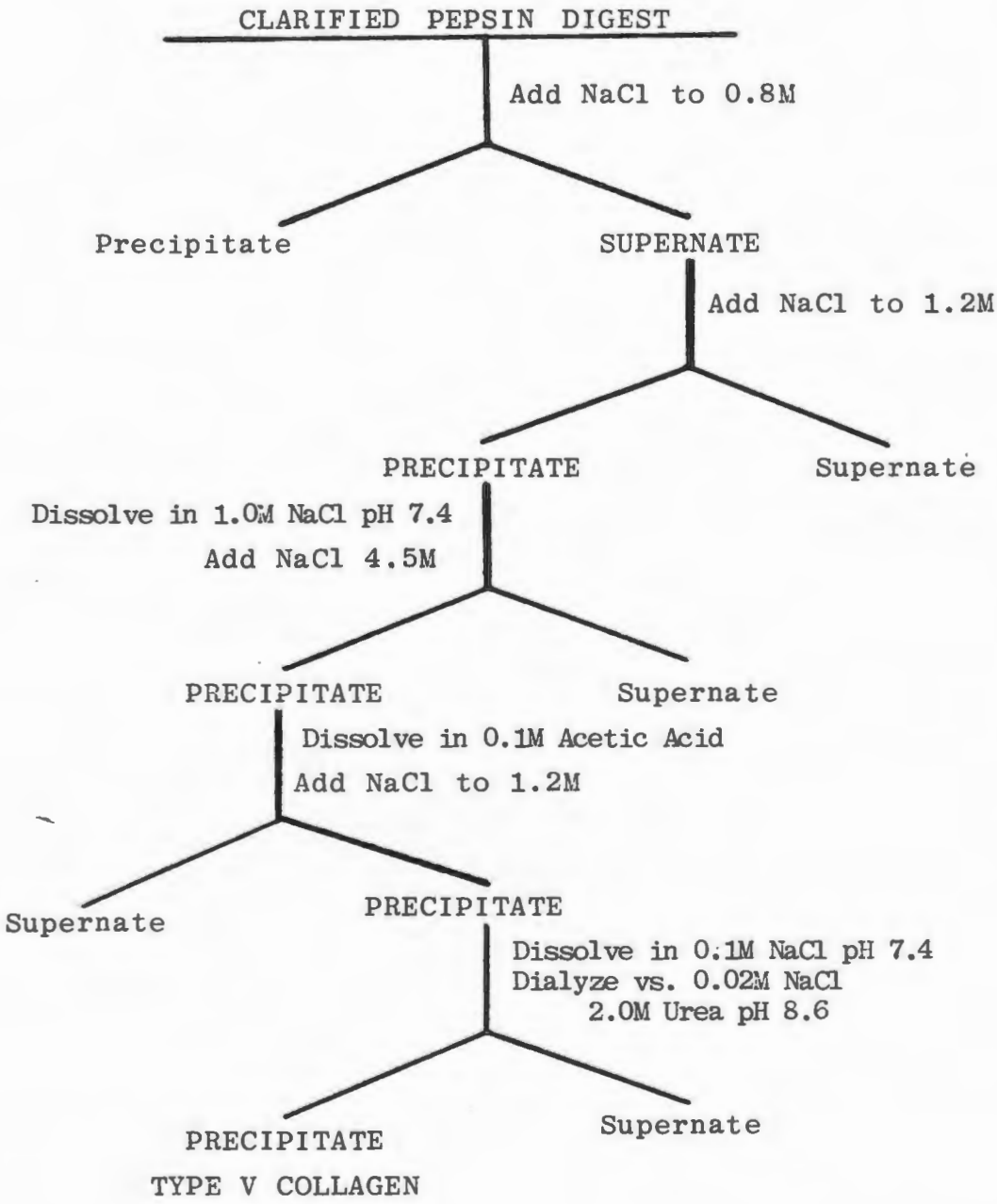


Figure 1: DIAGRAM OF THE PURIFICATION PROCEDURE USED TO ISOLATE TYPE V COLLAGEN. Type V collagen was purified from amnionic pepsin digests by the outlined procedure.

stirred for 24 hours. The precipitate which formed was collected by centrifugation and resuspended in 0.05 M Tris-HCl pH 7.4 containing 1 M NaCl. The Type V collagen in this solution was precipitated by adjusting the NaCl concentration to 4.5 M. The precipiate collected by centrifugation was dissolved in 0.1 M acetic acid and the collagen was reprecipitated by adding NaCl to 1.2 M. The precipitate formed was collected and resuspended in 0.05 M Tris-HCl pH 7.4 containing 0.1 M NaCl and was dialyzed against 0.01 M Tris-HCl pH 8.6 containing 0.02 M NaCl and 2 M urea. The precipitate formed during dialysis was collected and dialyzed against 0.05M acetic acid and lyophilized. Aliquots of each purification step were analyzed by SDS-PAGE by the method of Neville (1971) using 6% polyacrylamide slab gels.

## $^{14}$C-Acetylation of Types I and V Collegens

Types I and V collagens were labeled with $^{14}$C-acetic anhydride. The labeling procedure utilizes an acetylation reaction which adds a $^{14}$C-acetate group to the epsilon amino groups of lysine residues in the triple helical region of the collagen molecule (Gisslow and McBride 1975). Purified collagen was suspended in 0.01% acetic acid at a concentration of 2 mg/ml; a typical reaction employed a

total of 200-300 mg of collagen. Prior to addition of the acetylating agent, the pH of the collagen solution was adjusted to 8 by the slow addition of 1 M $K_2HPO_4$. 1-$^{14}$C-acetic anhydride, 1 mCi in 1.5 ml of benzene, was added dropwise over the course of 2 hours, the pH was maintained at 8 during the course of the reaction by addition of 1N NaOH. After this time the solution was acidified with glacial acetic acid to pH 4, dialyzed exhaustively against deionized water and lyophilized. A 2 mg sample of the dried protein was hydrolyzed in 0.5 mls of Digestol at 60°C for 3 hours and the specific activity of the substrate was determine. Acetylation of Type I collagen yielded a substrate with a specific activity of $1.41 \times 10^6$ dpm/mg; the Type V substrate had a specific activity of $1.12 \times 10^6$ dpm/mg. All substrates were stored at -20°C.

## Type I Collagenase Assay

Type I collagenase activity was measured according to Lindblad and Fuller (1982). Lyophilized $^{14}$C-labeled Type I collagen was suspended in 0.01% acetic acid at a concentration of 2 mg/ml. Immediately prior to use, this solution was diluted with 0.1 M Tris-HCl pH 7.6 containing 0.4 M NaCl and 0.01 M $CaCl_2$ so that 0.05 ml contained approximately 20,000 counts per minute (cpm). In a typical assay,

0.2 ml of activated culture medium was mixed with 0.1 ml of 0.05 M Tris-HCl pH 7.5 containing 0.005 M $CaCl_2$. Each assay contained 0.05 ml of the diluted substrate solution and was incubated at 35°C for 1 hour. The assay was terminated by addition of 0.1 ml of 0.1 M EDTA containing 150 µg of carrier Type I collagen. This mixture was incubated for an additional 30 minutes to assure complete denaturation of degraded substrate, and then cooled to 15°C for 5 minutes. Native substrate was precipitated by the addition of 0.8 ml of a 4:1 (V/V) dioxane/methanol solution. This mixture was centrifuged for 25 minutes at 6000 Xg and 0.8 ml of the supernatant was mixed with 5 ml of Atomlight to determine soluble radioactivity.

## Preparation of a Type V Collagen Degrading Activity

Type V collagen degrading activity was obtained from *in vivo* activated alveolar macrophages maintained in culture using the method of Mainardi *et al* (1980a). An adult female albino rabbit (3 kg) was injected with 0.2 ml of Freunds complete adjuvant in the ear vein. Two weeks later the rabbit was anesthetized and its lungs were surgically removed. Alveolar macrophages were washed out of the lungs by lavage using sterile PBS, the lavage treatment was repeated eight times using a total of 200 ml of PBS. The

collected cells were washed three times in DME media containing 100 U penicillin/ml and 100 µg streptomycin/ml. Cells were plates out in 100 x 20 mm culture dishes at densities of 1 x $10^7$ cells/dish in 10 ml of DME media supplemented with 10% FCS, 100 U penicillin/ml, 100 µg streptomycin/ml and 2.5 µg Fungizone/ml. Cells which adhered to the plates after 24 hours of growth in serum containing medium were used for the remainder of the procedure. Plates were maintained at 37°C in a humidified environment containing 5% $CO_2$ and 95% air. Each plate contained 10 ml of serum-free DME media which was collected and replaced with fresh media every 2 days for 2 weeks. The collected media was concentrated to 10 ml by pressure dialysis using an Amicon PM-10 ultrafiltration membrane. A 0.05 ml aliquot of this preparation degraded approximately 4 µg of Type V collagen substrate in 5 hours at 30°C pH 7.5.

## Type V Collagenase Assay

Type V collagen degrading activity was measured using an improved assay which utilized a soluble substrate, non-denaturing conditions, and dioxane/methanol which precipitates native substrate molecules. Lyophilized $^{14}C$-labeled Type V collagen was suspended in 0.01% acetic

acid at a concentration of 1 mg/ml. Immediately prior to use, this solution was diluted with 0.1 M Tris-HCl pH 7.6 containing 0.2 M NaCl and 0.01 M $CaCl_2$ so that 0.05 ml contained 20,000 cpms (20 µg substrate). Activated culture medium or other enzyme solutions were mixed with 0.05 M Tris-HCl pH 7.5 containing 0.005 M $CaCl_2$ in a final reaction volume of 0.3 ml. A volume of 0.05 ml of the diluted substrate solution was added to each assay tube and incubated at 30°C for 12-24 hours. The assay was terminated by cooling the samples to 15°C for 5 minutes and adding 0.1 ml of ice cold dioxane/methanol (4:1 V/V). Precipitated native substrate was separated from degraded substrate by centrifugation at 6000 Xg for 25 minutes at 4°C. A 0.2 ml aliquot of the supernatant was mixed with 5 ml of Atomlight to determine soluble radioactivity.

## Elastase Assay

Insoluble elastin powder, purchased from Sigma Chemical Company, was labeled using $^3H$-sodium borohydride by the method of Stone *et al* (1982). The lyophilized labeled elastin was suspended in 0.05 M Tris-HCl pH 7.5 containing 0.005 M $CaCl_2$ at a concentration of 1 mg/ml; 0.25 ml of this suspension was added to 1 ml microcentrifuge tubes. Aliquots of the cell sonicates (0.1 to 0.5 ml) and 0.05

M Tris-HCl pH 7.5 containing 0.005 M $CaCl_2$ were added to the microcentrifuge tubes in a final reaction volume of 1.0 ml. The reaction mixtures were incubated at 37°C for 24 hours without agitation, cooled on ice, and centrfuged in a Fischer Model 59 microcentrifuge for 2 minutes at 7000 Xg. A 0.2 ml aliquot of the supernatant was removed and mixed with 5 ml of Atomlight to determine soluble radioactivity.

## Ion Exchange Chromatography

DEAE cellulose ion exchange chromatography was utilized to separate Types I and V collagen degrading activities secreted by DLD-1 colon carcinoma cells. DEAE cellulose (Whatman DE-52) was suspended in 0.05 M Tris-HCl pH 8.3 containing 0.005 M $CaCl_2$ and 0.02% $NaN_3$, packed into a column 1.6 x 13 cm and allowed to equilibrate at 4°C by washing with several volumes of buffer. Pooled serum-free media from 900 100 x 20 mm culture dishes of late log phase DLD-1 cells were concentrated by pressure dialysis using an Amicon PM-10 ultrafiltration membrane and dialyzed against 10 liters of column buffer. The sample, approximately 100 ml, was trypsin activated as previously described and centrifuged for 10 minutes at 8000 Xg to remove any precipitate which had formed during concentration and dialysis. The supernatant was then applied to

the column at a flow rate of 25 ml/hour and the column was washed with buffer until absorbance at 280 nm of the effluent returned to baseline. Bound proteins were eluted in a linear gradient from 0.0 to 1.0 M NaCl in column buffer. The gradient was run over the course of 16 hours using an Ultrograd Gradient Mixer (LKB Bromma, Sweden). The total gradient volume was 480 mls. Six ml fractions were collected and monitored for absorbance at 280 nm and assayed for Type I and V collagenase activity.

## Gel Filtration Chromatography

Molecular weights of Type I and V collagenolytic activities were determined using Ultrogel AcA44 gel filtration medium. The column material was packed into a column 1.6 x 90 cm at 4°C and equilibrated in 0.05 M Tris-HCl pH 7.6 containing 0.01 M $CaCl_2$ and 0.02% $NaN_3$. The flow rate of the column was kept constant at 8 ml / hour. The column was calibrated using the globular protein standards bovine serum albumin, ovalbumin, carbonic anhydrase and ribonuclease, 1 mg of each standard was chromatographed separately. Blue Dextran 2000 was used to determine the void volume ($V_o$). Fractions of 2 ml were collected and the absorbance at 280 nm was monitored. Peaks of Type I or Type V collagenolytic activity from the DE-52 chromatogram were concentrated to

1 ml by pressure dialysis using an Amicon PM-10 ultrafiltration membrane. These samples were applied to the column. Fractions of 2 ml were collected, the absorbance monitored at 280 nm was monitored, and the fractions were assayed for Types I and V collagenase activity.

## Statistical Analysis

The statistical methods used in this study were obtained from the statistics textbook, "Introduction to Applied Statistics", (Lentner, M., 1976).

1. Mean $(\bar{X}) = \Sigma X_i/n$ $\quad$ n = sample size, $X_i$ = ith sample value

2. Standard Deviation(s) = $\left[\Sigma(X_i - \bar{X})^2/(n-1)^{\frac{1}{2}}\right]$

3. Unpaired Student t test = $t = \dfrac{(\bar{X}_1 - \bar{X}_2)}{\left[\dfrac{(S_1^2 - S_2^2)}{n}\right]^{1/2}}$

n = sample size of 1 group

degrees of freedom = 2(n-1)

# RESULTS

## Purification of Type V Collagen

Collagenous proteins present in an insoluble form in the extracellular matracies of many tissues can be extracted in a soluble form by limited digestion of the tissue with pepsin. This procedure was employed to solubilize Type V collagen from human amnionic membranes. Pepsin soluble collagens can be separated by differential precipitation with NaCl. This procedure takes advantage of solubility differences between the five genetically distinct collagen types. Interstitial collagen Types I and III in the amnionic pepsin digest were precipitated at low ionic strength (0.8M NaCl). Type V collagen precipitated when the ionic strength of the solution was raised to 1.2M NaCl. This precipitate was suspended in neutral salt buffer to inactivate any residual pepsin and the Type V collagen was reprecipitated by the addition of NaCl to 4.5 M. The 4.5 M NaCl precipitate was suspended in neutral salt buffer and dialyzed against a buffer of low ionic strength. This step separates Type V collagen from low molecular weight protein fragments which are soluble in low ionic strength solutions.

The precipitate formed during dialysis contained purified Type V collagen. The purification began with 745 grams of amnionic membranes (wet weight) and produced 350 mg of Type V collagen, a yield of 0.05%. The purity of the preparation was monitored by SDS-PAGE at various stages of the purification as shown in Figure 2. The electrophoretic pattern of purified Type I collagen is shown for comparison (Lane 1). The whole pepsin digest was found to contain both interstitial and Type V collagens (Lane 2). The precipitate which formed at 0.8M NaCl contained predominately Type I collagen and was devoid of Type V collagen (Lane 3). Pepsin added at the start of the purification was separated from Type V collagen when the NaCl concentration was raised to 1.2M. Type V collagen precipitates at this ionic strength and the pepsin remains soluble (Lane 5). The Type V collagen which was purified by pepsin digestion and differential NaCl precipitation consisted of two $\alpha$ chains, $\alpha$1(V) and $\alpha$2(V) present in an approximate 2:1 ratio (Lane 7). The molecular weights of these $\alpha$ chains were estimated to be 117,000 and 105,500 using molecular weight standards (Lane 8).

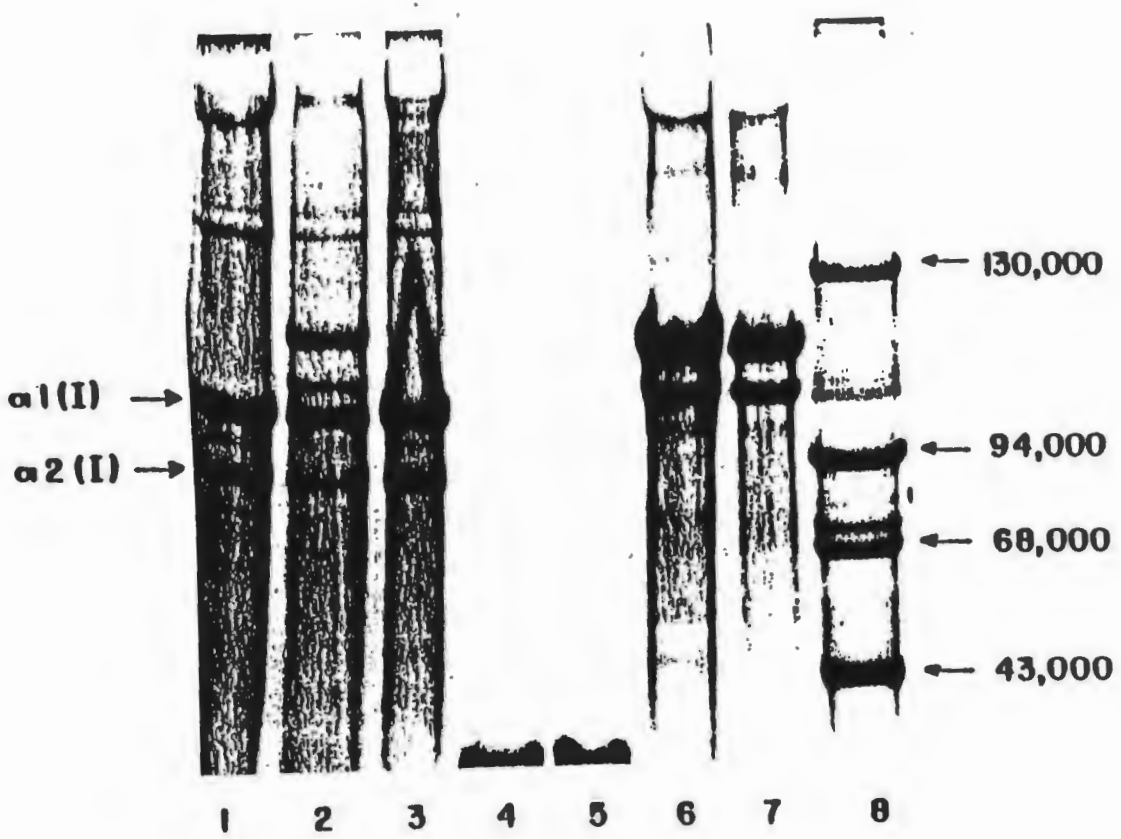


Figure 2. SDS Polyacrylamide Slab Gel Electrophoresis of Type V Collagen Purification. Aliquots of different stages of the purification were electrophoresed and protein bands were visualized by staining with Coomaisee Brilliant Blue. (1) Purified Type I collagen standard, (2) amnionic pepsin digest, (3) 0.8M NaCl precipitate, (4) pepsin standard, (5) 1.2M NaCl supernate, (6) 4.5M NaCl precipitate, (7) isolated Type V collagen, (8) molecular weight standards.

## Type V Collagenase Assay

An assay for measuring Type V collagen degrading activity was developed. Purified Type V collagen was labeled with $^{14}C$-acetic anhydride. This compound acetylates the epsilon amino groups of lysine residues in collagen. The labeled Type V collagen had a specific activity of $1.12 \times 10^6$ dpm/mg. The assay was performed at an incubation temperature of 30°C. This is several degrees below the point at which the Type V collagen triple helix begins to denature (Rhodes and Miller, 1978). The precipitating solvent dioxane/methanol (4:1, v/v) was used to separate native substrate molecules from degraded substrate molecules. This solvent has been used previously in Type I collagenase assays (Lindblad and Fuller, 1982).

The solubility of native and denatured Type V collagen substrate in dioxane/methanol was determined. Increasing volumes of the precipitating solvent were added to native substrate (30°C) and substrate which had been denatured by heating at 60°C for 30 minutes. The solutions were centrifuged and the amount of substrate which precipitated was determined (Figure 3). Native substrate molecules precipitated at much lower dioxane/methanol concentration

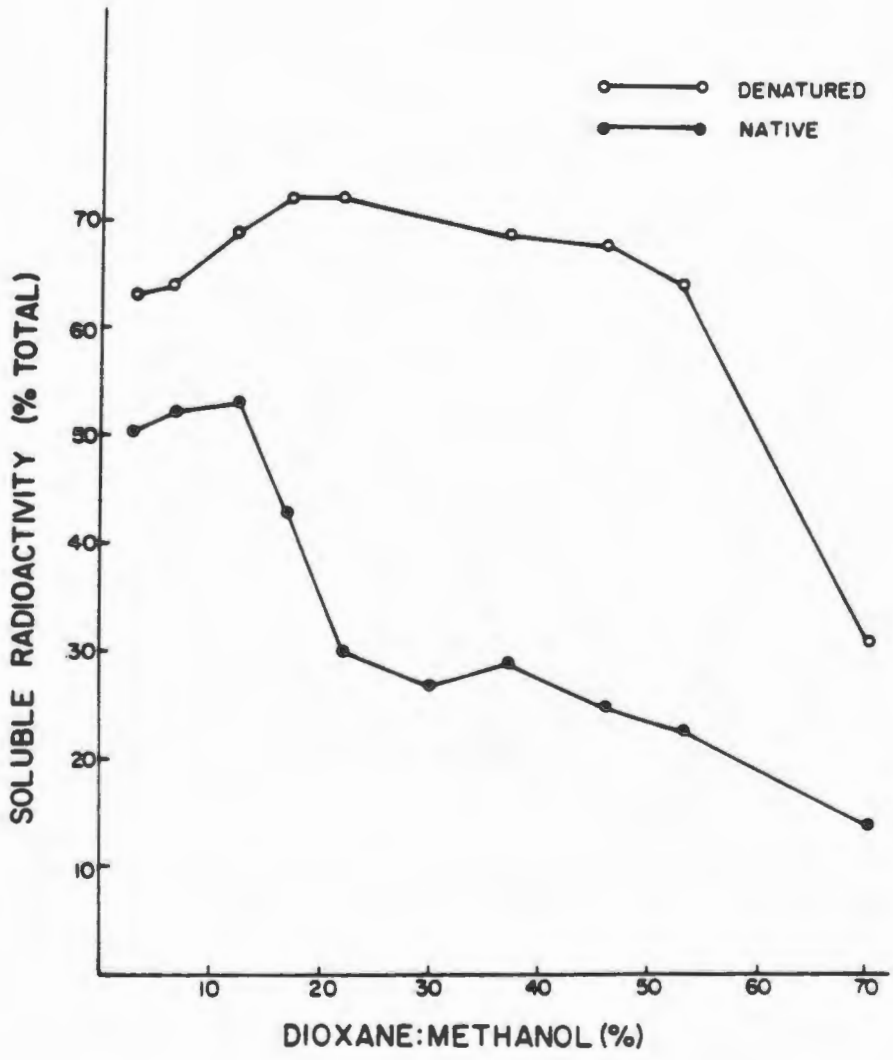


Figure 3. Solubility of Native and Denatured Radiolabeled Type V Collagen in Dioxane/Methanol/Water. Increasing volumes of a dioxane/methanol solution (4:1, v/v) were added to native (30°C) and heat denatured (60°C for 30 mins.) $^{14}C$-Type V collagen, centrifuged and soluble radioactivity was determined.

than denatured substrate molecules. The difference in their solubilities was greatest between 23%-46% dioxane/methanol.

The solubility of enzymatically produced Type V collagen reaction products in dioxane/methanol were determined. Native substrate was degraded by three different collagenases, Clostridium histolytium collagenase (E.C. 3.4.24.3), rabbit alveolar macrophage collagenase, and collagenase obtained from the media of cultured DLD-1 colon carcinoma cells. The enzyme-substrate solutions were incubated at 30°C for six hours then increasing volumes of dioxane/methanol were added. Following centriguration, the solubility of the reaction products were determined (Figure 4). When the substrate was degraded by bacterial collagenase soluble radioactivity was highest at 29% dioxane/methanol. Following incubation with alveolar macrophage and DLD-1 collagenases soluble radioactivity was highest at 13% dioxane/methanol. To optimize the detection of enzyme activity while precipitating the majority of the native substrate molecules 23% dioxane/methanol was chosen as the standard concentration to be used in the assay. At this concentration 70% of the native substrate is precipitated and appreci-

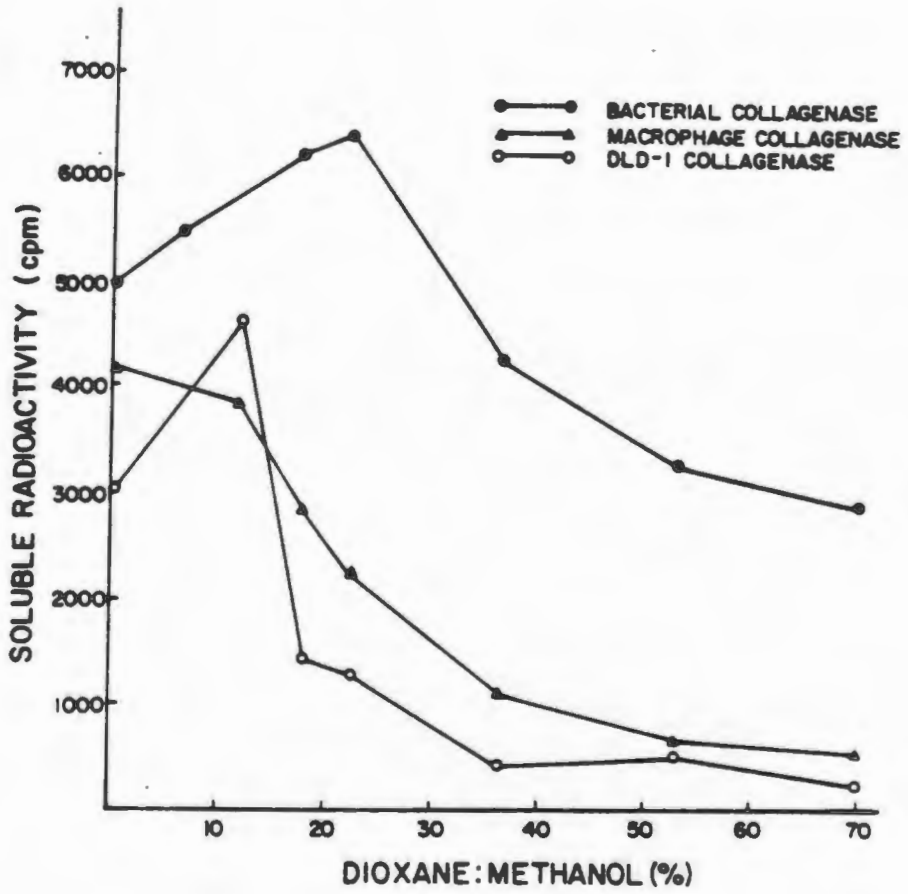


Figure 4. Solubility of Radiolabeled Type V Collagen Degradation Products Formed by Three Collagenases in Dioxane/Methanol/Water. $^{14}$C-Type V collagen (20 µg) was incubated with three different collagenases; bacterial collagenase, alveolar macrophage collagenase and DLD-1 colon carcinoma collagenase at 30°C. Increasing volumes of dioxane/methanol (4:1, v/v) were added, centrifuged and soluble radioactivity was determined.

able amounts of enzymatic activity can be detected.

As shown on Figure 5, degradation of the substrate by alveolar macrophage collagenase was linear with time. Approximately 20% of the added substrate (20 µg) had been degraded after five hours of incubation at 30°C and pH 7.5.

## Levels of Collagenolytic and Elastinolytic Activities In Normal Fibroblasts, DLD-1, and B16-F10 Cells

Late log phase cultures of DLD-1 colon carcinoma cells, B16-F10 murine melanoma cells, and normal dermal fibroblasts were maintained in serum-free media for 24 hours. The cells and media were processed for enzyme assays, protein and DNA determinations as described in the Experimental Section. DLD-1 cells and normal fibroblasts produced comparable levels of Type I collagenolytic activity (Table 1). B16-F10 cells produced approximately half as much activity. Normal fibroblasts produced the highest level of Type V collagenolytic activity (0.48 ± 0.14 µg collagen degraded/mg protein/hr), DLD-1 cells produced about half this amount of activity while B16-F10 cells only degraded 0.13 ± 0.06 µg collagen/mg protein/hr.

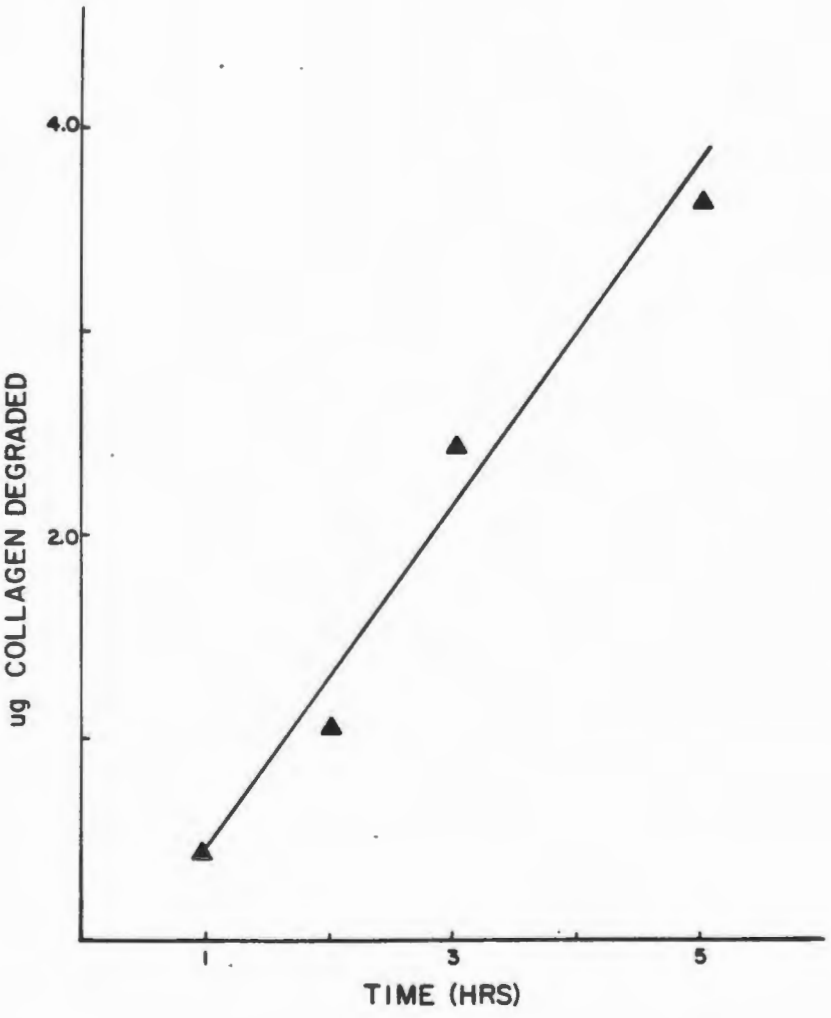


Figure 5. Degradation of Radiolabeled Type V Collagen by Alveolar Macrophage Collagenase As A Function of Time. $^{14}$C-Type V collagen (20 µg) was incubated with alveolar macrophage collagenase at 30°C for various lenghts of time and the amount of substrate degraded was determined.

Table 1. Levels of Type I Collagenase, Type V Collagenase, Cellular Elastace, and Media Elastase Produced by DLD-1 Colon Carcinoma Cells, B16-F10 Melanoma Cells, and Normal Dermal Fibroblasts.

| CELL LINE | ENZYME ACTIVITY | | | |
|---|---|---|---|---|
| | μg substrate degraded/hr/mg protein | | | |
| | TYPE I COLLAGENASE | TYPE V COLLAGENASE | CELLULAR ELASTASE | MEDIA ELASTASE |
| DLD-1 Colon Carcinoma | 7.18 ± 0.80 | 0.25 ± 0.03 | 0.25 ± 0.04 | 31.97 ± 2.80 |
| B16-F10 Melanoma | 3.70 ± 0.26 | 0.13 ± 0.06 | 0.38 ± 0.09 | 0.55 ± 0.09 |
| Normal Dermal Fibroblast | 7.27 ± 1.66 | 0.48 ± 0.14 | 0.77 ± 0.26 | 1.17 ± 0.32 |

Results are expressed as the mean ± S.D. with an n = 4.

The Triton X-100 cell extracts and culture media from the three cell lines were assayed for elastin degrading activity. DLD-1 cells produced the highest levels of elastinolytic activity of the three cell lines, this activity was found predominately in the media (>99% of the total). Fibroblasts contained the second highest level of elastinolytic activity, approximately 40% of the activity was cell-associated and 60% was in the media. The elastin degrading activity from B16-F10 cells was distributed between the cellular compartment and the media in the same ratio as the fibroblast activity.

## DEAE Ion Exchange Chromatography

Media containing DLD-1 collagen degrading activities was collected and several attempts were made to separate Type I and Type V collagen degrading activities using gel filtration chromatography. These attempts proved to be unsuccessful, each chromatogram revealed numerous peaks of Type I and Type V collagen degrading activity and adequate resolution of the two activities was not possible. DEAE cellulose ion exchange chromatography was then employed to separate Type I and Type V collagen degrading

activities. DEAE cellulose chromatography was used to separate these two activities produced by cultured macrophages (Mainardi et al, 1980a). Serum-free media from 900 plates of late log phase DLD-1 co-on carcinoma cells was used as the source of collagenase activity. As seen in Figure 6 approximately half of the media proteins bound to the DE-52 column and half passed through the column. The fractions containing the protein which did not bind to the resin were analyzed and only Type V collagen degrading activity was detected. The proteins which bound to the resin were eluted with a 400 ml linear NaCl gradient (0-0.3M). This was found to be insufficient to elute all the proteins, a second gradient of 0.3M to 1.0M NaCl was used to elute the remainder of the proteins. The first peak of protein eluted between 0.2M and 0.3M NaCl, these fractions were found to contain only Type I collagen degrading activity. The proteins which eluted in the second gradient between 0.3M and 0.5M NaCl were analyzed for collagenase activity and both Type I and Type V collagen degrading activity were detected.

## Gel Filtration Chromatography

The three peaks of collagen degrading activity generated by DEAE cellulose chromatography were further

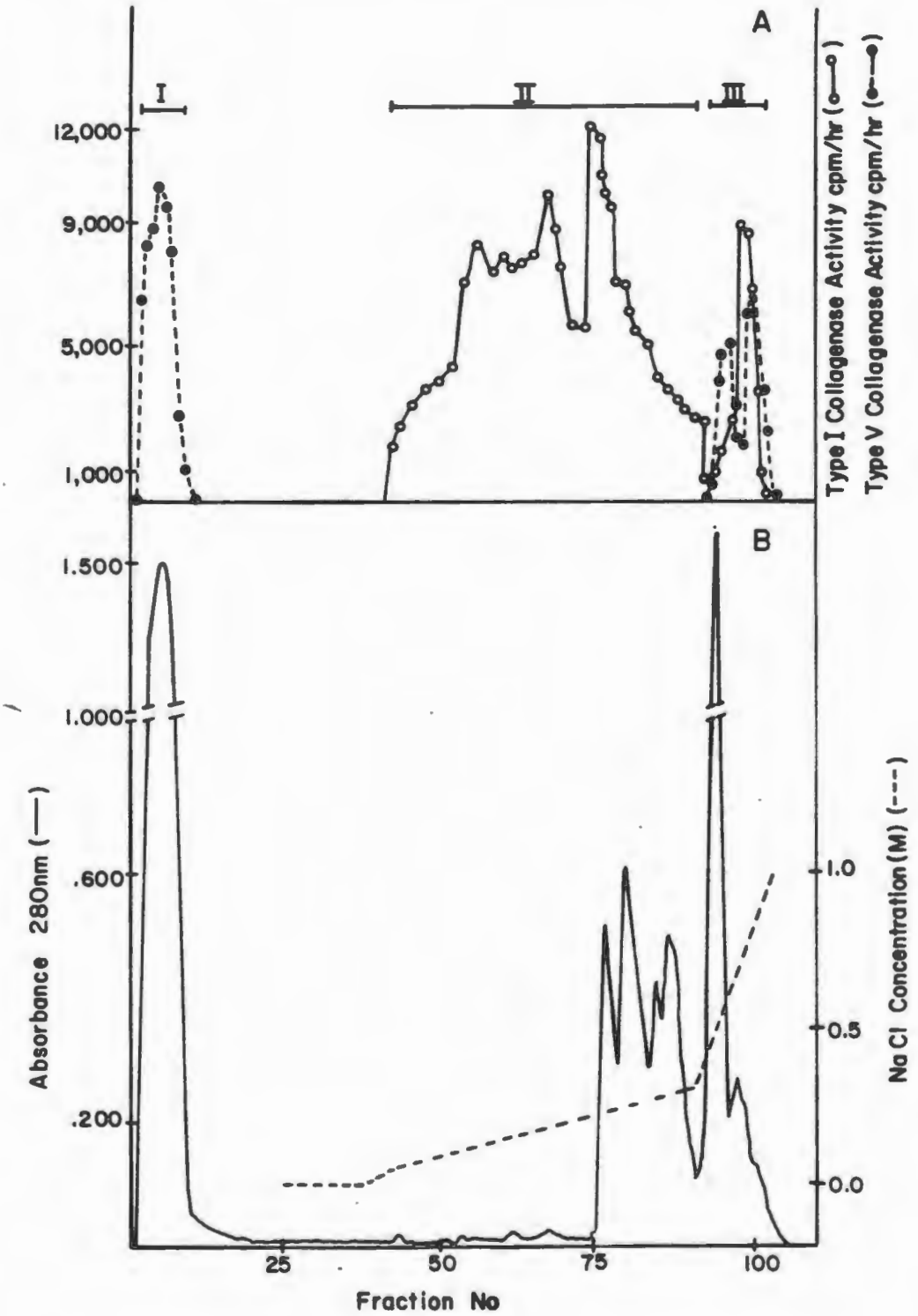
A
I
II
III
12,000
9,000
5,000
1,000
Type I Collagenase Activity cpm/hr (o—o)
Type V Collagenase Activity cpm/hr (•—•)
B
1.500
1.000
.600
.200
Absorbance 280nm (—)
1.0
0.5
0.0
NaCl Concentration (M) (---)
25
50
75
100
Fraction No

Figure 6. DEAE Cellulose Chromatography of Concentrated DLD-1 Colon Carcinoma Cell Serum-Free Media. Serum-free media from 900 100 x 20 mm culture dishes of late log phase DLD-1 cells was concentrated and applied to a 1.5 x 13 cm column of DE-52 equilibrated in 0.05M Tris-HCl pH 8.3 containing 0.005M $CaCl_2$ and 0.02% $NaN_3$. The bound proteins were eluted in two NaCl gradients, one from 0 to 0.3M NaCl and the second gradient from 0.3M to 1.0M. Six ml fractions were collected and monitored for absorbance at 280 nm (Panel B) and analyzed for Type I and Type V collagen degrading activity (Panel A). The fractions were combined to form three pools according to enzyme activity (I, II, III) as indicated.

fractionated by gel filtration chromatography on Ultrogel AcA44. The column was calibrated for molecular weight determination using Blue Dextran, bovine serum albumin, ovalbumin, pepsin, carbonic anhydrase, and ribonuclease A. The fractions containing the protein which did not bind to the DE-52 column, Pool I, were concentrated and applied to the Ultrogel AcA44 column. This pool was fractionated into two peaks of Type V collagen degrading activity (Figure 7). The first peak eluted in the void volume ($V_o$), this peak represented a minor portion of the total protein and enzyme activity. Its molecular weight (M.W.) was larger than 200,000 daltons, the exclusion limit for Ultrogel AcA44. The second peak of Type V collagen degrading activity generated by Ultrogel chromatography had an elution volume corresponding to a M.W. of 54,000 daltons. This peak constituted the majority of the enzyme activity added and only a minor portion of the total protein in Pool I. The majority of the protein in Pool I was of low M.W., approximately 10,000 daltons.

Pool II, the major peak of Type I collagen degrading activity from the DE-52 chromatogram, was also chromatographed on Ultrogel. It was separated into two peaks of protein and both contained Type I collagen degrading activity (Figure 8). The $V_o$ contained approximately half

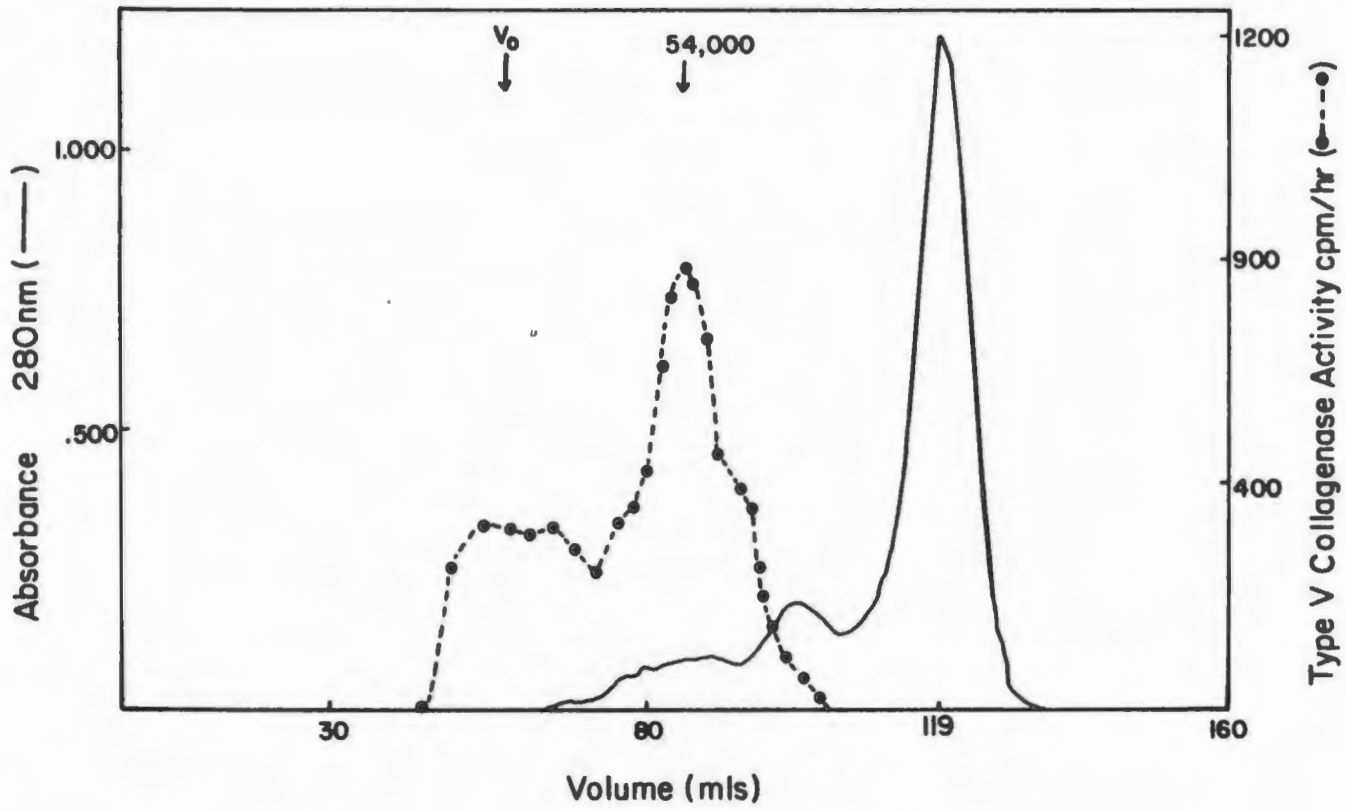


Figure 7. Ultrogel AcA44 Gel Filtration Chromatography of Pool I. All fractions in Pool I of the DE-52 chromatogram were concentrated by pressure dialysis to 1.5 mls and applied to a column (1.6 x 90 cm) of Ultrogel AcA44 equilibrated in 0.05M Tris-HCl pH 7.5 containing 0.01M $CaCl_2$ and 0.02% $NaN_3$. Fractions of 1.7 mls were collected and monitored for absorbance at 280 nm and for Type V collagenase activity.

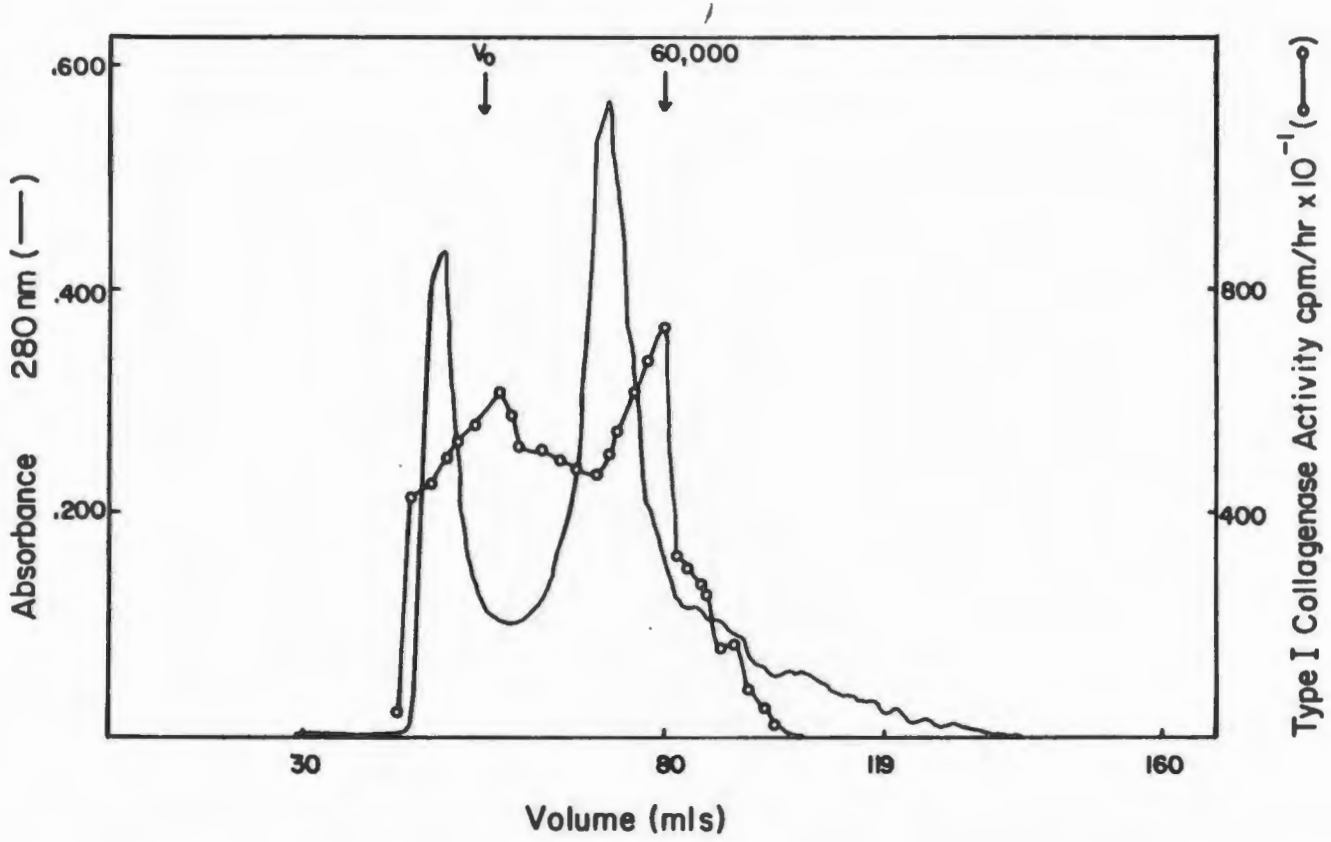


Figure 8. Ultrogel AcA44 Gel Filtration Chromatography of Pool II. All fractions in Pool II of the DE-52 chromatogram were concentrated by pressure dialysis to 1.5 mls and applied to a column (1.6 x 90 cm) of Ultrogel AcA44 equilbrated with 0.05M Tris-HCl pH 7.5 containing 0.01M $CaCl_2$ and 0.02% $NaN_3$. Fractions of 1.7 mls were collected and monitored for absorbance at 280 nm and for Type I collagenase activity.

of the total enzyme activity and about 30% of the total protein applied to the column. The second peak of enzymatic activity eluted in the 60,000 dalton M.W. range.

Pool III which eluted from the DEAE cellulose column during the 0.3M to 1.0M NaCl gradient, was fractioned into three peaks of enzyme activity by chromatography on Ultrogel AcA44 (Figure 9). The $V_o$ fractions from this chromatogram contained the majority of the protein that was loaded on the column. Both Type I and Type V collagen degrading activities were detected in these fractions. The second of the three peaks generated contained only Type V collagen degrading activity and had a M.W. of approximately 80,000 daltons. The final peak which eluted in the 35,000 dalton M.W. range contained Type I collagen degrading activity.

## Inhibition of Types I and V Collagen Degrading Activities

Concentrated serum-free media from DLD-1 colon carinoma cells were used as the source of Type I and Type V collagen degrading activities. Fetal calf serum, EDTA, PMSF, dithiothreital (DTT), and N-ethylmaleimide (NEM) were tested for their ability to inhibit DLD-1 collagen degrading activities using the standard enzyme assays

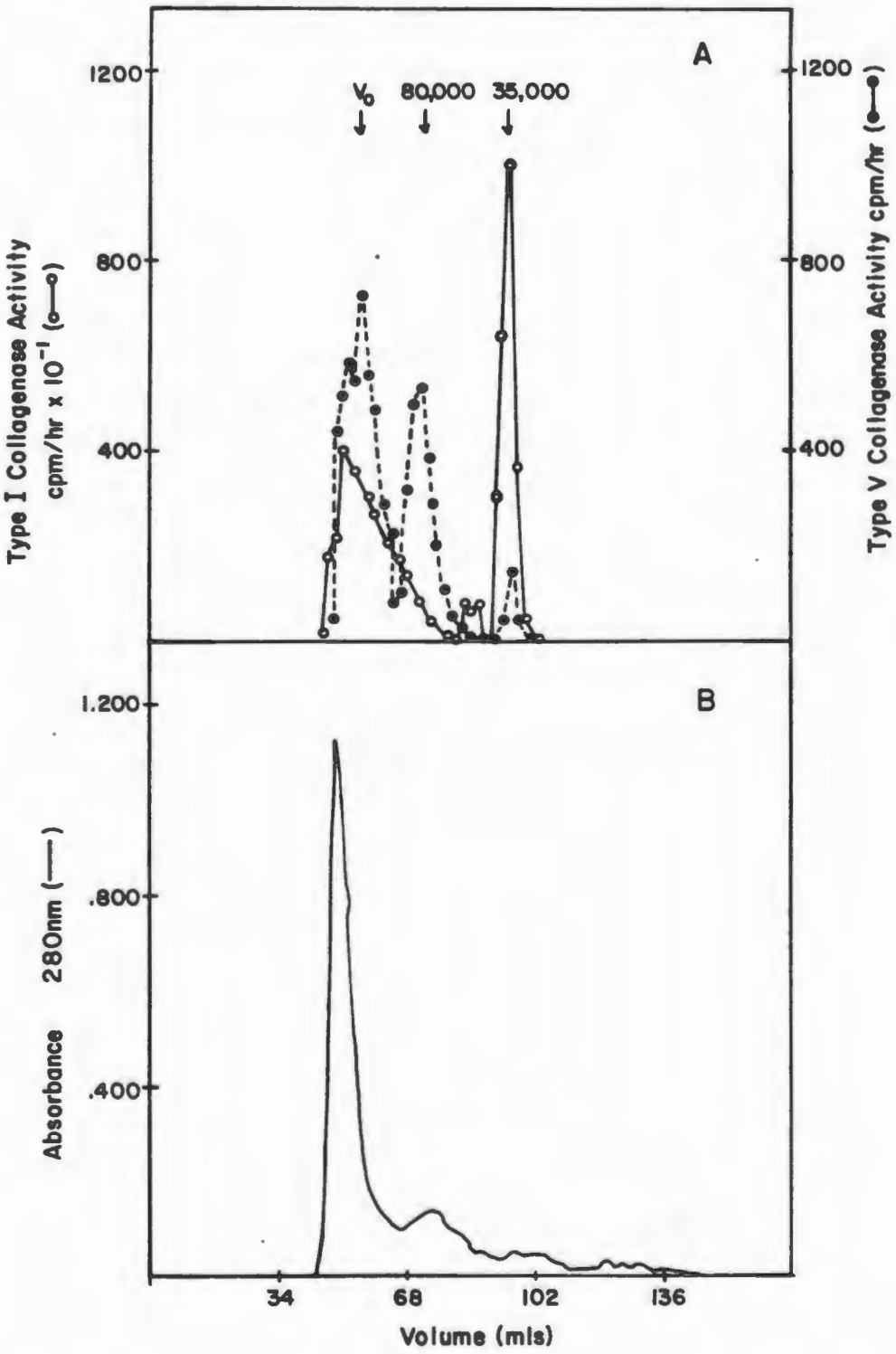
A
B
1200
800
400
1.200
.800
.400
$V_o$
80,000
35,000
Type I Collagenase Activity
cpm/hr x $10^{-1}$ (o—o)
Type V Collagenase Activity cpm/hr (●—●)
Absorbance 280nm (——)
34
68
102
136
Volume (mls)

Figure 9. Ultrogel AcA44 Gel Filtration Chromatography of Pool III. All fractions in Pool III of the DE-52 chromatogram were concentrated by pressure dialysis to 1.5 mls and applied to a column (1.6 x 90 cm) of Ultrogel AcA44 equilibrated with 0.05M Tris-HCl pH 7.5 containing 0.01M $CaCl_2$ and 0.02% $NaN_3$. Fractions of 1.7 mls were collected and monitored for Type I and Type V collagenase activity (Panel A) and absorbance at 280 nm (Panel B).

(Table 2). Both Type I and Type V collagen degrading activities were inhibited by serum and EDTA, the metalloproteinase inhibitor. The serine protease inhibitor PMSF had no effect on Type V collagen degrading activity and reduced the Type I collagen degrading activity by only 18%. DTT, which reduces disulfide bonds and maintains sulfhydryl groups in a reduced state, blocked 41% and 32% of Type I and Type V collagen degrading activities, respectively. NEM, which reacts with free sulfhydryl groups, had no effect on either enzyme activity.

## Effects of DMF on the Production of Type I and Type V Collagenase, Cellular, and Media Elastase Activities By DLD-1 Colon Carcinoma Cells

The effect of DMF on the production Type I and Type V collagenase and elastase activities by DLD-1 cells was determined using late log phase cultures. The production of these enzymatic activities was measured over a 24 hour period. Enzyme activity was normalized using total cellular protein and DNA values (Table 3). DMF caused a 20% increase in Type I collagenase activity over control cultures when the activity was expressed per mg of protein.

Table 2. Inhibition of Type I and Type V Collagen Degrading Activities by Protease Inhibitors.

| INHIBITOR | CONCENTRATION | PERCENT INHIBITION | |
|---|---|---|---|
| | | TYPE I COLLAGENASE[1] | TYPE V COLLAGENASE[2] |
| SERUM | 5% | 78 | 70 |
| EDTA | 10 MM | 73 | 68 |
| PMSF | 1 MM | 18 | 0 |
| DTT | 10 MM | 41 | 32 |
| NEM | 10 MM | 0 | 0 |

Crude DLD-1 colon Carcinoma Serum-Free Media was used as the source of collagenase activity in both assays.

1. Type I collagenase assay was incubated at 35°C for 1 hour.

2. Type V collagenase assay was incubated at 30°C for 12 hours.

Table 3. Effect of DMF[(1)] on Type I Collagenase, Type V Collagenase, Celluar Elastase, and Media Elastase Production by DLD-1 Colon Carcinoma Cells.

| ACTIVITY MEASURED | ENZYME ACTIVITY | | | |
|---|---|---|---|---|
| | µg substrate degraded/hr/mg protein | | µg substrate degraded/hr/mg DNA | |
| | CONTROL | DMF | CONTROL | DMF |
| Type I Collagenase | 7.18 ± 0.80 | 8.60 ± 0.50* | 311 ± 33 | 773 ± 167* |
| Type V Collagenase | 0.25 ± 0.03 | 0.37 ± 0.06* | 10 ± 1 | 34 ± 5* |
| Cellular Elastase | 0.25 ± 0.04 | 0.32 ± 0.02* | 10 ± 1 | 30 ± 4* |
| Media Elastase | 31.97 ± 2.80 | 22.38 ± 11.9 | 1345 ± 108 | 2066 ± 1034 |

Results are expressed as the mean ±S.D. with an n = 4.

(1) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control, $p < 0.05$ determined by unpaired Student's t test.

The increase due to DMF was 148% when DNA was used to normalize the enzyme values. Both values for Type I collagenase activity in DMF cultures were significantly higher than control values ($p < 0.05$). Type V collagenase activity increased 44% and 220% due to DMF treatment when enzyme activity was expressed per amount of protein and DNA, respectively. DLD-1 cellular elastase activity also increased as a result of DMF treatment. Cellular elastase activity per mg protein increased 31% while elastase activity per μg DNA increased 192%. Both values were significantly higher than control ($p < 0.05$). Media elastase activity per mg protein dropped by 30% in response to DMF. This decrease was not found to be significant ($p < 0.05$). Media elastase activity expressed per μg DNA increased 53% when the cells were treated with DMF. This value for enzyme activity in DMF treated culture was significantly higher than control cultures ($p < 0.05$).

To determine if these increases in activity were associated with alterations in the levels of cellular protein, DNA and RNA the levels of these cellular constituents were determined. Cells were grown in media containing 0.8% DMF for four passages prior to analysis. DMF caused a significant rise in the cellular levels of protein and RNA over controls ($p < 0.05$). DMF cells contained significantly less

DNA than control cells ($p < 0.05$) (Table 4).

## Effect of DMF on the Incorporation of $^{3}$H-glycine into Protein

The effect of DMF on the capacity of DLD-1 cells to synthesize and secrete protein was analyzed by measuring the rate of incorporation of $^{3}$H-glycine into TCA insoluble cell and media proteins. Determinations were made after three and six hours of growth in media containing 5 μCi $^{3}$H-glycine/ml. The results of these experiments were normalized in two ways; per amount of cellular protein and DNA. DMF treated cells incorporated $^{3}$H-glycine into cellular protein at a slower rate than control cells after three hours of growth in $^{3}$H-glycine containing media (Table 5). This decrease was significant only when the values were expressed per unit of DNA ($p < 0.05$). However, after six hours of incubation, the DMF treated cells were incorporating label into cellular protein at a faster rate than controls. The DMF values were significantly greater than controls by both units of measure ($p < 0.05$).

Analysis of media proteins revealed that DMF treated cells were secreting $^{3}$H-glycine containing protein into

Table 4. Effect of DMF(1) on Protein, DNA, and RNA Content of DLD-1 Colon Carcinoma Cells.

| TREATMENT | CELLULAR CONTENTS | | |
|---|---|---|---|
| | mg PROTEIN/CELL x $10^{-7}$ | µg DNA/CELL x $10^{-5}$ | µg RNA/CELL x $10^{-6}$ |
| Control | 6.89 ± 0.80 | 1.64 ± 0.22$^{x}$ | 6.11 ± 2.20 |
| DMF | 8.13 ± 0.50* | 0.87 ± 0.12 | 9.52 ± 0.83* |

Results are expressed as the mean ±S.D. with an n = 4.

(1) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control, $p < 0.05$ determined by unpaired Student's t test.

x Significantly greater than DMF, $p < 0.05$ determined by unpaired Student's t test.

Table 5. Effect of DMF(1) on the Incorporation of $^{3}$H-glycine into Cellular Protein of DLD-1 Colon Carcinoma Cells.

| LENGTH OF INCUBATION IN LABEL CONTAINING MEDIA | RATE OF INCORPORATION OF $^{3}$H-GLYCINE | | | |
|---|---|---|---|---|
| | dpm/mg Protein/hr x $10^{-3}$ | | dpm/μg DNA/hr x $10^{-3}$ | |
| | CONTROL | DMF | CONTROL | DMF |
| 3 Hours | 553 ± 28 | 539 ± 122 | 433 ± 2$^{x}$ | 331 ± 6 |
| 6 Hours | 528 ± 41 | 634 ± 57* | 324 ± 2 | 404 ± 3* |

Results are expressed as the mean ±S.D. with an n = 3.

(1) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control, $p < 0.05$ determined by unpaired Student's t test.

x Significantly greater than DMF, $p < 0.05$ determined by unpaired Student's t test.

the media at a slower rate than controls at three hours (Table 6). The rate of secretion of labeled proetin by DMF treated cells was significantly slower than control when expressed per unit of DNA but was not significantly different per unit of protein ($p < 0.05$). At six hours, the situation had reversed, DMF treated cells were secreting labeled protein at a faster rate than controls. This increase was significant only when the data was expressed per unit of DNA ($p < 0.05$).

## Effect of Cycloheximide on the Synthesis of Connective Tissue Degrading Enzymes by Control and DMF Treated DLD-1 Cells

Control and DMF treated DLD-1 cells were treated with cycloheximide to determine if the effects of DMF were dependent on the synthesis of protein. Cells were exposed to 1 µg cycloheximide/ml for the 24-hour period during which enzyme production was quantified. Treatment of DMF treated DLD-1 cells with cycloheximide resulted in a small decrease in Type I collagenolytic activity expressed per unit of protein and an insignificant increase in activity per unit of DNA ($p < 0.05$) (Table 7). Cycloheximide had the same effect on control cells; synthesis of Type I

Table 6. Effect of DMF(1) on the Incorporation of $^{3}$H-glycine into Media Protein of DLD-1 Colon Carcinoma Cells.

| LENGTH OF INCUBATION IN LABEL CONTAINING MEDIA | RATE OF INCORPORATION OF $^{3}$H-GLYCINE | | | |
|---|---|---|---|---|
| | dpm/mg Protein/hr | | dpm/μg DNA/hr | |
| | CONTROL | DMF | CONTROL | DMF |
| 3 Hours | 4811 ± 455 | 3450 ± 1078 | 374 ± 46 x | 213 ± 65 |
| 6 Hours | 5750 ± 1014 | 7309 ± 1110 | 353 ± 65 | 464 ± 47* |

Results are expressed as the mean ±S.D. with an n = 3.

(1) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control, $p < 0.05$ determined by unpaired Student's t test.

x Significantly greater than DMF, $p < 0.05$ determined by unpaired Student's t test.

Table 7. Effect of Cycloheximide(1) on Type I Collagenase Production by Control and DMF Treated(2) DLD-1 Colon Carcinoma Cells.

| TREATMENT | TYPE I COLLAGENASE ACTIVITY | |
|---|---|---|
| | µg collagen degraded/hr /mg protein | µg collagen degraded/hr /mg DNA |
| Control | 7.18 ± 0.8 | 311 ± 33 |
| Control + Cycloheximide | 6.27 ± 0.7 | 458 ± 145 |
| DMF | 8.60 ± 0.5* | 773 ± 167* |
| DMF + Cycloheximide | 8.54 ± 2.7 | 867 ± 200* |

Results are expressed as the mean ± S.D. with an n = 4.

(1) Cells were treated with 1 µg cycloheximide/ml.

(2) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control or control + cycloheximide, $p < 0.05$ determined by unpaired Student's t test.

collagenolytic activity was diminished but not completely abolished. Cycloheximide treatment could not inhibit the increase in Type I collagenolytic activity produced by DMF treatment.

Treatment of DMF and control DLD-1 cells with cycloheximide effected the synthesis of Type V collagenolytic activity in a manner similar to that seen with Type I collagenolytic activity. Increases in Type V collagenolytic activity due to DMF were still present when DMF and control cells were treated with cycloheximide; however, the magnitude of the increase was less (Table 8). DMF treated cells treated with cycloheximide produced significantly more Type V collagenolytic activity than control cells treated with cycloheximide ($p < 0.05$).

A similar pattern of reduction in the DMF effect upon treatment with cycloheximide was seen with cellular elastase activity (Table 9). DMF treated cells treated with cycloheximide produced significantly more cellular elastase activity than control cells treated with cycloheximide when the activity was expressed per unit of DNA ($p < 0.05$). The increase was not significant when expressed per unit of protein.

Table 8. Effect of Cycloheximide(1) on Type V Collagenase Production by Control and DMF Treated(2) DLD-1 Colon Carcinoma Cells.

| TREATMENT | TYPE V COLLAGENASE ACTIVITY | |
|---|---|---|
| | µg collagen degraded/hr /mg protein | µg collagen degraded/hr /mg DNA |
| Control | 0.25 ± 0.03 | 10.67 ± 1.24 |
| Control + Cycloheximide | 0.22 ± 0.01 | 16.52 ± 4.40 |
| DMF | 0.37 ± 0.06* | 34.20 ± 5.20* |
| DMF + Cycloheximide | 0.30 ± 0.06* | 31.30 ± 5.16* |

Results are expressed as the mean ± S.D. with an n = 4.

(1) Cells were treated with 1 µg cycloheximide/ml.

(2) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control or control + cycloheximide, $p < 0.05$ determined by unpaired Student's t test.

Table 9. Effect of Cycloheximide(1) on Cellular Elastase Production by Control and DMF Treated(2) Colon Carcinoma Cells.

| TREATMENT | ELASTASE ACTIVITY | |
|---|---|---|
| | µg elastin degraded/hr /mg protein | µg elastin degraded/hr /mg DNA |
| Control | 0.25 ± 0.04 | 10.30 ± 1.70 |
| Control + Cycloheximide | 0.23 ± 0.03 | 13.15 ± 1.90 |
| DMF | 0.32 ± 0.02* | 30.14 ± 4.10* |
| DMF + Cycloheximide | 0.24 ± 0.06 | 24.77 ± 4.50* |

Results are expressed as the mean ± S.D. with an n = 4.

(1) Cells were treated with 1 µg cycloheximide/ml.

(2) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control or control + cycloheximide, $p < 0.05$ determined by unpaired Student's t test.

A markedly different result due to cycloheximide treatment was obtained when media elastase activity was measured. Treatment of control cells with cycloheximide resulted in a small decrease in media elastase activity per unit of protein and a small but insignificant increase in activity per unit of DNA ($p < 0.05$) (Table 10). These results were analogous to those seen for Type I and Type V collagenase and cellular elastase. However, when DMF treated DLD-1 cells were treated with cycloheximide media elastase activity increased above the levels seen in cultures treated with DMF alone. These increases were significant when the activity was expressed per unit of protein and DNA ($p < 0.05$).

Table 10. Effect of Cycloheximide(1) on Media Elastase Production by Control and DMF Treated(2) DLD-1 Colon Carcinoma Cells.

| TREATMENT | ELASTASE ACTIVITY | |
|---|---|---|
| | µg elastin degraded/hr /mg protein | µg elastin degraded/hr /mg DNA |
| Control | 31.97 ± 2.8 | 1345 ± 108 |
| Control + Cycloheximide | 30.70 ± 11.0 | 1959 ± 914 |
| DMF | 22.38 ± 11.9 | 2066 ± 1034 |
| DMF + Cycloheximide | 52.68 ± 20.6 | 5151 ± 814* |

Results are expressed as the mean ± S.D. with an n = 4.

(1) Cells were treated with 1 µg cycloheximide/ml.

(2) Cells were treated with 0.8% DMF for four passages prior to analysis.

* Significantly greater than control + cycloheximide, $p < 0.05$ determined by unpaired Student's t test.

# DISCUSSION

## Type V Collagen Purification and Type V Collagenase Assay

In order to study the degradation of Type V collagen by tumor proteases a fairly large quantity of the protein in purified form was needed. Human placentas were a good source of Type V collagen since they were easily obtainable and contained fairly large quantities of collagenous proteins. Initial attempts to purify Type V collagen utilized the whole placenta. It was later found advantageous to use only the amnionic membrane as the starting material since Type V collagen comprises a greater portion of the total collagen present in anmions than in whole placentas (Sage and Bornstein, 1979). The exact molecular configuration of Type V collagen is not established, many preparations are composed of two different $\alpha$ chains, $\alpha$ 1(V) and $\alpha$ 2(V) in a 2:1 ratio (Rhodes and Miller, 1978; Bentz et al, 1978; Burgeson et al, 1976). A third $\alpha$ chain $\alpha$ 3(V) has been isolated from whole placenta but is absent from amnionic membranes (Sage and Bornstein, 1979). The Type V collagen purified in this study was of the configuration $\alpha$ 1(V)$_2$, $\alpha$ 2(V). The molecular weights of the $\alpha$ chains are in close agreement with those reported by Rhodes and Miller (1978). The purified material was

labeled via an acetylation reaction and used as the substrate in the Type V collagenase assay. Due to the high specific activity of the substrate ($1.12 \times 10^6$ dpm/mg) only 20 µg of collagen per assay was required to maintain a linear rate of degradation for the duration of the assay.

The Type V collagenase assay developed offered several improvements over existing methods. This assay used 30°C as an incubation temperature. This temperature is several degrees below the temperature at which the Type V collagen triple helix begins to unfold (~33°C)(Rhodes and Miller, 1978). Other assays used to measure Type V collagen degrading activity have used incubation temperatures of ~32.5°C and 37°C (Mainardi et al, 1980a; Liotta et al, 1980). At these temperatures the Type V collagen helix may be starting to unfold or may be partially denatured and non-specific degradation of the molecule may occur. Using 30°C as the incubation temperature, degradation of the substrate by non-collagenase proteases is minimized.

The degradation products formed during the reaction between enzyme and substarte were separated from non-degraded substrate molecules using dioxane/methanol. This solvent precipitates native substrate molecules but not degraded denatured molecules. Dioxane was first used in

in Type I collagenase assay by Terato et al (1976). The precipitating solvent was later modified by adding methanol (4:1 dioxane/methanol)(Lindblad and Fuller, 1982). They found the addition of methanol increased the precipitating efficiency of the solvent. The degradation products formed by three different collagenases exhibit differences in their solubility in dioxane/methanol. Mammalian collagenases are known to make one cleave in the collagen helix producing two specific reaction products (Gross and Nagai, 1965) where as bacterial collagenase digests the molecule into many low molecular weight peptides (Nagai et al, 1960). The larger reaction products produced by the mammalian collagenases precipitated at lower dioxane/methanol concentrations than the more extensively degraded bacterial collagenase reaction products. The solubility differences between mammalian and bacterial collagenase reaction products are most likely due to the size of the fragment formed and the degree to which the fragment retains a helical structure. 23% dioxane/methanol was chosen as the standard concentration for the assay because native and denatured substrate molecules are maximally separated at this concentration and 25%-50% of the total enzyme activity produced by the mammalian collagenases could be detected. Others have used 50% dioxane to precipiate non-degraded Type V collagen (Mainardi et al, 1980a). In this assay,

the addition of 50% dioxane would allow the detection of less than 15% of the total radioactivity solubilized by DLD-1 and alveolar macrophage collagenases. The addition of small quantities of highly radioactive collagen and the improved precipitating solvent increases the sensitivity of the assay. If one unit of enzyme activity is defined as 1 μg of collagen degraded per minute at 30°C, the assay can detect as little as 13 mU of enzyme activity. Similar substrates and precipitating solvents have been used in Type I collagenase assays and detection limits of 15 mU of enzyme activity were reported (Lindblad and Fuller, 1982).

## Levels of Connective Tissue Degrading Enzymes in Normal Fibroblasts, DLD-1 Colon Carcinoma, and B16-F10 Melanoma Cells

The levels of collagenolytic and elastolytic activity measured in these three cell lines are not easily compared to published values for other cell lines because of variations in the type of substrate used and the temperatures at which the assays were performed. Fibroblasts are one of the highest collagenase producers, collagenase protein represents 6% of the total protein exported by these cells (Valle and Bauer, 1979). DLD-1 colon carcinoma cells produced levels of collagen degrading activity that were com-

parable to those produced by normal dermal fibroblasts. DLD-1 cells produced considerably more collagenolytic activity than highly metastatic B16-F10 murine melanoma cells. B16-F10 cells are a subline of the B16 melanoma parent line which have been selected *in vivo* and *in vitro* for their ability to form experimental metastases (Fidler, 1973).

The location of elastase activity appears to vary with different cell types (Werb *et al*, 1982). The elastin degrading activity produced by DLD-1 cells was found almost exclusively in the culture medium. Elastases from pancreatic cells and macrophages are also secreted into the growth medium (Barrett *et al*, 1980; Banda and Werb, 1981). Elastolytic activity has also been detected in the media of cultured human breast carcinoma cells (Kao *et al*, 1982). The elastin degrading activity produced by the normal fibroblasts and B16-F10 cells was found in both the cellular compartment and the media, with a greater portion being cell-associated. Hornebeck *et al* (1980) has detected elastase activity in the Triton X-100 extracts of fibroblasts and smooth muscle cells. Neutrophil elastase is also known to be localized intracellularly (Werb *et al*, 1982).

## Characterization of DLD-1 Collagen Degrading Activities

One of the objectives of this study was to analyze the collagen degrading activities produced by DLD-1 cells to determine if Type I and Type V collagen were degraded by the same or different enzymes. DEAE cellulose partially separated Type I and Type V collagen degrading activities. All of the Type I collagen degrading activity added to the column bound and eluted in two peaks. DLD-1 Type I collagen degrading activity appears to be an anionic protein since it bound to the positively charged resin. The small peak of activity which eluted during the second gradient may be the same Type I collagenolytic activity complexed to a more strongly anionic media protein allowing a stronger interaction between the column and protein to occur. Protein-protein interactions involving collagenase and other culture media proteins has been documented (Fiedler-Nagy et al, 1976). Huang and Abramson (1975) and Woolley et al (1973) have characterized Type I collagenase from guinea pig and human skin cells as anionic proteins, the DLD-1 Type I collagenolytic activity appears to be similar to these enzymes. Several other Type I collagenases have been characterized and exhibit opposite charge properties. Rheumatoid synovial colla-

genase and rabbit $V_2$ ascites tumor collagenase fail to bind to anionic resins (Woolley et al, 1975a, McCroskery et al, 1975). Type I collagenase from human skin fibroblasts appears to have the capacity to bind to both anionic and cationic resin under similar conditions (Stricklin et al, 1977; Woolley et al, 1973).

The majority of the Type V collagen degrading activity in DLD-1 media did not bind to the DEAE column while a minor portion bound tightly. This protein appears to be cationic in nature. The minor fraction of the Type V collagenolytic activity which did bind to the column may be a complex of enzyme and another media protein which binds tightly to column. These results were different from those obtained by Mainardi et al (1980a) who separated macrophage Type I and Type V collagenolytic activities. All the Type V collagen degrading activity from macrophages bound to DEAE cellulose. The difference in the physiochemical properties of these two Type V collagenolytic activities may simply reflect a difference in enzymes derived from macrophages and neoplastic epithelial cells. Although the majority of Type V collagenolytic activity from DLD-1 cells failed to bind to the column, DEAE cellulose chromatography was an effective method for partially separating Type I and Type V

collagen degrading activities.

The two partially separated collagenase activities were further purified and characterized by gel filtration chromatography on Ultrogel AcA44. The Type V collagen degrading activity which did not bind to the DEAE column was fractionated into two peaks of enzymatic activity by gel filtration chromatography. The major peak of enzyme activity from the Ultrogel chromatogram eluted in the 54,000 dalton M.W. range. A small peak of enzymatic activity was present in the $V_o$, indicating a M.W. of 200,000 daltons or greater. Large M.W. peaks of enzyme activity have been encountered during collagenase purifications (Fidler-Nagy, 1977; Woolley et al, 1973; Huang and Abramson, 1975; Brikedal-Hansen et al, 1976). These peaks may be polymers of enzyme molecules or complexes of enzyme molecules and fragments of the collagen substrate (Woolley et al, 1973; Fidler-Nagy, 1977). Presently, only three Type V collagen degrading activities have been reported (Liotta et al, 1980; Mainardi et al, 1980a; Rich et al, 1983), and only one study has reported a M.W. Reticulium cell sarcoma Type V collagenolytic activity has a M.W. of 80,000 daltons (Liotta et al, 1980). The difference in M.W. between DLD-1 Type V collagen degrading activity and the reticulium cell sarcoma activity may

represent a difference in the enzyme produced by two different cell types. Alternatively, the activity reported by Liotta et al (1980) may be a complex of enzyme and a collagen degradation fragment.

The major peak of Type I collagen degrading activity from the DEAE chromatogram was also fractionated into two peaks of enzymatic activity by Ultrogel chromatography. One peak eluted in the $V_o$ and may be a complex of collagenase and collagen. Native collagens have M.S.'s of approximately 300,000 daltons and would elute from the Ultrogel column in the $V_o$. Collagenases are known to bind tightly to their substrates (Stricklin et al, 1978). The second peak of activity eluted in the 60,000 dalton M.W. range. This M.W. is in agreement with M.W.'s reported for Type I collagenases isolated from two other carcinoma cell lines. A M.W. of 63,000 daltons was reported for a rat prostate carcinoma collagenase (Huang et al, 1979) and 68,000 daltons has been cited as the M.W. of Type I collagenase from a squamous cell carcinoma of the parotid gland (Huang et al, 1982). These minor differences in M.W. may be due to the different types of gel filtration media used in each study. A third Type I collagenase isolated from an ascitic carcinoma cell line

has been characterized and a M.W. of 42,000 daltons was reported (Wolf and Wirl, 1982). The differences in M.W. between the ascites cell enzyme and the other Type I collagenases may be due to the presence of substrate fragments complexed with the enzyme or the same collagenases originating from different species or tissues may simply be heterogeneous.

The third pool of collagen degrading activity generated by DEAE cellulose was fractionated into three peaks of activity by gel filtration chromatography. A high M.W. peak of activity was found in the $V_o$ and contained both Type I and Type V collagenolytic activities. As in the two previous gel filtration chromatograms this peak was probably an enzyme-substrate complex. A peak of Type V collagenolytic activity eluted at a volume corresponding to 80,000 daltons. Liotta et al (1980) has also reported 80,000 daltons as the M.W. of a reticulium cell sarcoma Type V collagenolytic activity. The third peak of activity generated by Ultrogel chromatography had a M.W. of 35,000 daltons and contained predominately Type I collagenolytic activity. This activity has a M.W. similar to Type I collagenase purified from rabbit $V_2$ ascites tumor homogenates (McCroskery et al, 1975).

Type I collagenase from cultured human skin fibroblasts has been extensively characterized and two species of enzyme of different M.W. (45,000 and 50,000 daltons) have been separated (Stricklin et al, 1977; Stricklin et al, 1978). The two enzyme were immunologically similar as determined by immunodiffusion. They were also found to have similar amino acid compositions, cyanogen bromide peptide analysis revealed 9 peptides from each enzyme, 8 of which were common to both activities. Trypsinization could not convert the higher M.W. enzyme into the smaller one, the two proteins were considered to be different species of the same enzyme. DLD-1 colon carcinoma cells may also synthesize two different Type I and Type V collagen degrading activities. Since these activities are not highly purified it is possible that the large M.W. forms of each collagenase may be a complex of enzyme and substrate fragment. The two types of chromatography used in this study were adequate methods for partially separating DLD-1 Type I and Type V collagenolytic activities and estimating their M.W. However, these methods were unable to completely separate the enzymes from a large M.W. protein, mostly likely their substrate. The complete separation of the enzymes from their substrates may require more extreme purification conditions. The addition of urea or Triton X-100 to the buffer systems or heating the enzyme

containing sample to 60°C prior to subsequent purification steps have all been used to completely dissociate collagenases from their substrates (Harris and Vater, 1982).

Type I and Type V collagenolytic activities from DLD-1 cells were found to have similar inhibition profiles. The Type V collagen degrading activities reported by Mainardi et al (1980a) and Liotta et al (1980) were characterized as metalloproteinases and had no serine protease activity. The DLD-1 Type V collagen degrading activity can be similarly characterized since it was inhibited by EDTA but not PMSF. All Type I collagenases have been characterized as metalloproteinases and are uneffected by serine protease inhibitors (Harris and Vater, 1982). The DLD-1 Type I collagenolytic activity is predominately a metalloproteinase. A non-collagenase enzyme having serine protease activity may also be present in the crude collagenase preparation. This activity could act on the substrate following the initial cleavage of the triple helix by the collagenolytic metalloproteinase activity. Collagenase from a rabbit $V_2$ ascites cell carcinoma was inhibited by DTT (McCroskery et al, 1975) as were both Types I and V collagen degrading activities from DLD-1 cells. These enzymes may require intact disulfide

bond for maximal activity since the reducing agent (DTT) diminished enzymatic activity.

## Effects of DMF on DLD-1 Colon Carcinoma Cells

The effects of DMF on the turmorigenicity, morphology and *in vitro* growth properties of DLD-1 colon carcinoma cells has been examined (Dexter *et al*, 1979; Dexter and Hager, 1980). *In vitro* DMF reduces the growth rate of DLD-1 cells and causes the cells to enlarge (Dexter *et al*, 1979). DMF treated cells appear less round and more "leafy" than untreated cells. Concommitant with these morphological changes phase optic microscopy has revealed that DMF treated cells have a higher cytoplasmic to nuclear ratio (Dexter *et al*, 1979). In this study cells treated with DMF contained more protein and RNA per cell and less DNA per cell than control cells. This finding is in agreement with those obtained by phase optic microscopy.

Treatement of DLD-1 cells with DMF resulted in the increased production of Type I and Type V collagenase and cell-associated elastase activities. It is unlikely that DMF had a selective effect stimulating the production of these particular proteases. The increases in enzymatic

activity probably resulted from a non-specific increase in the rate of protein synthesis. This hypothesis, however, cannot explain the decrease in media elastase activity seen in DMF treated cultures. This decrease was not statistically significant and the value may be erroneous since the corresponding standard deviation was very large.

To determine if the effect of DMF on protease production was the result of an increased rate of protein synthesis, the rate of incorporation of $^{3}$H-glycine into protein was measured. After three hours of growth in label containing media, the DMF cultures appeared to be incorporating label slower than control cultures, although the decrease was significant only when the data were expressed per unit of DNA. After six hours of growth, the DMF cultures were synthesizing protein faster than controls. This increased rate of protein synthesis could explain the increased levels of Type I and Type V collagenases and cellular elastase activities present in DMF treated cultures. The decreased rate of incorporation of $^{3}$H-glycine seen in DMF treated cells at the three hour point may not be a true indicator of decreased macromolecule synthesis, the decrease could have resulted from an initial lag in the rate of glycine penetration into the cells. DMF treatment may have altered the cell membrane configuration

resulting in a decreased rate of amino acid uptake. After longer growth periods in label-containing media sufficient quantities of $^{3}$H-glycine may have entered the DMF treated cells and allowed their rate of protein synthesis to exceed controls.

If the DMF effect on enzyme production was due to increased enzyme biosynthesis, blockade of protein synthesis with cycloheximide should inhibit the effect. Control and DMF treated cultures were treated with 1 μg cycloheximide/ml, a dose which inhibited collagenase production by endotoxin-activated macrophages (Wahl et al, 1974). The effect of DMF on Type I and Type V collagenolytic activities was still apparent however, in the presence of cycloheximide. This result indicated that DMF could produce its effect independent of protein synthesis. It has been reported that collagenases are synthesized and stored intracellularly as zymogens (Harper et al, 1971). The DMF treated cells may have synthesized and stored intracellularly greater quantities of these enzymes prior to the inhibition of protein synthesis. The increased levels of these enzymes seen in DMF treated cells treated with cycloheximide could have been the result of the release of greater quantities of stored enzyme.

When DMF treated cells were exposed to cycloheximide for 24 hours, the cells secreted large amounts of elastase into the media. Control cells did not respond to cycloheximide in the same manner. There was no significant difference in media elastase levels in control and control cells treated with cycloheximide, this was analogous to the effects on collagenolytic activities. The elastase activity secreated by DMF treated cells following cycloheximide treatement most likely originated from a preexisting intracellular store of enzyme. Cycloheximide has been shown to effect the turnover of tyrosine transaminase in cultured liver cells leading to increased levels of transaminase activity (Kenney, 1967; Hershko and Tomkins, 1971). It was postulated that the synthesis of proteases responsible for the degradation of intracellular proteins are more sensitive to cycloheximide treatment than the synthesis of tyrosine transaminase (Kenney, 1967). DLD-1 elastase levels may have been increased due to decreased intracellular degradation of the enzyme.

# CONCLUSIONS

1. Type V collagen was purified from human amnions, labeled and used as a substrate in an improved assay for measuring Type V collagen degrading activity. Non-degraded substrate molecules were separated from degraded substrate fragments by precipitation with 23% dioxane/methanol.

2. DLD-1 colon carcinoma cells, B16-F10 murine melanoma cells, and normal dermal fibroblasts produced proteases which could degrade Type I and Type V collagen and elastin.

3. Type I and Type V collagen degrading activities secreted by DLD-1 cells were characterized as metalloproteinases. These activities were partially separated using DEAE cellulose chromatography.

4. Two forms of DLD-1 Type I collagenolytic activity were partially purified and had molecular weights of 60,000 and 35,000 daltons.

5. Two forms of DLD-1 Type V collagenolytic activity were partially purified and had molecular weights of 80,000 and 54,000 daltons.

6. DMF treatment increased the production of Type I and Type V collagenolytic activities and cell-associated elastinolytic activity by DLD-1 cells.

7. DMF treatment increased the cellular protein and RNA content and decreased the DNA content of DLD-1 cells.

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