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An inhibitor of angiogenesis isolated from bovine cornea

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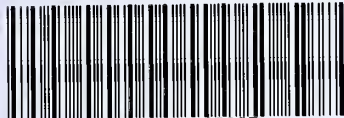Follow this and additional works at: http://elischolar.library.yale.edu/ymtdl

AN INHIBITOR OF ANGIOGENESIS
ISOLATED FROM BOVINE CORNEA

ELIZABETH ANNE MULLEN

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AN INHIBITOR OF ANGIOGENESIS

ISOLATED

FROM BOVINE CORNEA

Elizabeth Anne Mullen

A thesis in partial fulfillment of the requirements for the degree

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DEDICATION

With gratitude and love to my parents, Katherine and John Mullen, for their boundless support and encouragement.

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ABSTRACT

AN INHIBITOR OF ANGIOGENESIS ISOLATED FROM BOVINE CORNEA. Elizabeth A. Mullen, Rosalind A. Rosenthal, *Peter V. Hauschka and Judah Folkman. Department of Surgical Research, Children's Hospital, Boston, MA, *Department of Orthopedic Research, Children's Hospital, Boston, MA. (Sponsored by Caren M. Gundberg, Department of Orthopaedics and Rehabilitation, Yale University School of Medicine, New Haven, CT).

The normally avascular cornea paradoxically contains stores of a potent stimulator of angiogenesis, basic fibroblast growth factor (bFGF) (Am. J. Path. 130:393, 1988). Mun *et al.* reported the partial purification from bovine cornea of a low molecular weight molecule which inhibits angiogenesis in the chick embryo (Inv Opth & Vis Sci. 30:151, 1989). This factor, termed corneal angiogenesis inhibitor (CAI), was also shown to antagonize certain bFGF induced effects on capillary endothelial cells, including cell migration, cell spreading and cell rounding. Endothelial cell rounding *in vitro* has been correlated with anti-angiogenesis *in vivo* (Endocrinology 119:1768, 1986). We report an improved purification method for CAI, which like Mun *et al.* used the ability of CAI to cause endothelial cell rounding to direct purification. We also measured the ability of CAI to inhibit DNA synthesis in endothelial cells, and block angiogenesis on the chorioallantoic membrane of chick embryos. Our improved

purification produced a sample sufficiently pure for preliminary structural analysis. This method includes: (1) Dissection of bovine corneas; (2) Extraction into 100ºC distilled water; (3) Filtration of corneas and centrifugation of filtrate; (4) Ultrafiltration through pressure dialysis, 1000 molecular weight (MW) cutoff, and lyophilization; (5) Extraction in 80% dimethylformamide (DMF), centrifugation and evaporation of solvent; (6) 100% DMF extraction, centrifugation and evaporation of solvent; (7) Reversed phase high performance liquid chromatography on both a C18 semi-preparative column and a C18 analytical column. Samples purified by this method were analyzed by desorptive chemical ionization and electospray mass spectroscopy. Possible candidates for the CAI molecule were identified at MW 228 and 172. CAI may play an important role in the regulation of vascularization in the cornea, perhaps acting as an endogenous inhibitor of bFGF.

INTRODUCTION

The regulation of vascularization is of great importance in many normal physiologic and pathologic processes. The term "angiogenesis" was introduced in 1935 to describe the formation of new blood vessels in the placenta (Hertig, 1935). It is currently used to denote the process of generating new capillary blood vessels from established vessels, resulting in "neovascularization". Under normal physiologic conditions, angiogenesis has an essential role in embryonic development, ovulation, placental maturation, and wound healing (Jakob et al., 1977; Gospodarowicz and Thakral, 1978; Wagner, 1980; Bär, 1980; Hunt et al., 1981).

Abnormal neovascularization dominates a number of serious pathologic conditions, both malignant and nonneoplastic. Much evidence exists that solid tumor growth is angiogenesis dependent (reviewed in Folkman, 1990; Klagsbrun and Folkman, 1990). Tumor angiogenesis and the incidence of metastasis in invasive breast carcinoma are highly correlated (Weidner et al., 1991). Many diverse diseases (Table 1) in which the dominant pathology is persistent neovascularization have been categorized as "angiogenic disease" (Folkman and Klagsbrun, 1987). Patients affected by angiogenic diseases span a wide range of ages, and include both sexes. Care and management of these conditions are divided among numerous medical and surgical specialties. For example, the excessive proliferation and shedding of the

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epidermis in psoriasis has been related to abnormal capillary growth in the dermis (Ryan, 1970). Hemangiomas can occur in newborn babies, with abnormal capillary proliferation persisting until up to two years of age, and which in rare cases may result in death from hemorrhage (Folkman, 1984; Folkman and Klagsbrun, 1987). All hemangiomas are known to eventually regress spontaneously; the mechanism through which this occurs is completely unknown. Atherosclerotic plaques can bleed secondary to capillary proliferation within the vessel wall, and contribute to sudden occlusion of coronary arteries (Barger *et al.*, 1984).

Abnormal neovascularization can also affect normally avascular tissue, as in rheumatoid arthritis, where the occurrence of capillary growth destroys joint cartilage (Matsubara and Ziff, 1987; Koch *et al.*, 1986; Crisp *et al.*, 1984). Neovascularization in avascular components of the eye leads to decreased vision or blindness. Diabetic retinopathy, retrolental fibroplasia, and neovascular glaucoma are pathologic conditions which involve a breach of the vascular/avascular barriers within the eye (Klagsbrun and Folkman, 1990). Disorders involving either the corneal epithelium or stroma (Table 2) can result in invasion of blood vessels into the avascular corneal stroma, and are a leading cause of blindness worldwide (Garner, 1986).

The control of the stimulation and inhibition of angiogenesis has been extensively investigated. A picture has

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emerged of a regulatory system analogous to other known physiologic processes, such as blood coagulation (Folkman and Klagsbrun, 1987). Both systems must be maintained in a state of inactivity, but constant readiness, over a long period of time. The proliferation of vascular endothelial cells in most normal adult tissue is measured with turnover time in years (Denekamp, 1984), congruent with a microvascular system designed to remain quiescent over periods of weeks to decades. The microvascular system, however, must be capable of prompt response when faced with pathologic conditions or physiologic demands which require the rapid growth of new vessels.

The search for factors which may stimulate or inhibit angiogenesis has been extensive. A number of factors, including polypeptides and nonpeptide low molecular weight compounds, have been shown to be angiogenic *in vivo* (Klagsbrun and Folkman, 1990) (Table 3). The process of angiogenesis *in vivo* is very complex. Many sequential steps are required: the degradation of capillary basement membrane, the migration and proliferation of endothelial cells, and tube formation. One of the earliest described stimulators of cell division, basic fibroblast growth factor (bFGF) has been found to be a potent stimulator of endothelial cell growth and angiogenesis. The term FGF was first used to describe a cationic polypeptide isolated from brain in 1974 (Gospodarowicz, 1974, 1975); its primary amino acid structure was described in 1985 (Esch *et al.*, 1985). An anionic species of FGF, which was termed acidic fibroblast growth factor

(aFGF) was also found in brain, and shown to have a 53% absolute sequence homology to the cationic polypeptide, which became known as bFGF (Thomas *et al.*, 1980; Lemmon and Bradshaw, 1983; Thomas 1987; Esch *et al.*, 1985). The FGFs have since been shown to be mitogenic and chemotactic for endothelial cells *in vitro*, to stimulate endothelial cells to produce proteases (collagenase and plasminogen activator) which result in degradation of basement membrane, and to induce capillary endothelial cells to migrate into three-dimensional collagen matrices and form tubes similar to capillaries (Gross *et al.*, 1983; Montesano *et al.*, 1986). These demonstrated *in vitro* properties of the FGFs are consistent with their observed angiogenic activity *in vivo*.

The FGFs are among the most ubiquitous growth factors in the body (Folkman and Klagsbrun, 1987). bFGF is found in brain (Lobb and Fett, 1984; Gospodarowicz *et al.*, 1984; Bohlen *et al.*, 1984), eye (Courty *et al.*, 1985), cartilage (Sullivan and Klagsbrun, 1985), bone (Hauschka *et al.*, 1986), reproductive tissue (Moscatelli *et al.*, 1986), adrenal gland (Gospodarowicz *et al.*, 1986), kidney (Baird *et al.*, 1985a), macrophages (Baird *et al.*, 1985b) and tumors (Shing *et al.*, 1984; Klagsbrun *et al.*, 1986), while aFGF is present mainly in neural tissue including brain (Lobb and Fett, 1984; Thomas *et al.*, 1984; Conn and Hatcher, 1984), retina (D'Amore and Klagsbrun, 1984), and eye (Courty *et al.*, 1985), and has also been identified in bone (Klagsbrun and Folkman, 1990). This extensive distribution raises the question of how rampant capillary proliferation is prevented in both

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normally vascular, and particularly, in normally avascular tissue. This question, in turn, has prompted the search for naturally occurring inhibitors of angiogenesis.

The first angiogenesis inhibitor was found in the normally avascular cartilage (Eisenstein *et al.*, 1973; Brem and Folkman, 1975), and has recently been characterized and identified as cartilage-derived inhibitor (CDI) (Moses *et al.*, 1990). CDI is a member of the tissue inhibitor of metalloproteinase (TIMP) family, and has been shown to inhibit angiogenesis *in vivo*, and capillary endothelial cell proliferation and migration *in vitro* (Moses and Langer, 1991; Moses *et al.*, 1990). This protein is also an inhibitor of mammalian collagenase. A number of other naturally-occurring inhibitors of angiogenesis have been identified. These factors include the 16 kDa fragment of prolactin (Ferrara *et al.*, 1991), thrombospondin (Good *et al.*, 1990; Bagavandoss and Wilks, 1990; Taraboletti *et al.*, 1990), platelet factor 4 (Maione *et al.*, 1990), and protamine (Taylor and Folkman, 1982). Transforming growth factor-beta (TGF-B) (Baird and Durkin, 1986; Müller *et al.*, 1987; Jennings *et al.*, 1988), and tumor necrosis factor-alpha (TNF-α) (Frater-Schroder *et al.*, 1987; Schweigerer *et al.*, 1987) are both polypeptides that inhibit endothelial cell proliferation *in vitro*, yet promote angiogenesis *in vivo* and tube formation *in vitro* . It is possible that TNF-α and TGF-B may inhibit the proliferative phase of endothelial cells, but promote the differentiation phase, e.g., matrix production and tube formation (Klagsbrun and Folkman, 1990).

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To date, none of these known angiogenesis inhibitors have been found in the avascular structures of the eye. However, much evidence exists to support the occurrence of endogenous inhibition of angiogenesis within the eye.

The eye contains both vascular and avascular structures (Figure 1). The sclera, which comprises the posterior four-fifths of the surface of the globe, is composed largely of collagen, proteoglycan and fibroblasts. Interestingly, although the sclera is interposed between two highly vascular tissues, the episclera and the choroid, it is avascular. Inflammatory diseases of the ocular surface involving macrophages and bFGF containing mast cells, which often result in corneal neovascularization, do not cause scleral neovascularization (Sunderkotter *et al.*, 1991). This could be attributed to the presence of a potent endogenous inhibitor(s) in the sclera. Alternatively, there could be physical or mechanical resistance to angiogenesis in the sclera.

The fetal lens contains hyaloid vessels during embryonic development; these regress shortly before birth. Human lens epithelium has been shown to contain angiogenic aFGF (Baudouin *et al.*, 1990). Paradoxically, extracts of both human and bovine lens demonstrate a reversible dose-dependent inhibition of bovine aorta endothelial cell proliferation *in vitro*. This result suggests that an extractable, endogenous inhibitor of angiogenesis also exists (Williams *et al.*, 1984). It has also been shown that the removal of lens and capsule in proliferative diabetic retinopathy

leads to an increased incidence of iris neovascularization (Aiello *et al.*, 1983). Numerous mechanisms to explain this phenomenon have been postulated, including facilitated diffusion of a retinal "vasoformative factor" (Ashton, 1957), decreased oxygenation of the iris and release of an "iris vasoformative factor" (Schulze, 1967), and reduced endogenous inhibition from the lens (Williams *et al.*, 1984).

The <u>aqueous</u> humor fills the anterior chamber, and physically contacts the cornea, lens, iris, ciliary body and trabecular meshwork. It has been demonstrated that normal aqueous humor inhibits capillary endothelial cell proliferation *in vitro*, while it promotes a dose-dependent proliferation of 3T3 smooth muscles cells and dermal fibroblast *in vitro* (Okamoto *et al.*, 1990). Interestingly, it was also found that this anti-angiogenic activity is absent in the aqueous humor of diabetic rabbits. The identity or source of the proposed endogenous inhibitor has not been elucidated.

The <u>vitreous</u> humor occupies the chamber anterior to the retina and posterior to the lens. It is composed of water, collagen, hyalocytes, and the proteoglycan hyaluronic acid. Clinically, the vitreous appears to be resistant to angiogenesis. In proliferative diabetic retinopathy, fronds of developing blood vessels grow onto the vitreous surface, but do not penetrate its substance. Interestingly, the vitreous from patients with diabetic proliferative retinopathy has been shown to contain

elevated levels of bFGF (Sivalingam *et al.*, 1990). This observation suggests the possibility of an increase in the ratio of angiogenesis stimulators to inhibitors in the vitreous in this disease. As evidence of the angiogenic potential of the vitreous, degradation products of vitreous hyaluronic acid assayed on the chick chorioallantoic membrane (CAM) were shown to induce neovascularization (West *et al.*, 1985). However, vitreous has also been shown to be inhibitory of angiogenesis. Angiogenic tumors implanted into the vitreous remain small and avascular - unless they happen into direct contact with the retina (Brem *et al.*, 1976). Extracts from the vitreous have been shown to inhibit both retinal extract-induced angiogenesis, and tumor-induced angiogenesis (Lutty *et al.*, 1985). These findings support the concept of regulatory interplay between both inhibitory and stimulatory factors of angiogenesis which, under normal conditions, maintain an avascular state in the vitreous.

Neovascularization of the retina is a serious consequence of several pathologic conditions; diabetes, sickle cell anemia, branch vein occlusion, and retrolental fibroplasia (Patz, 1982). Blindness results from vitreous hemorrhage and retinal detachment following the development of new blood vessels in the retina (Ashton, 1983). Studies have shown that retina contains endothelial cell growth factors, including aFGF and bFGF (Baird *et al.*, 1985), as well as serum factors such as platelet release factors (Miyazono *et al.*, 1987), and insulin-like growth factors (Grant *et al.*, 1987; King *et al.*, 1985). However, more

recently McIntosh and co-workers showed that retinal extract inhibited the serum-induced proliferation of retinal capillary endothelial cells (McIntosh *et al.*, 1989). This finding suggests a regulatory system for vascularization within the retina involving a balance of angiogenesis stimulators and inhibitors.

The normally avascular cornea is paradoxically known to contain large stores of angiogenic bFGF (Folkman *et al.*, 1988). The bFGF has been shown to bind to Bowman's layer, the acellular, most anterior portion of the collagenous corneal stroma, which is rich in proteoglycan (Adamis *et al.*, 1991). BFGF is also found in Descemet's membrane, a true basement membrane produced by corneal endothelial cells on the posterior surface of the cornea (Figure 2). Folkman and co-workers demonstrated that bFGF could be released from the cornea by treatment with heparin, heparan sulfate, or heparanase (Folkman *et al.*, 1988). Their work suggested that sequestration of this angiogenic endothelial mitogen in the basement membrane could be a general mechanism for controlling the accessibility of bFGF to vascular endothelium, therefore regulating vascularization. The abnormal release of bFGF could be the cause of corneal vascularization in a number of ocular diseases. In support of this concept, injury-induced release of bFGF from corneal epithelial cells has recently been demonstrated (Adamis *et al.*, 1991).

As an alternate, or additional explanation for the maintenance of avascularity of the cornea in the presence of

angiogenic bFGF, the existence of an angiogenesis inhibitor in the cornea was hypothesized. A number of studies supported this hypothesis. Corneal extracts which were mitogenic for endothelial cells were found to be inhibitory of cell growth at high concentrations (Folkman *et al.*, 1988). When pieces of cornea from several animal species were placed onto the chorioallantoic membrane (CAM) of a developing chick embryo (Folkman, Casey, Levenson, unpubl. results), avascular zones appeared around the corneas. These corneas could be moved to another chick embryo, and again, an avascular zone on the CAM surrounding the cornea would be produced. This suggested the production of a diffusible angiogenesis inhibitor by the cornea.

A systematic search for this corneal angiogenesis inhibitor, termed CAI, was undertaken. A partial purification scheme was established by Susan Doctrow, Edward Mun, and Judah Folkman, and many characteristics of the molecule defined. The inhibitor was found to stable to boiling and pronase digestion. Sensitive amino acid analysis techniques revealed no amino acids, indicating the inhibitor is not a peptide. Bioactivities of the partially purified CAI were also characterized. In addition to the inhibition of angiogenesis on the CAM assay, CAI was shown to cause inhibition of DNA synthesis in endothelial cells, and inhibition of bFGF stimulated cell migration, in a Boyden chamber model (Mun *et al.*, 1989). Partially purified CAI blocks both attachment (cell number analysis) and cell spreading of bovine capillary endothelial (BCE) cells in response to bFGF

(morphometric analysis) (Carter, Ingber, Folkman, unpubl.). Staining with antibodies to vimentin demonstrates disconnection of the intermediate filaments from the plasma membrane, followed by the depolarization of actin. Associated with the inhibition of cell spreading, corneal extracts can also cause endothelial cells to "round", in a dose-dependent manner. This rounding effect can be reversed by titration with bFGF. Rounded cells exclude trypan blue, and stain with fluorescein diacetate, indicating viability. Inhibition of cell migration was observed with the lowest quantities of CAI, followed by DNA synthesis inhibition, and endothelial cell rounding. Angiogenesis inhibition on the CAM required the most material.

A semi-quantitative screening assay based on the cell rounding effect of CAI was designed to guide the further purification of this molecule (Mun *et al.*, 1989). In large part, the purification was hampered by inconsistent behaviors and reproducibility of results, particularly in achieving binding to high-pressure liquid chromatography (HPLC) column resins.

In our work, we have further improved the purification of CAI, while retaining its bioactivity. Aqueous extraction, ultrafiltration, organic extraction, and reverse phase high-pressure liquid chromatography were used to reproducibly obtain a partially pure preparation of CAI, suitable for preliminary analysis by mass spectroscopy.

METHODS

Cell Culture - Bovine capillary endothelial (BCE) cells were isolated from bovine adrenal glands as described by Folkman *et al*., (1979), except 5 μ/ml crude retinal factor (Gitlin and D'Amore, 1983) was used instead of mouse sarcoma conditioned medium. BCE cells are usually maintained in Dulbecco's modified Eagle's medium (DMEM), containing 25 μg/ml endothelial mitogen (BTI), 10% calf serum, 2 mM glutamine, and antibiotics.

Endothelial Cell Rounding Assay - The samples to be tested on the rounding assay were first dried in a model TM 100 SpeedVac Concentrator (Savant), and then dissolved in rounding assay media (DMEM, 10% calf serum, 20 mM HEPES pH 7.4, 2 mM glutamine and antibiotics). Bovine capillary endothelial cells were seeded onto 96 well half-area plates (0.16 cm^2 wells) (CoStar), at a density of 2000 cells per 25 μl per well, in rounding assay medium. After a 6 - 12 hour attachment period, the cells were treated with serial dilutions of inhibitor fractions (Figure 3). Cells were examined by phase microscopy after 6 - 24 hours, and judged for cell rounding. One unit of cell rounding is defined as the amount of sample which is sufficient to cause rounding in 50% of the cells in a given well (Figure 4). The number of rounding units in a given sample is calculated from the greatest dilution of sample which will produce one unit of cell rounding.

DNA Synthesis Assay - Bovine capillary endothelial cells are plated on 48 well plates (CoStar) at a density of 7500 cells/well in DMEM containing 2% calf serum, 3 μM thymidine, 2 mM glutamine, and antibiotics. After a 24 hour attachment period, the medium is exchanged with rounding assay medium, the samples are added, and the cells are incubated for an additional 18 hours at 37°C. The cells are then incubated with 1 μCi ^{3}H-thymidine/well (82.2 Ci/mmol) for 6 h. The cells are washed once with phosphate-buffered saline (PBS). The cells are subjected twice to a wash of cold methanol for 5 minutes each wash, and twice to cold 5% trichloroacetic acid for 10 minutes each. The plates are washed briefly with water and O.3 M NaOH is added. The NaOH-solubilized material is transferred to vials containing scintillation fluid. The sample is neutralized with glacial acetic acid, and the vials are counted in a Beckman model LS 3801 scintillation counter.

Chick Chorioallantoic Membrane (CAM) Assay - The CAM assay is performed as described by Crum *et al.* (1985). The sample to be tested for stimulatory or inhibitory properties is combined with a carrier of 0.45% methylcellulose (10 μl), and air dried to form a disc of 2 mm diameter. This disc is applied to the shell-less embryo in a cultured petri dish (Auerbach *et al.*, 1974; Klagsbrun *et al.*, 1976; Folkman, 1985). After incubation at 37°C in 3% CO_2 for 48 hours, the embryos are examined under a dissecting microscope (6X power). An embryo is scored as negative (indicating inhibition) if an avascular zone larger than 4

mm^2 is observed (Figure 5).   Positive angiogenic activity is revealed by the radical ingrowth of new vessels.   Inhibition or stimulation of neovascularization can be confirmed by histologic microsections of India-ink-injected specimens (Haudenschild, 1980).

PURIFICATION SCHEME

Dissection   -   Bovine eyes (-70°C), with the eyelids removed, were obtained from BioResources, Texas, and the corneas were dissected immediately upon arrival.   The eyeballs were rinsed briefly in cold distilled H_2O, and the corneas carefully excised from the frozen eyes, using a number 15 scalpel blade (Figure 6).   The corneas were placed on dry ice, and stored at -80°C until use.

Extractions   -   To begin the extraction of CAI, frozen corneas (500) were placed in a 1 liter Erlenmeyer flask, with 500 ml of HPLC grade H_2O (Pierce HPLC grade solvents were used exclusively throughout the purification).   The flask was put into a hot water bath, brought to 100°C, and boiled for 20 minutes.   The hot extract was filtered through coarse Whatman filter paper under vacuum, to remove the corneas and debris.   The filtrate was centrifuged for 15 minutes at 10,000xg, and the supernatant retained.   The supernatant was subjected to ultrafiltration using an Amicon YM2 (1000 molecular weight cut-off) membrane under 55 psi nitrogen pressure, overnight at 4°C.   The filtrate was

collected and lyophilized. The lyophilized powder was reconstituted with 10 ml of H_2O. Dimethylformamide (DMF, 90 ml) was added, and the solution stirred at room temperature for 1 hour. The material was then centrifuged in glass tubes for 20 minutes at IO,OOOxg. The supernatant was dried in a model EL 131 RotoVapor (Buchi). DMF (25 ml) was added directly to the round bottom RotoVapor flask, and the flask agitated at room temperature for 1 hour. This material was centrifuged for 20 minutes, and the supernatant retained and stored at 4°C.

High-Pressure Liquid Chromatography

C18 Analytical HPLC column - Initial investigation of the binding and elution of CAI was performed using a Supelco octadecylsilica (C18 reversed phase) analytical (15 cm X 4.6 mm) HPLC column. DMF extracted material (1 ml) was dried in 12 X 75mm borosilicate tubes in a SpeedVac rotor (Savant), and resuspended in 100 μl of H20, 0.1% trifluoroacetic acid. This sample was injected onto the column, and washed with a complex gradient of O -100% acetonitrile, 0. 1% trifluoroacetic acid (TFA) (Figure 7). Fractions (2 ml) were collected, and dried in a Speed Vac. Absorbance of the fractions eluting from the column was monitored at 220nm. These fractions were saved for assay in the endothelial cell rounding assay, DNA synthesis inhibition, and CAM assay.

C18 Semi-preparative HPLC column - A similar technique was employed when using the C18 semi-preparative HPLC column.

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The 100% DMF extracted material (3ml) was dried and resuspended as above, in 0.1% TFA (200 µl). This sample was injected onto a C18 semipreparative (25 cm X lOmm) HPLC column (Supelco), and absorbance of eluted fractions again measured at 220 nm (Figure 8). Fractions were collected and assayed for bioactivity.

C18 Analytical HPLC column - second HPLC purification step. A single fraction (fraction 13) obtained from the C18 semi-preparative column which demonstrated the greatest number of units of endothelial cell rounding activity was selected and dried. This fraction was reconstituted in 100 µl of H20, injected onto a Supelco C18 analytical (15 cm X 4.6mm) HPLC column (Supelco), and eluted with a complex gradient of O - 50% acetonitrile, in the absence of trifluoroacetic acid (Figure 9). Fractions were collected by hand, as individual peaks of absorbance seen at 220 nm. These samples representing individual peaks of absorbance were assayed for bioactivity using the endothelial cell rounding assay, and the elution fractions which contained bioactivity were determined. This second HPLC purification step was repeated under exact conditions, using identical starting sample material. Identical (superimposable) chromatographs were obtained. These fractions were similarly collected by hand, as individual peaks, and retained for mass spectroscopy investigations (Figure 10).

Mass Spectroscopy - As detailed in the previous section, fractions which eluted from the C18 analytical column

purification step identically to those which had demonstrated bioactivity were collected in pre-weighed 12 x 75 baked glass tubes, dried in a SpeedVac, and submitted for structural analysis to SmithKline Beecham Pharmaceuticals (Michael Huddleston, Steven A. Carr, Ph.D.). Additionally, the fractions representing absorptive peaks which did not demonstrate bioactivity were submitted for comparative studies. All samples were analyzed by electrospray mass spectroscopy (ESMS), and bioactive fractions were analyzed by desorptive chemical ionization (DCI) in both positive and negative ion modes, using both ammonia and isobutane as the reagent gases (Steven Carr, personal communication).

RESULTS

Previously, a method of extraction and partial purification of CAI from bovine cornea was established by Susan Doctrow and Ed Mun. A schematic of their method is given in <u>Table 4,</u> and in <u>Table 5,</u> a schematic of the improved purification method which we have established is presented. In the previous scheme, corneas purchased from BioResources were extracted in 10% ethanol, overnight in the cold. Ethanol was employed to control bacterial overgrowth. A second two hour extraction followed, and the extract was boiled for 15 minutes after which the corneas were removed by filtration. The extract was passed through a membrane with a MW cut-off of 1000, and the filtrate lyophilized. Two chloroform : methanol (50:50) extractions were performed, and the soluble fraction was retained and dried on a RotoVapor. Several conditions for high pressure liquid chromatography were employed, but successful binding and elution of the CAI activity could not be reproducibly achieved with the chloroform:methanol extracted material.

We have developed an improved purification method for CAI. In order to better control for the preparation and storage of our starting material, we obtained frozen whole bovine eyes, and dissected the corneas in our laboratory directly upon arrival from BioResources. Next, the corneas were boiled in HPLC H_2O for 20 minutes. The resultant extract yielded the same or higher amount of rounding activity/cornea as the longer cold extraction of the

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previous method, but had a lower protein content, and less visible debris. Significant advantages of this step are also the reduction in time from 28 to 2 hours, and elimination of the need for ethanol, which must be removed later in the purification.

The ultrafiltration step remains unchanged in the current method. This step yields an 80% recovery of rounding activity after filtration (Table 6). We have recently found that a second ultrafiltration step, using a membrane with a 500 MW cut-off and identical conditions, yields an 80% recovery of activity that had been recovered after the first ultrafiltration step (1000 MW cut-off filter).

The major modification in the current method is the organic extraction performed. In the current method, DMF is added to a concentrated aqueous solution of the CAI, to produce a final concentration of 90% DMF. This instantly produces a large, flocculent precipitate. After centrifugation, almost 90% of the rounding activity is recovered in the supernatant, and none is detected in the pellet. A subsequent 100% DMF extraction yields more precipitated material, and 85 - 90% recovery of rounding activity. The results of these extractions compare favorably with the result of the organic extraction of the previous purification methods. The chloroform:methanol extraction yielded only a 50% recovery of activity in the soluble fraction, with a nearly equal amount of activity detectable in the insoluble material, even after two overnight extractions.

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The material obtained from the DMF extractions also demonstrated the ability to inhibit DNA synthesis, as partially purified CAI by the previous method did (Figure 11). There is significant inhibition of DNA synthesis, at concentrations of CAI much less than required for rounding activity. Similar results had been found with the chloroform: methanol extracted material.

The most important outcome of the DMF extractions was that with this material, we were able to establish conditions to consistently bind and elute CAI activity from HPLC C18 reverse phase columns. Figure 7 shows a chromatograph obtained by using a C18 analytical column, and running a complex gradient from O to 100% acetonitrile, with 0.1% trifluoroacetic acid. Fractions were collected and assayed for rounding activity. Fractions 2 - 14 all showed some rounding activity, but two major peaks of activity were demonstrated. The early fraction were very acidic, with a pH of 5 in the most concentrated samples in the cell rounding assay. In order to test if all the rounding activity seen in these fractions correlated with anti-angiogenic activity, we tested the fractions on the CAM assay. The results are presented in Table 7. Fractions from the C18 column were pooled into four groups. Pool 1-4 and 10-13 showed similar amounts of endothelial cell rounding activity, the other pools less. Pool 1-4 was clearly toxic to the embryos, killing 4/5. The one surviving egg had no avascular zone. In contrast, the pool of fractions 10-13 showed dramatic anti-angiogenic activity, with 5/5 large avascular

zones. The other pools showed no toxicity, and both demonstrated some weak anti-angiogenic activity.

In order to increase the amount of CAI purified, we scaled up the purification using a C18 semipreparative column. The larger column yielded better resolution and separation, again, with two major peaks of rounding activity seen (Figure 8). Interestingly, the fractions collected from this column which were highly acidic were fraction 3 and 4, and no rounding activity was seen in these fractions.

As the next step in the purification, a single fraction from the semipreparative column containing high rounding activity was selected, and run on the C18 analytical column. A more gradual gradient of acetonitrile, without TFA, was utilized, in an attempt to obtain further separation of the bioactive material. The resultant chromatograph contained only a few well-delineated peaks, and was found to be highly reproducible, run under identical conditions, using identical starting material (Figure 9). The peaks seen on the C18 analytical chromatograph were collected by hand, and dried in a SpeedVac. Presumably identical fractions were obtained from consecutive runs: one set was devoted towards assay for endothelial cell rounding activity, and was set was prepared and retained for analysis by mass spectroscopy. The first and fifth peak (Figure 10) both showed rounding activity.

Because of the concern of possible toxicity in the bioactivity assays from the use of organic solvents throughout the purification, a blank run of the entire purification scheme was performed, and samples from each stage of the purification were tested on the endothelial cell rounding assay. All samples demonstrated no cell rounding, and no toxicity to the cells was observed. These blank samples, produced by following the purification scheme, were also submitted with the active samples for mass spectroscopy analysis, to rule out artifactual results.

The mass spectroscopy analysis showed a consistent species of molecular weight 228 in electrospray mass spectroscopy (ESMS) and desorptive chemical ionization (DCI). A gas phase exchange experiment indicated that the molecule has three exchangeable hydrogens (H). The electrospray data was clouded by very strong signals derived from sodium trifluoroacetate. A unique peak was seen at m/z 173 (possible Mr = 172). This signal, however, was not observed in the DCI, and could not be pursued further.

Electrospray and DCI mass spectra was also performed on blanks which had been submitted. These blanks were obtained by performing the entire purification scheme, without the corneas. It was concluded that the extraction process and solvents were not contributing to the observations which had been made about the active samples.

Scanning high resolution was next attempted on these partially analyzed samples, however, the sample was found to be inadequate, secondary either to insufficient quantity, or degradation of the material.

Scanning high resolution was next attempted on these partially analysed samples, however, the sample was either inadequate, secondary either to insufficient quantity or degradation of the material.

DISCUSSION

This paper describes the further characterization and purification of a novel corneal angiogenesis inhibitor, initially described by Mun *et al.* (1989). Three different assays were used to assess the bioactivity of partially purified samples of CAI. Primarily, BCE rounding activity has been used to guide the purification. Retention of the ability to inhibit DNA synthesis by BCE cells, and to generate avascular zones on the CAM has also been demonstrated throughout the purification.

A major difficulty in the previous purification scheme was the inability of the CAI partially purified by chloroform:methanol extraction to reproducibly bind to and elute from an HPLC column. The finding that CAI was soluble in DMF enabled the use of HPLC for further purification of the molecule. There are several reasons why the DMF extraction facilitated HPLC purification. The DMF extraction could have removed contaminating impurities from the CAI and enabled binding to the C18 column. Alternatively, DMF extraction could have caused a chemical alteration of the CAI molecule, of a carrier, or an associated molecule. This alternative seems less likely, since BCE DNA synthesis inhibition, BCE rounding activity, and CAM angiogenesis inhibitory activity were retained after both the DMF extraction and the HPLC C18 column steps.

Two clearly distinct peaks of rounding activity eluted from both the analytical and semi-preparative C18 HPLC columns (Figures 7-9). Pools of the fractions from the peaks observed on the first analytical column separation were also analyzed for DNA synthesis inhibition and angiogenesis inhibition on the CAM. The material from the later eluting peak of rounding cell activity clearly retained both the ability to inhibit BCE DNA synthesis, and the ability to cause avascular zones in the CAM assay. The early eluting peak from the C18 column was found to have rounding activity, and it inhibited BCE DNA synthesis. This material did not cause avascular zones on the CAM assay, however 4/5 of the embryos died with this treatment, suggesting toxicity. Investigation of the ability to inhibit angiogenesis in the CAM assay has not yet been carried out with material from the C18 semi-preparative column. The first peak of rounding cell activity from this column is better separated from the bulk of UV-absorbing material than with the analytical column. It is possible that the semi-preparative column might provide separation of cell-rounding activity from the material which was toxic on the CAM assay. If isolated from toxic substances, the material from early peak of rounding cell activity may also inhibit angiogenesis on the CAM assay. This early eluting material could represent an alternate form of the CAI. It is possible that the CAI exists as a dimer, or multimer. Alternatively, a carrier molecule could be associated with the CAI molecule.

The second peak of cell rounding activity which eluted from the C18 column also demonstrated inhibition of DNA synthesis, and inhibition of angiogenesis on the CAM, therefore this material was selected for further purification. Because of difficulty in finding another HPLC resin to which the CAI would bind, the ability of CAI to bind to C18 was further exploited. A single semi-preparative column fraction which demonstrated the highest endothelial cell rounding activity (fraction 13) was selected, and run on the C18 analytical column under slightly different solvent conditions. Selection of a single fraction of the CAI activity peak for the next stage of the purification entailed a significant sacrifice of active material. If all active fractions from the HPLC column were recombined, this step involved about a 30% loss of activity from the material loaded onto the column. By selecting only a single active fraction, only 5-10% of this activity was utilized in the next step of purification. Accepting such a significant sacrifice of active material is an obvious disadvantage to the purification scheme. However, this method does yield a high degree of purification which we hoped to be sufficient for structural analysis. The chromatograph obtained from the C18 analytical run of fraction 13 showed few well-delineated peaks (Figure 10). Multiple runs of the C18 analytical column, using fraction 13 from multiple runs of the C18 semi-preparative column, provided identical chromatographs. These peaks were collected as separate fractions for examination of bioactivity. Endothelial cell rounding activity was observed in two of the fractions collected from the column (peaks 1 and 5). Because the

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peaks from the analytical column eluted sharply and seemed to represent single species, it was decided that this material might be pure enough for further analysis by mass spectroscopy and that valuable structural information might be obtained from this analysis. There was not enough material accumulated from the C18 analytical column to also establish anti-angiogenic activity on the CAM assay. This work is in progress.

By mass spectroscopy, possible candidates for the active CAI molecule were found at MW 228 and 172. The 228 species was also found in desorptive chemical ionization. Gas exchange was employed to show that this molecule had 3 exchangeable hydrogens (by comparison with NH_3 or ND_3 as the reagent gases), and possible alcohol functions (mass 18 losses). The molecule 2-deoxyuridine fits this description. Therefore this compound was assayed for BCE rounding activity at concentrations up to 10 mM. No rounding activity was observed. However, it is possible that CAI may be co-purified with a carrier or associated molecule which may be required for bioactivity. One approach to test this theory would be to add 2-deoxyuridine to samples of CAI with known activity, and look for enhancement of the bioactivity.

Characterization of the CAI molecule has revealed new information. It is a molecule of very low molecular weight. It is stable to boiling and freezing. It has shown resistance to pronase digestion. Furthermore, sensitive amino acid analysis indicates that it is not a peptide. On the other hand, solubility and

chromatography behaviors of the CAI molecule are consistent with those of a polar lipid. Based on these characteristics, possible candidates for the identity of CAI include sphingolipids, glycolipids, or their metabolites. Recent reports demonstrate that these molecules can perform roles in regulation of vascularization.

Sphingolipids and sphingolipid breakdown products have been shown to have important roles in cell regulation and physiology (Hannun and Bell, 1989). Breakdown products and metabolites of these cellular lipids include arachadonic acid, prostaglandins, leukotrienes eicosanoids, thromboxanes (Needleman *et al.*, 1986; Samuelsson *et al.*, 1987), and diacylglycerol (Nishizuka, 1986). These molecules may function as endogenous modulators of cell function (Hannun and Bell, 1989). Prostaglandins E_1 and E_2 have been demonstrated as nonpeptide angiogenic factors (BenEzra, 1978: Ziche *et al.,* 1982; Form and Auerbach, 1983), and proposed to have a role in rheumatoid arthritis, tumor induced angiogenesis, and lymphocyte induced angiogenesis (Form and Auerbach, 1983). The role of prostaglandins in the eye is not well known; however a recent study of the influence of a fish oil dietary supplement on immunogenic keratitis has looked indirectly at this question (Verby and Van Haeringen, 1990). In this study, rabbits were given either a fish oil diet, or a sunflower seed oil diet, before immune complex keratitis was induced by corneal intrastromal injection of human serum albumin. Fish lipids are known to contain large amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid.

These fatty acids are known to have an effect on prostaglandin and leukotriene synthesis. The sunflower seed diet is rich only in linoleic acid, which does not influence the production of either prostaglandins or leukotrienes. The animals which were given the fish oil diet showed significantly less neovascularization, leukocyte infiltrate, and corneal edema, as compared to the animals fed a sunflower seed oil diet. This study provides a provocative inference that eicosanoids, either prostaglandins or leukotrienes, may play an important role in the inhibition of angiogenesis in the cornea.

An arachadonic acid metabolite produced by the cornea has been recently described as a potent, dose dependent, chemotactic and angiogenic factor (Masferrer et al., 1991). Cytochrome P450 from human and bovine corneal epithelium have been shown to convert arachadonic acid to 12(R)-hydroxy-5,8,14(Z,Z,Z)-eicosatrienoic acid (compound D) (Masferrer et al., 1989) (Murphy et al., 1988). This metabolite has inflammatory properties including stimulation of vasodilation and breakdown of the blood-aqueous barrier (Masferrer et al., 1989). Human, bovine, and rabbit corneas have also been shown to metabolize arachadonic acid to 12(R)-hydroxy-5,8,10,14 eicosatetraenoic acid [12(R)HETE], which is an inhibitor of Na^+-K^+-ATPase (Schwartzman et al., 1987). The actual role of this molecule in the cornea has not been described. Again, given the characteristics of the partially purified CAI, further investigation in the potential relationship to the corneal

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cytochrome P450 arachadonic acid metabolites is certainly warranted.

In summary, a reproducible and straightforward method of extraction and partial purification of CAI from bovine cornea has been established. Successful purification of an angiogenesis inhibitor from cornea is of great potential significance. Because it is of low molecular weight and naturally occurring, this compound has the potential to serve as a diffusible, non-antigenic treatment of corneal neovascularization and possibly, of other angiogenic-dependent diseases. Another potential use of purified CAI might be to improve the storage of corneas prior to corneal transplant. It is well known that there is a high incidence of endothelial rejection and graft failure in corneal transplants in which the recipient beds are vascularized is high (Khodadoust, 1973). A storage solution containing added CAI might help tp prevent neovascularization of the transplanted corneas. This exogenously added CAI could diffuse into the cornea and make it less angiogenic. In the current aqueous solutions, endogenous CAI can presumably diffuse out of the stored cornea and into the storage solution, leaving bFGF in Bowman's layer and Descemet's membrane, which can lead to increased neovascularization. Finally, discovery of the structure of this anti-angiogenic compound may help elucidate the mechanism of its role in counteracting angiogenic factors such as bFGF. It is quite possible that the same or very similar compounds may be found in

many other tissues of the body, functioning in the regulation of vascularization.

TABLE 1

DISEASES MANIFESTING PATHOLOGIC ANGIOGENESIS

Ophthalmology
    Corneal graft neovascularization
    Diabetic retinopathy
    Neovascular glaucoma
    Trachoma
    Retrolental fibroplasia
Internal Medicine
    Arthritis
    Scleroderma
Pediatrics
    Hemophiliac joints
    Angiofibroma
    Hemangioma
Radiology
    Arteriovenous malformations

Orthopedics
    Nonunion fractures
Oncology
    Solid tumors
Surgery
    Granulations-burns
    Vascular adhesions
    Hypertrophic scars
    Delayed wound healing
Dermatology
    Psoriasis
    Pyogenic granuloma
Neurology
    Osler-Weber Syndrome
Cardiology
    Atherosclerotic plaques

TABLE 1 - This table represents a group of diseases previously thought to be unrelated, however, it has been recognized that the principle pathologic feature of each of these conditions is the abnormality of capillary blood vessel growth. Furthermore, there is the possibility that therapeutic control of the abnormal capillary growth could eliminate other manifestations of the disease. (Adapted from Klagsbrun and Folkman, Angiogenesis, Handbook of Experimental Pharmacology. Vol. 95(II), Chapter 37, 1990).

TABLE 2

DISORDERS ASSOCIATED WITH CORNEAL NEOVASCULARIZATION

Atopic keratitis
Sjogren's disease
Acne roseaca
Contact lens overwear
Pterygium
Phylectenulosis
Keratoconjunctivitis
Superior limbic keratitis
Epithelial keratoconjunctivitis
Vitamin A deficiency
  - xerophthalmia
  - keratomalacia

Lues
Lipid degeneration
Mycobacteria
  - T.B.
  - leprosy
     - fortuitum
Pemphigoid
Mooren's Ulcer
Post-keratoplasty
Radial keratotomy
Alkali burns
Scleritis

Bacterial Ulcers
Herpes simplex virus
Protozoans - acanthamoeba
Fungal ulcers
Trachoma
Kaposi's sarcoma
Sarcoidosis
Lymphocytic hyperplasia
Systemic lupus erythema
Rheumatoid arthritis
Stevens-Johnson syndrome
Wegner's granulomatosis
Polyarteritis nodosa

TABLE 2 - Capillary growth into the normally avascular cornea can result in decreased vision or blindness in the above disease states. Disorders can involve either the corneal epithelium, corneal stroma, or both tissues.

TABLE 3

PURIFIED ANGIOGENIC FACTORS

GROWTH FACTOR	MOLECULAR WEIGHT
Basic FGF	18 000
Acidic FGF	16 500
VEGF	45 000
Angiogenin	14 100
TGF - α	5 500
TGF - β	25 000
TNF - α	17 000
Platelet-derived ECGF	45 000
Angiotropin	4 500
1-Butyryl-glycerol	306

TABLE 3 - These factors have been shown to be angiogenic *in vivo*, and include polypeptide and nonpeptide low molecular weight compounds (Dobson *et al.*, 1990; Rosenthal *et al.*, 1990; Klagsbrun and Folkman, 1990).

TABLE 4

CAI PREVIOUS PURIFICATION METHOD

Extract frozen bovine corneas in 10% ethanol, 24 hrs,4ºC

Filter to remove corneas

Re-extract corneas in 10% ethanol, 2 hrs, 4ºC, filter

Boil filtrates, 10 min, centrifuge

Evaporate ethanol on Rotovapor, 1 hr

Ultrafiltration of supernatant, 1000MW cut-off filter

Lyophilize filtrate

Extract in 1:1 chloroform:methanol, 18-24 hrs, 25ºC, centrifuge

Re-extract pellet in 1:1 chloroform:methanol, 2 hrs, 25ºC, centrifuge

Combine supernatants from chloroform:methanol extractions

Dry on RotoVapor

TABLE 4 - A method of extraction and partial purification of CAI from bovine cornea established by Susan Doctrow and Ed Mun is presented above.

TABLE 4 - A method of extraction and partial
purification of CAT from bovine corneas, established by
Susan Decker and Ed Mun is presented above.

TABLE 5

CAI CURRENT PURIFICATION METHOD

Extract frozen bovine corneas in 100°C distilled H_2O, 20 min

Filer to remove corneas, centrifuge filtrate

Ultrafiltration of supernatant, 1000MW cut-off filter

Lyophilize filtrate

Extract in 90% dimethylformamide (DMF), 1hr, 25°C

Centrifuge, dry supernatant on RotoVapor

100% DMF extraction, 1 hr, 25°C

Dry extract in Speed Vac

HPLC C18 Semi-preparative column
Elute with 0 - 100% acetonitrile, 0.1% trifluoroacetic acid

HPLC C18 Analytic column
Elute with 0 - 50% acetonitrile

TABLE 5 - This schematic represents the current CAI partial purification method. This method has demonstrated improvement in reproducibility, and more significantly, DMF extraction of CAI has allowed for consistent utilization of HPLC to obtain further purification.

TABLE 6

ACTIVTY YIELDS OF THE CURRENT CAI PURIFICATION

Boiling Extraction	100%
Ultrafiltration	80%
90% DMF Extraction	90%
100% DMF Extraction	85%
Semi-preparative C18 HPLC - all active fractions totaled	70%
Single fraction (13) - selected for further HPLC purification	5-10%
C18 analytical HPLC column - total of active fractions	80%

TABLE 6 - The above table presents the approximate yields of activity of each step in the purification scheme. Activity is based on observed bioactivity in the semi-quantitative BCE rounding cell assay. Activities were calculated as the % of activity retrieved from the total activity of the starting material from the previous step.

TABLE 7

ANTI-ANGIOGENIC ACTIVITY OF C18 HPLC FRACTIONS

CHICK CHORIOALLANTOIC MEMBRANE ASSAY

C18 Fractions	Rounding Units	% Avascular Zones	Observations
1 - 4*	126	0 %	Toxic to 4/5 eggs
5 - 9*	81	20 %	1/5 small zone
10 - 13*	114	100 %	5/5 large avascular zones
14 - 16*	18	40 %	2/5 small zones

* Fractions pooled from 6 runs of C18 HPLC analytical column

TABLE 7 - Fractions collected from a sample of 100% DMF extracted CAI material run on a C18 analytic HPLC column (**Figure 7**) were pooled into four groups: Fractions 1-4, 5-9, 10-13, 14-16. The samples were assayed for BCE rounding activity and angiogenesis inhibition on the CAM. The pool of fraction 10-13 showed dramatic anti-angiogenic activity, and high BCE rounding activity.

FIGURE 1

SCHEMATIC REPRESENTATION
OF THE VASCULAR AND AVASCULAR STRUTURES
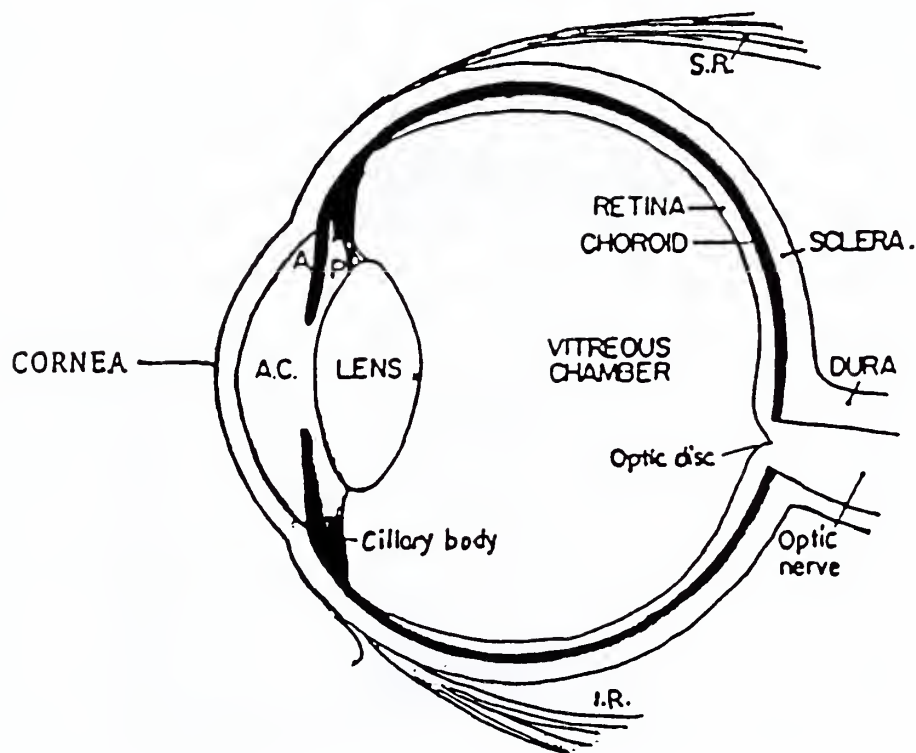## OF THE BOVINE AND HUMAN EYE

FIGURE 1 - The eye is divided into vascular and avascular zones. The cornea, lens, sclera, aqueous humor and vitreous humor are all normally avascular zones. Infringement of blood vessel growth into these tissues can lead to decreased vision or blindness (Diagram adapted from Goldberg, 1984).

FIGURE 2

SCHEMATIC REPRESENTATION OF HISTOLOGIC SECTIONS OF THE CORNEA
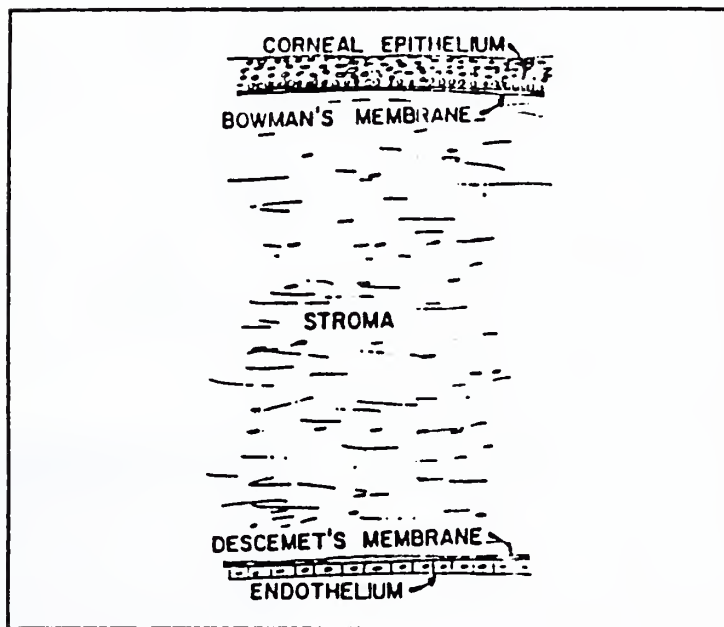
FIGURE 2 - Bowman's layer is the acellular, anterior-most portion of the collagenous corneal stroma, rich in heparan-sulfate proteoglygan. Descemet's membrane is a true basement membrane produced by corneal endothelial cells on the posterior surface of the cornea. Both layers bind bFGF (Adamis *et al.*, 1991; Diagram adapted from Goldberg, 1984).

FIGURE 3

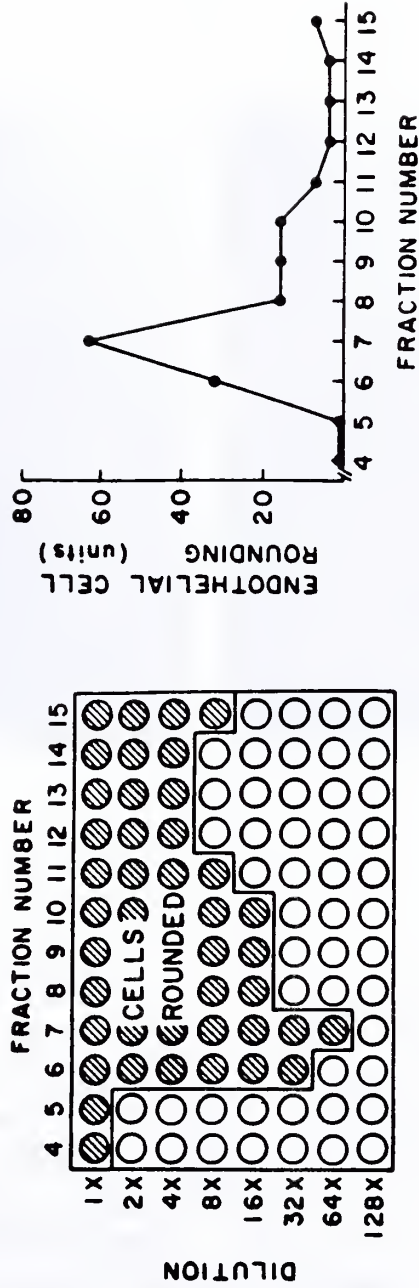## ENDOTHELIAL CELL ROUNDING ASSAY

FIGURE 3 - The units of CAI activity in a given sample are based on the ability of the sample to cause endothelial cell rounding. For this assay, BCE cells were seeded onto 96 well 1/2 area plates at density of 2000 cells/25µl/well. After a 12 h attachment period, cells were treated with varied concentrations of inhibitor fractions, by making two-fold serial dilutions vertically, as indicated in the sample diagram above. After 24 hours, the cells were scored as either positive (shaded) or negative (open) for cell rounding. A positive sample is one in which at least 50% of the cells in the well are rounded (see **Figure 4**). One unit of cell rounding is defined as the amount of sample sufficient to cause rounding in only 50% of the cells in a given well. The number of rounding units in a given sample is calculated from the greatest dilution of that sample which will produce one unit of cell rounding. A graphic profile of rounding units calculated for the fractions in this sample assay is also shown. (Adapted from Doctrow and Mun, unpubl.)

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FIGURE 4

CAI TREATED BOVINE
CAPILLARY ENDOTHELIAL CELLS
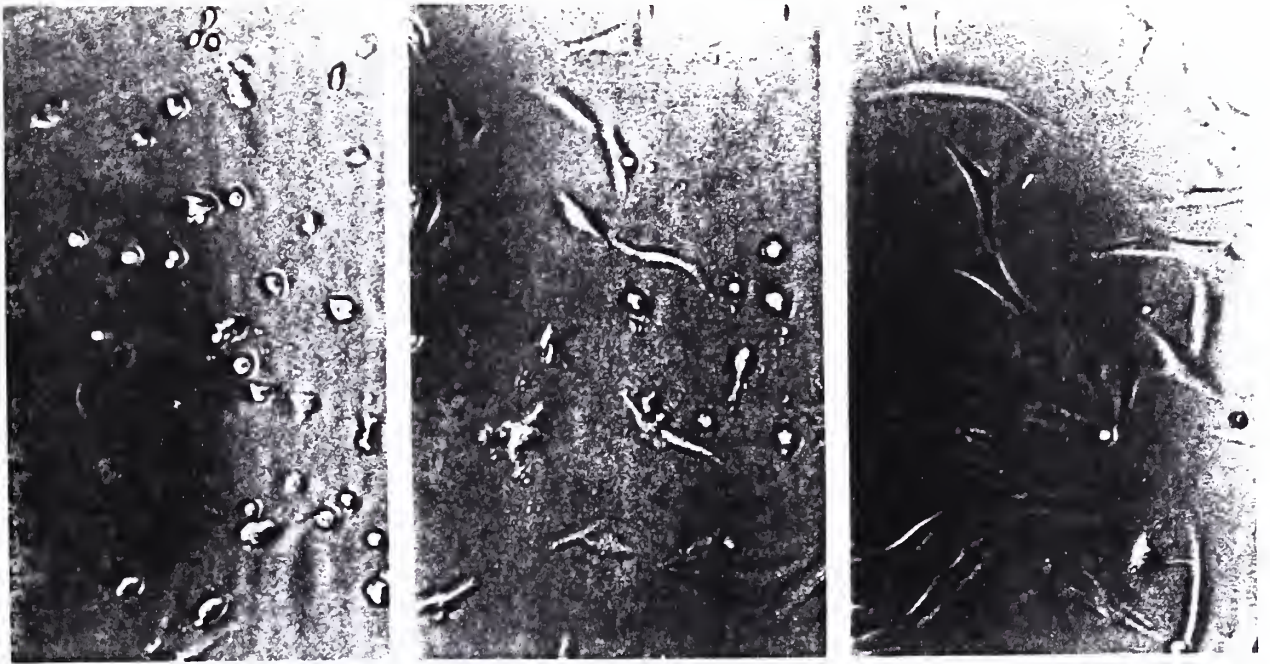
FIGURE 4 - Bovine capillary endothelial cells were plated at a density of 2000 cells per 25 µl per well, in 96 well 1/2 area plates. These cells were treated with serial dilutions of CAI inhibitor samples (decreasing concentrations from left to right). The inhibitor causes rounding of the cells after 10 - 24 hours, in a concentration dependent manner. One unit of cell rounding as defined as the amount of sample sufficient to cause rounding of 50% of the cells in a given well. The cells in the middle well above would be scored as 50% round. The rounded cells exclude trypan blue and stain with the viability marker fluorescein diacetate

FIGURE 5

CAI INHIBITION OF ANGIOGENESIS ON THE CHICK EMBRYO CHORIOALLANTOIC MEMBRANE

FIGURE 5 - Application of a sample of partially purified CAI to the chorioallantoic membrane of a six-day chick embryo causes capillary regression and produces a 4 mm avascular zone at 48 hrs, indicating inhibitory activity. The chick embryo was injected with India ink prior to dissection to improve visualization of the vessels. (Photo courtesy of Susan Doctrow and Ed Mun).

FIGURE 6

BOVINE CORNEA DISSECTION
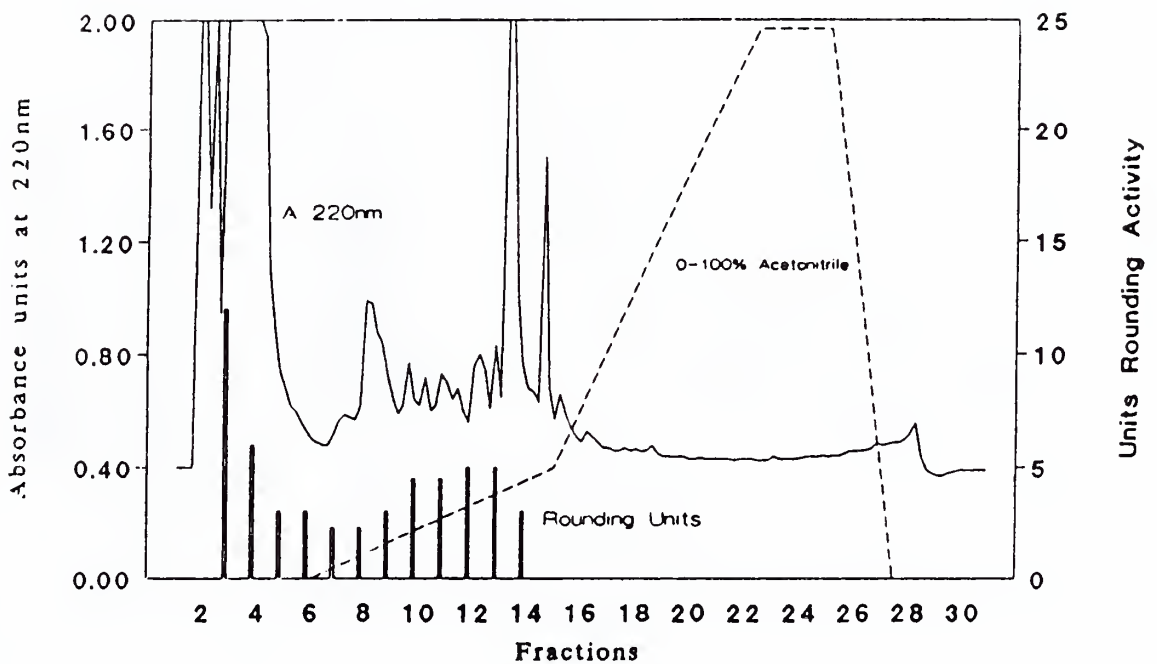

FIGURE 6 - Bovine eyes (-70ºC) were rinsed briefly in cold distilled H_2O, and the cornea carefully excised from the frozen eye using a number 15 scalpel blade. The corneas were placed on dry ice and stored at -80ºC.

FIGURE 7

C18 ANALYTIC HPLC COLUMN
100% DMF EXTRACTED CAI SAMPLE

C18 Analytical Column

FIGURE 7 - This chromatograph was obtained with a sample of CAI which had been extracted in 100% DMF. CAI rounding cell activity was found to consistently bind and elute from the C18 analytic column as shown above. Two major peaks of rounding cell activity were observed, and fractions from both peaks also inhibited BCE DNA synthesis. Only the second peak, however, was shown to have angiogenic inhibitory capability on the CAM assay.

FIGURE 8

C18 SEMIPREPARATIVE HPLC COLUMN
100% DMF EXTRACTED CAI SAMPLE

C18 SEMI-PREPARATIVE COLUMN
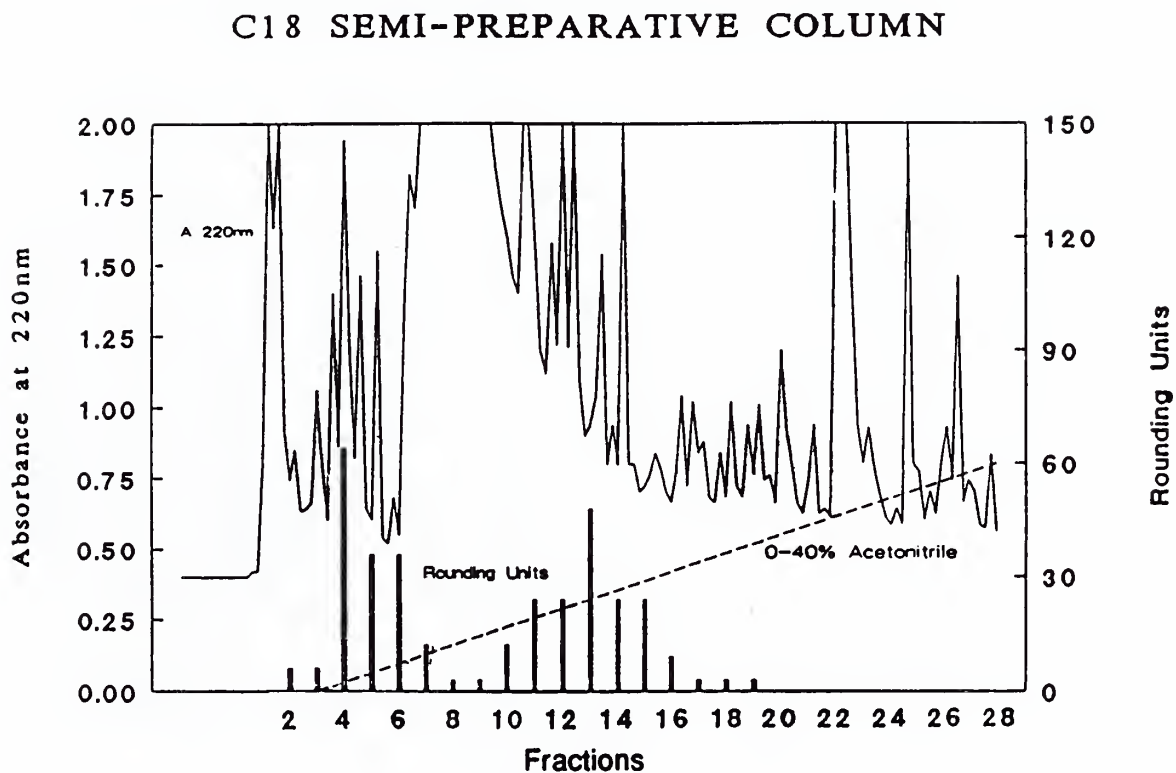
FIGURE 8 - In order to increase the amount of CAI purified using C18 HPLC, a semipreparative C18 HPLC column was employed. Using the larger column, better resolution and separation of CAI bioactivity was obtained. As with the analytical column, two major peaks of BCE rounding activity were observed. Notably, fractions 3 and 4 collected from this column were highly acidic, but contained no rounding activity.

FIGURE 9

C18 ANALYTICAL HPLC COLUMN
SECOND HPLC PURIFICATION STEP

C18 Analytical HPLC Column
C18 semi-preparative column fraction 13
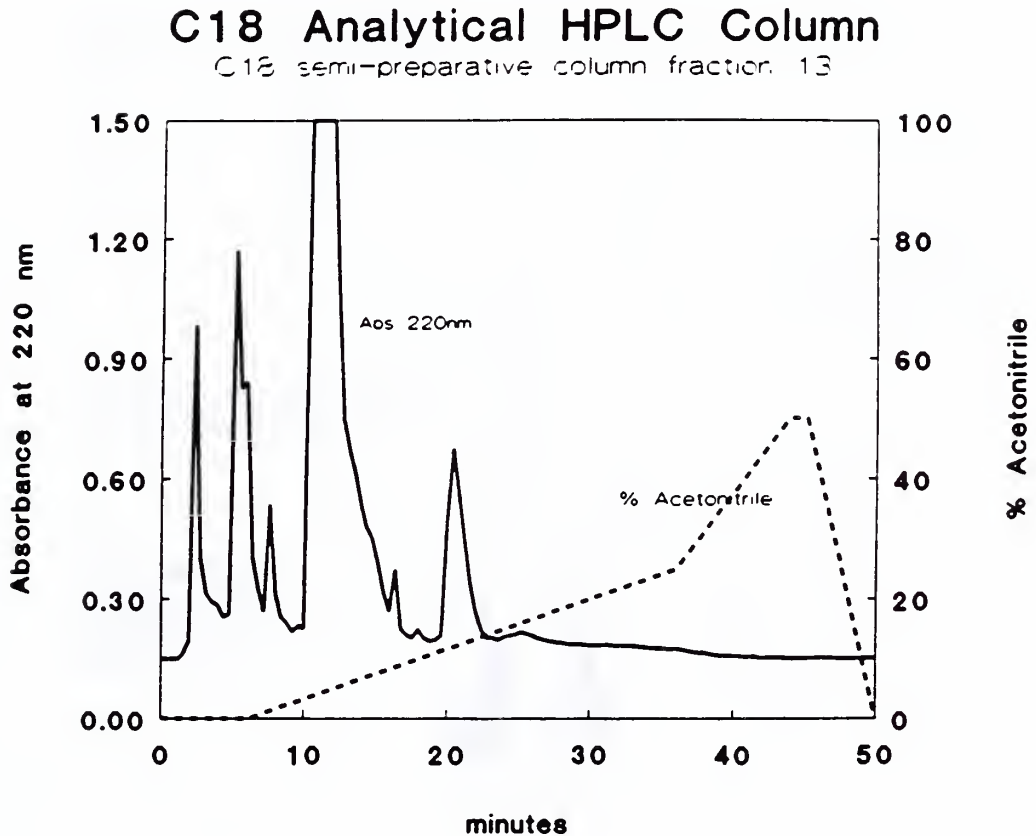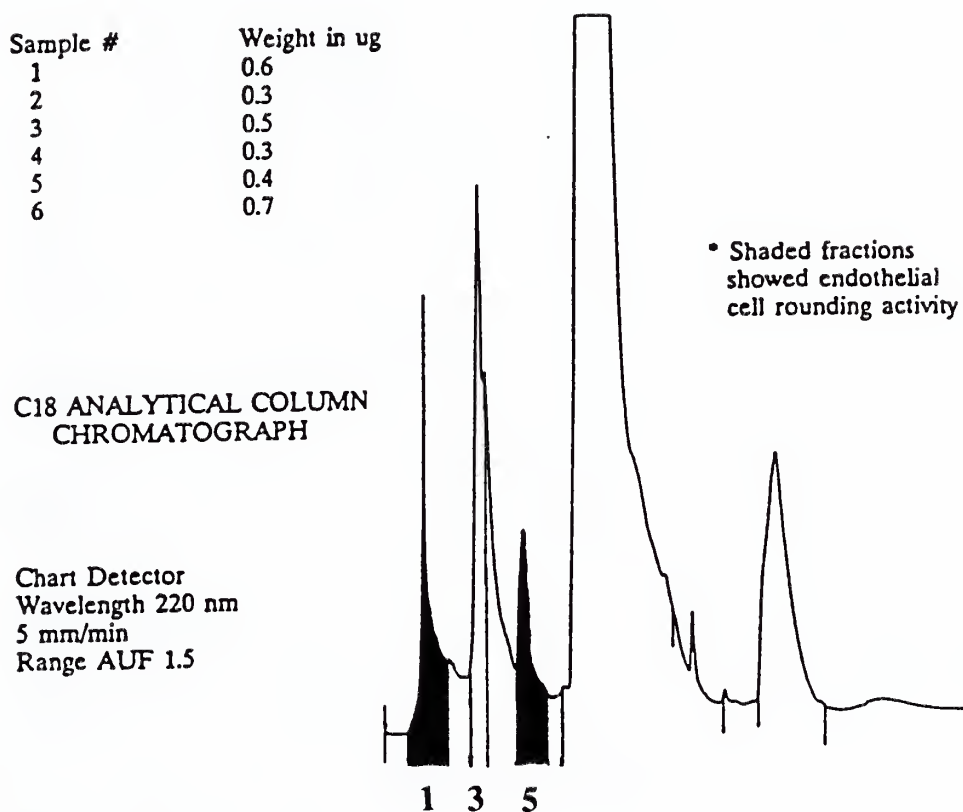

FIGURE 9 - To further the purification of CAI, a single fraction from the C18 semi-preparative column (Fraction 13) which contained high BCE rounding activity (Figure 8) was selected, and run on a C18 analytical column, with a 0 - 50% gradient of acetonitrile (no TFA). The above chromatograph was obtained, and the individual peaks collected as separate fractions and assayed for bioactivity.

FIGURE 10

C18 ANALYTICAL HPLC COLUMN BIOACTIVITIES AND WEIGHTS OF SAMPLES SUBMITTED FOR MASS SPECTROSCOPY

Sample #	Weight in ug
1	0.6
2	0.3
3	0.5
4	0.3
5	0.4
6	0.7

C18 ANALYTICAL COLUMN
CHROMATOGRAPH

Chart Detector
Wavelength 220 nm
5 mm/min
Range AUF 1.5

* Shaded fractions
showed endothelial
cell rounding activity

1 3 5

FIGURE 10 - Fractions were collected as marked on the chromatograph (1 - 5), and assayed for rounding activity. Fraction 1 contained 7 units of rounding activity, fraction 5 contained 6 units.

Another run was performed under identical conditions, and these same fractions were collected in baked glass tubes, dried in the SpeedVac, and weighed. These samples were submitted for mass spectroscopy, with samples 1 and 5 corresponding to active fractions.

FIGURE 11

EFFECT OF CAI ON BCE DNA SYNTHESIS
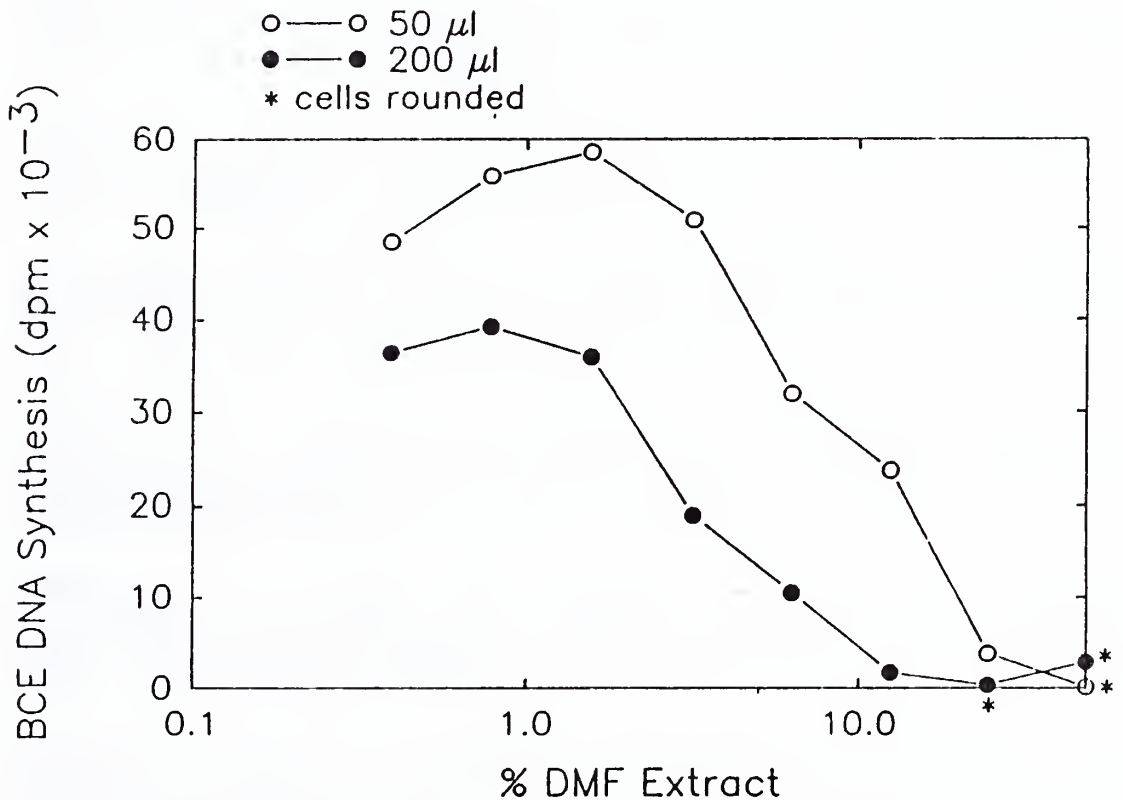
FIGURE 11 - CAI partially purified by DMF extraction which had demonstrated bioactivity in the BCE rounding assay was assayed for bioactivity in a DNA synthesis experiment. The DMF extracted CAI sample demonstrated significant inhibition of DNA synthesis, at concentrations of CAI much less than required for rounding activity. Similar results had been obtained with the chloroform:methaol extracted material from the previous purification scheme.

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