Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 3068–3072, May 1981
Cell Biology

Angiogenic activity from bovine retina: Partial purification and characterization

(endothelial cells/growth factor/angiogenic factor/neovascularization/retinopathy)

PATRICIA A. D'AMORE*, BERT M. GLASER*, SANDRA K. BRUNSON*, AND ALLAN H. FENSELAU†

*Wilmer Ophthalmological Institute and the †Department of Physiological Chemistry, The Johns Hopkins University School of Medicine and The Johns Hopkins Hospital, Baltimore, Maryland 21205

Communicated by Daniel E. Koshland, Jr., February 23, 1981

ABSTRACT **Bovine retinas contain a factor that stimulates proliferation of aortic endothelial cells in culture as well as neovascularization on the chicken chorioallantoic membrane. The stimulatory activity has been partially purified from a balanced salt solution extract of bovine retinas. The stability of the activity to acid pH was utilized as the first step in purification. The acid-treated material was subjected to ion-exchange chromatography on DEAE Bio-Gel A. The material that was eluted with 0.1 M NaCl/50 mM Tris·HCl (pH 7.6) stimulated both the proliferation of vascular endothelial cells in culture and angiogenesis *in vivo*. A molecular weight of between 50,000 and 100,000 for the native molecule is suggested by ultrafiltration and gel chromatography; gel electrophoresis of partially purified material under reducing and denaturing conditions revealed the presence of two major components with apparent molecular weights of 50,000 and 70,000. The endothelial cell stimulatory activity was stable to pH 4–9 and to heating at up to 60°C for 30 min, to incubating for 1 hr with DNase or RNase, to incubating for 2 hr with immobilized Pronase or immobilized or soluble trypsin, and to treating with 1 mM 2-mercaptoethanol, 4 M urea, or 2 M guanidine. Heating at 65–75°C for 30 min, boiling for 2 min, extreme acidic (pH 2) or basic (pH 12) conditions, treating with 0.02% $NaDodSO_4$, or incubating (5 hr) with soluble Pronase destroyed the activity. The significance of an angiogenic factor derived from retina stems from the fact that neovascularization is a serious complication in a number of ocular diseases.**

Angiogenesis, the formation of new blood vessels, plays a critical role in a number of biological processes, both normal and pathological (1–4). For most of these processes, however, neither the stimuli for angiogenesis nor their modes of action have been identified. Pathologic neovascularization occurs in several ophthalmic diseases, including diabetic retinopathy (4–7). The complications of neovascularization in these disorders are so destructive that the proliferative retinopathies constitute a major cause of permanent blindness. A feature common to all of these conditions is the development of areas of retinal capillary closure. It has been postulated that the nonperfused retinal tissue liberates a diffusible angiogenic substance (8–10). This hypothesis is supported by the fact that destruction of retinal tissue by laser photocoagulation (the present treatment for proliferative retinopathy) results in regression of the newly grown vessels.

We have examined the retina as a source of angiogenic activity and have demonstrated vasoproliferative activity in extracts of retinas from a number of mammalian species (11). In the following we report studies on the partial purification and characterization of a bovine retina-derived angiogenic factor, which reveal significant differences from other reported angiogenic materials (12–14).

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MATERIALS AND METHODS

Thymidine Incorporation Assay. Fetal bovine aortic endothelial cells (EC) were harvested and cultured as reported (15). The cells grow to confluence, forming the characteristic monolayer of polygonal cells, and stain positively with anti-Factor VIII antibody, a marker unique to vascular EC (16).

The incorporation of [^{3}H]thymidine into perchloric acid-insoluble material was utilized to measure stimulation of EC proliferation by retinal extract. This assay system was developed by Mello (17) and was utilized as we have previously described (11). Briefly, the cells were plated in multiwell plates and incubated in medium 199 (GIBCO) containing 1% fetal bovine serum (Sterile Systems, Logan, UT) with additions of test materials. After 48 hr at 37°C the cells were pulse labeled for 1 hr with [^{3}H]thymidine at 0.625 μCi/ml of medium (Amersham; 1 mCi/ml, 6.7 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) in serum-free Eagle's minimal essential medium (GIBCO) and then processed for scintillation counting.

Chicken Chorioallantoic Membrane (CAM) Assay. The angiogenic activity of the fractions yielded by each purification step was determined by using the chicken CAM assay. The assay was performed by using the procedure of Folkman (18) with modifications that we have described (11). Bovine serum albumin was added to the more purified fractions before their incorporation into Elvax (an ethylene/vinyl acetate polymer) pellets. This was done to standardize the level of protein incorporated into each pellet, thereby equalizing the release rates (19). Activity was quantitated by determining the number of new vessel loops that had grown toward an Elvax pellet containing the test or control materials. The response was considered negative if the number of vessel loops induced was 1 or less and positive if the number of new vessel loops induced was 2 or more.

Retinal Extract. Bovine eyes were obtained from a local abattoir. The retinal extract was prepared as reported (11). More than 100 preparations (utilizing more than 1500 retinas) of bovine retinal extract have been prepared in this manner and have provided highly reproducible results. The average protein concentration of the crude extract was 6.36 mg/ml (range 3.6–8.65 mg/ml).

Characterization Studies. Samples (1–2 ml) of bovine retinal extract were subjected to various physical, chemical, or enzymatic treatments. Any precipitate that was formed under these conditions was removed by centrifugation (40,000 × *g*, 15 min, 4°C). The soluble material was sterilized by filtration and tested for activity.

Abbreviations: EC, endothelial cells; CAM, chorioallantoic membrane.

Heat treatment was carried out at temperatures from 56°C to 75°C for 30 min or 100°C for 2 min. The extract was titrated with stirring to various pH values, using dilute solutions of NaOH and HCl.

Bovine retinal extract was dialyzed overnight against urea (4 M) or guanidine·HCl (2 M) (Schwarz/Mann) followed by dialysis against 50 mM Tris·HCl (pH 7.2). The extract was also treated with urea (4 M), guanidine·HCl (2 M), $NaDodSO_4$ (0.02%) (Sigma), 2-mercaptoethanol (1 mM), ethanol (90%, vol/vol, or absolute), trichloroacetic acid (5%), or $MgCl_2$ (2 M) for 30 min at 37°C with gentle stirring. The clarified extract was then dialyzed extensively against several changes of 50 mM Tris·HCl (pH 7.2), sterilized, and tested for activity.

DNase and RNase (both bovine pancrease, Sigma) treatment used the conditions outlined in the *Worthington Enzyme Manual* (20). An excess of nucleic acid (DNA, salmon type III; RNA, calf liver type IV; both from Sigma) was used to terminate the RNase and DNase treatment of the retinal extract. Appropriate DNA and RNA controls were performed to determine the effect of the various components of the enzyme assay system on the growth and viability of the EC.

Enzite agarose trypsin (Miles) or insoluble Pronase (*Streptomyces griseus;* Sigma) was incubated with 2-ml samples of acid-treated retinal extract (10 units of enzyme per mg of Lowry protein in the extract per ml) or balanced salt solution (10 units/ml) for 2 hr at room temperature. The immobilized enzyme was removed by centrifugation. The enzyme-treated retinal extract was filtered through a 0.8-μm-pore-diameter Millipore filter, sterilized, and tested for activity. The activity of the immobilized protease was determined by monitoring its action on the activity of aldolase. The treated extract and control sample (balanced salt solution) were assayed for the presence of free protease by monitoring their effect on a similar preparation of aldolase.

Because an extended protease treatment was required, a procedure was devised to permit the incubation of retinal extract with soluble Pronase with subsequent removal of the protease. Pronase (*S. griseus,* Calbiochem–Behring) was added to acid-treated retinal extract to a final concentration of 15 units/ml and incubated at room temperature. At various times during the incubation (0, 1, 3, 5, and 7 hr) 2 ml of the retinal extract/protease mixture was removed, added to 10 ml of buffer (0.01 M Tris·HCl, pH 7.2), and pressure filtered until the original 2-ml volume was reached. A Diaflo XM-50 membrane (Amicon; M_r cut-off 50,000) was used during the filtration to assure retention of the activity and removal of the protease. The dilution and filtration step was repeated until no measurable protease activity (using inactivation of aldolase, as above) remained in the retentate. A control sample of retinal extract with no added protease was kept at room temperature and then pressure-filtered as the experimental samples were. The retentate was then sterilized by filtration and tested for activity. Gel samples were taken at each time point.

Chromatography. The retinal extract was adjusted to pH 4.0 by adding 0.5 M HCl dropwise with stirring at room temperature. The acid-precipitable material was removed by centrifugation (40,000 × *g*, 15 min at 4°C) and the remaining supernatant was adjusted to pH 7.2 with 0.5 M NaOH.

The acid-treated material was applied to a Sephadex G-50 (Pharmacia) column (1.6 × 26 cm) and eluted at 4°C with 0.1 M Tris·HCl, pH 7.2. The fractions from each peak were pooled and tested for activity.

The acid-treated material was dialyzed overnight at 4°C in Spectrapor 3 dialysis tubing (M_r cut-off 3500) against two changes of 200 vol of 50 mM Tris·HCl (pH 7.2). Five milliliters (1.0 mg/ml) of the dialyzed material was applied to a DEAE Bio-Gel A column (Bio-Rad) (0.7 × 8 cm) (disposable polypropylene column, Kontes) that had been equilibrated in 50 mM Tris·HCl (pH 7.2). The material was eluted in two steps with 0.05 M and 0.10 M NaCl/50 mM Tris·HCl. The fractions from each step were pooled and the volume was adjusted to that of the starting material. A range of doses from each peak was tested for activity on EC cultures, using the [^{3}H]thymidine incorporation assay.

Gel Electrophoresis. The purification steps as well as the products from the extended Pronase treatment of the bovine retinal extract were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis, using a modification of the procedures of Laemmli (21). The slab gel consisted of an 8% resolving gel (9 × 14 × 0.15 cm) with a 6% stacking gel (9 × 2 × 0.15 cm). Samples for electrophoresis were lyophilized and redissolved in sample buffer containing 50 mM Tris·HCl, 2% (wt/vol) $NaDodSO_4$, 10% (vol/vol) 2-mercaptoethanol, 5% (vol/vol) glycerol, and 0.005% bromphenol blue. Electrophoresis was performed at room temperature for 3–4 hr at a constant voltage of 100 V, while current varied from 25 to 13 mA. The gels were stained by using 0.025% Coomassie blue R-250 in 25% (vol/vol) isopropyl alcohol and 10% (vol/vol) acetic acid.

RESULTS

The addition of crude bovine retinal extract to EC in culture stimulated an increase in [^{3}H]thymidine incorporation. The dose-dependent stimulation of thymidine incorporation by four typical preparations of extract is illustrated in Fig. 1. Maximal stimulation was elicited by approximately the same protein concentration, 125 μg/ml, of each extract tested. The addition of crude retinal extract in protein concentrations above 350 μg/ml resulted in cell death as evidenced by rounding of the cells and their detachment from the substrate. This may be due to the presence of cytotoxic or inhibitory cell materials in the retinal extract. Acid treatment of the crude extracts abolishes this effect (data not shown).

The nature of the EC-stimulating activity was investigated by testing its stability to a variety of physical, chemical, and enzymatic treatments. The results of these experiments are shown in Table 1. The activity was stable at 55–60°C for 30 min

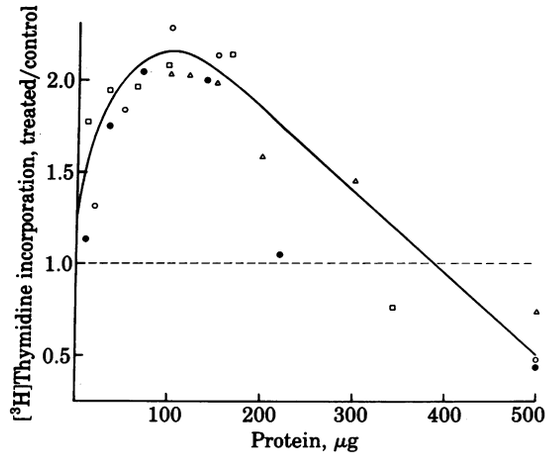

FIG. 1. Effect of various concentrations of crude bovine retinal extract on [^{3}H]thymidine incorporation by EC in culture. Crude bovine retinal extract was added to wells containing 15,000–20,000 EC in 1 ml of medium 199 with 1.0% fetal bovine serum. The volumes of the additions were 10–100 μl. Control wells received an equal volume of the appropriate buffer. The protein concentrations as measured by the Lowry assay are given on the abscissa. Each set of symbols represents a different preparation of retinal extract. Each point is the mean of values from triplicate wells.

Table 1. Stability of EC-stimulatory activity from bovine retinal extract

Treatment	Stability of activity*	[^{3}H]Thymidine incorporation, treated/control
Heat		
56°C, 30 min	+	2.32 ± 0.16
60°C, 30 min	±	1.36 ± 0.12
65°C, 30 min	−	1.04 ± 0.03
70°C, 30 min	−	1.07 ± 0.09
75°C, 30 min	−	0.98 ± 0.02
100°C, 2 min	−	0.94 ± 0.13
pH		
4–9	+	2.05 ± 0.11
2	−	0.85 ± 0.06
12	−	0.98 ± 0.04
Chemical		
2-Mercaptoethanol (1 mM)	+	1.72 ± 0.06
Urea (4 M)	+	1.53 ± 0.05
Guanidine (2 M)	+	1.49 ± 0.07
$NaDodSO_4$ (0.02%)	−	1.18 ± 0.08
$MgCl_2$ (2 M)	+	1.83 ± 0.06
Ethanol (90%)	−	0.96 ± 0.06
Trichloroacetic acid (5%)	−	0.91 ± 0.005
Enzyme		
DNase	+	2.17 ± 0.24
RNase	+	1.61 ± 0.19
Trypsin, immobilized (2 hr)	+	1.58 ± 0.04
Trypsin, soluble (5 hr)	+	2.06 ± 0.04
Pronase, immobilized (2 hr)	+	1.99 ± 0.10
Pronase, soluble (5 hr)	−	1.05 ± 0.01

Samples (1–2 ml) of bovine retinal extracts were subjected to various conditions. The treated material was then tested for growth-stimulatory ability on cultured EC, using the [^{3}H]thymidine incorporation assay. The results are expressed as the ratio of [^{3}H]thymidine incorporation by cells with additions of treated retinal factor (treated) to cells receiving an equivalent volume of buffer (control). Each value is the mean (±SD) of at least six determinations.

* +, activity is stable to the treatment; −, activity is destroyed by the treatment.

and at pH 4–9. Between 60°C and 65°C about half of the activity was lost. Heating at 65–75°C for 30 min or at 100°C for 2 min, titration to extreme acidic (pH 2) or basic (pH 12) conditions, and trichloroacetic acid precipitation destroyed the activity. Neither incubation for 30 min at 37°C with urea (4 M) or guanidine·HCl (2 M) nor overnight dialysis against urea or guanidine, both treatments followed by dialysis against 50 mM Tris·HCl (pH 7.2), inactivated the factor. Incubation for 30 min at 37°C with 2-mercaptoethanol (1 mM) or $MgCl_2$ (2 M) did not affect the activity, but treatment with $NaDodSO_4$ at 0.02% did inactivate the factor. The activity cannot be extracted from the lyophilized extract into 90% or absolute ethanol. A 1-hr treatment with DNase or RNase did not alter the extract's stimulatory ability.

The effect of protease treatment on the EC growth-stimulatory activity in the retinal extracts was examined by using both immobilized and soluble proteases. After a 2-hr incubation with either immobilized Pronase or trypsin, using conditions sufficient to inactivate aldolase, no loss of activity was detected (Table 1). Likewise, soluble trypsin over a 5-hr incubation period failed to alter the activity of the extract. However, more vigorous proteolytic conditions using the nonspecific protease Pronase did lead to a loss in growth-stimulatory activity. Selective filtration allowed removal of Pronase and degraded material yet permitted retention of the higher molecular weight retina-derived species. Analysis of the activity of the protease-treated materials after 1, 3, 5, and 7 hr of treatment showed a progressive loss of EC growth-stimulatory activity during the first 5 hr. Concomitant with this loss of activity was the loss of high molecular weight components detectable by $NaDodSO_4$/polyacrylamide gel electrophoresis. After only a 1-hr period, activity appeared undiminished. Under identical incubation conditions the activity of an equivalent protein concentration of aldolase was decreased by 45% in the first 15 min and was totally destroyed by 1½ hr. The 1-hr treatment with Pronase did substantially degrade the acid-treated retinal extract as monitored by gel electrophoresis. This preparation, which showed no loss of activity, was enriched with respect to components of molecular weights 50,000 and 70,000.

The stability of the active material to acid pH was utilized as a first step in purifying the active factor. Adjusting the pH of the crude extract (6 mg/ml) to 4.0 precipitated approximately 85% of the total protein as measured by the Lowry assay. All of the original activity was recovered in the soluble fraction. Half of the acid-precipitable protein was irreversibly denatured, and the precipitated material that could be redissolved in aqueous solutions did not display any stimulatory activity (data not shown).

The acid-treated material was applied to a Sephadex G-50 column. Only two peaks of absorbance (A_{280}) were recovered. The first was eluted just after the void volume, contained 38% (A_{280}) of the applied material, and was active in the cell culture assay. The second peak eluted near the low molecular weight marker, [^{3}H]adenine, contained 66% (A_{280}) of the applied material, and was not active in the cell culture assay. Since this method did not afford a significant purification it was not incorporated into the purification scheme. Because the active material eluted just after the void volume of the column we estimate the molecular weight to be 30,000 or greater. Furthermore, retention of the stimulatory activity by an XM-50 Diaflo membrane but not by an XM-100 (M_r cut-offs 50,000 and 100,000, respectively) indicates that the molecular weight of the native material is in the range between 50,000 and 100,000.

The soluble fraction of the acid-treated retinal extract was subjected to ion-exchange chromatography and the elution profile of a typical experiment is shown in Fig. 2. When acid-treated retinal extract (5 mg; 1 mg/ml) was applied to a DEAE Bio-Gel A column, half of the protein did not bind and was recovered in the flow-through. The rest of the protein was divided equally between the 0.05 M NaCl and 0.1 M NaCl eluates. The stimulatory activity was recovered, exclusively and quantitatively,

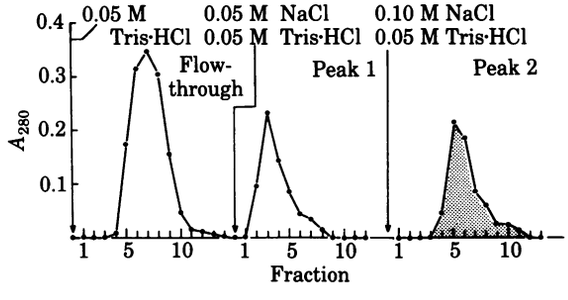

FIG. 2. Elution profile of bovine retinal extract from DEAE Bio-Gel A. The column (0.7 × 8 cm) was equilibrated in 50 mM Tris·HCl (pH 7.2). The soluble fraction of the acid-treated extract was applied to the column and eluted in two steps with NaCl/50 mM Tris·HCl. Absorbance at 280 nm was monitored and the stimulatory activity of the fractions in [^{3}H]thymidine incorporation assay and on the CAM assay was determined. The shaded area indicates the location of the stimulatory activity.

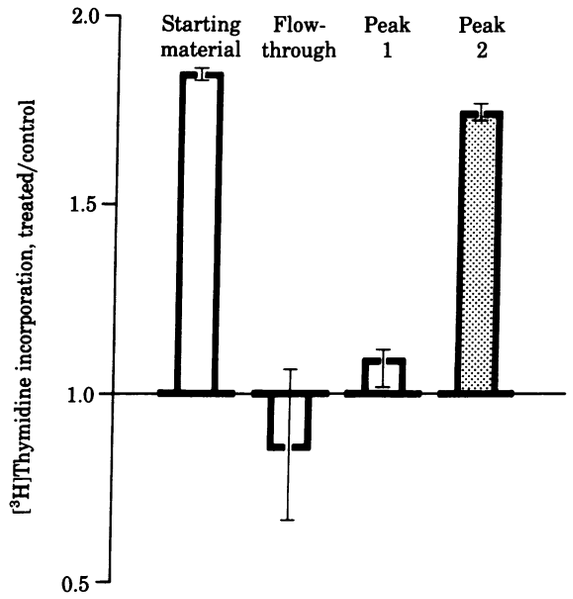

FIG. 3. Quantitation of recovery of stimulatory activity from a typical DEAE Bio-Gel A column. Recovery of the stimulatory activity was determined by pooling all of the fractions in each peak and adjusting the volume to that of the starting material. A range of doses from each peak was tested for activity in the [^{3}H]thymidine incorporation assay. The plot illustrates the effect of 33-μl additions from each peak. Activity was recovered in peak 2, which is shown as shaded. Error bars indicate SD.

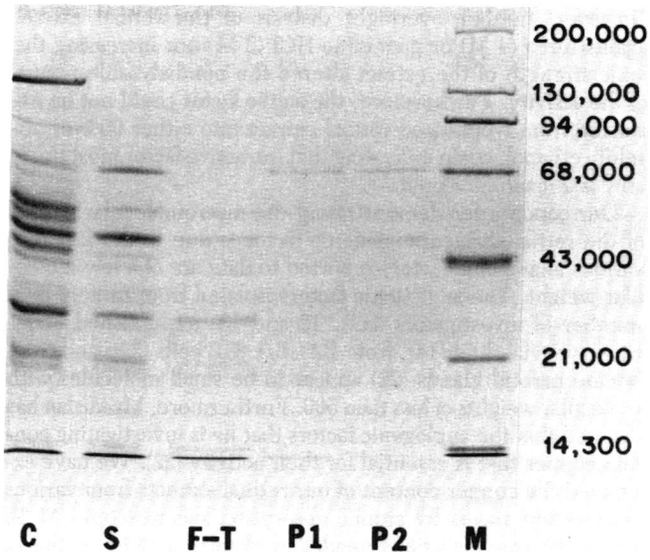

FIG. 4. NaDodSO$_4$/polyacrylamide gel electrophoresis of the bovine retinal extract purification. Each channel contains 20 μg of protein. C is the crude retinal extract and S is the soluble fraction of the acid-treated material. The next three lanes are the eluates of the DEAE Bio-Gel A column: F-T is the flow-through; P1 is the 0.05 M NaCl eluate, and P2 is the 0.1 M NaCl eluate. Standard molecular weight markers (channel M) included: myosin, 200,000; β-galactosidase, 130,000; phosphorylase B, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,300.

in the 0.1 M NaCl eluate. As Fig. 3 indicates, maximal growth stimulation obtained from an addition (33 μl) of the acid-treated material was 1.84 times that of buffer controls. No activity was elicited by additions (33 μl) of either the flow-through or peak 1. Addition of an equivalent volume of peak 2 resulted in a 1.74-fold increase in [^{3}H]thymidine incorporation by the EC. Maximal stimulation by crude extract required addition of an average of 125 μg of protein (Lowry assay). Equivalent stimulation by the partially purified material required an average of 4 μg, representing a 31-fold purification.

NaDodSO$_4$/polyacrylamide gel electrophoresis of the fractions obtained during purification is shown in Fig. 4. The channel labeled P2 is the 0.1 M NaCl eluate from which the activity was recovered. Two major bands and several minor bands are evident. Calculation of the molecular weights from the relative migration of the bands indicates that the molecular weights of these two proteins are approximately 50,000 and 70,000. Bands of identical molecular weights appear on gels of the preparation arising from partial Pronase digestion of the acid-treated retinal extract.

The angiogenic activity of the column eluates was assayed on the chicken CAM. In two experiments utilizing a total of 40 eggs, 10 per group, the activity was found to reside in peak 2. Using Fisher's exact test for a 2 × 2 table, the P value for significance of the CAM data was found to be 0.034. These results confirm those obtained by using the EC [^{3}H]thymidine incorporation assay. In both cases stimulatory activity was found to reside exclusively in the 0.1 M NaCl/50 mM Tris·HCl eluate (peak 2).

DISCUSSION

We have partially purified from an extract of bovine retinas a factor that stimulates the proliferation of EC in culture as well as neovascularization on the chicken CAM. The results from these two assay systems not only indicate the angiogenic nature of the retina-derived factor but also demonstrate the feasibility of the cell culture system as an assay for angiogenic factors.

The EC culture assay, therefore, was relied upon as the major technique for following purification and for characterizing the active material in the retinal extracts. These studies have demonstrated the stability of the active materials to various treatments, such as heating up to 60°C, nuclease digestion, and exposure to mild denaturing conditions. Loss in activity was achieved by using extreme conditions of pH, heating at 65–75°C for 30 min, boiling for 2 min, or treating with 0.02% NaDodSO$_4$ or with soluble Pronase. The findings from the protease treatment deserve special mention because they reveal an unusual stability of the retina-derived angiogenic factor to proteolytic degradation. These active materials were inert to the more moderate proteolytic actions of trypsin (in either the immobilized or the soluble form) or to a 2-hr treatment with the less specific, immobilized Pronase. The significance of these results will undoubtedly become apparent when the nature of the active materials has been determined.

The inference from the purification and stability studies is that the active substances in the bovine retinal extracts are proteinaceous macromolecules. The nondialyzable nature of the retina-derived activity, its elution volume from a Sephadex G-50, and its retention in the Amicon ultrafiltration system by an XM-50 Diaflo membrane (M_r cut-off 50,000) but not by an XM-100 (M_r cut-off 100,000) indicate an apparent molecular weight for the native material of between 50,000 and 100,000. In agreement with this estimate of molecular size are the results from gel electrophoretic analysis of active materials obtained by two disparate methods. Both partial Pronase digestion and column chromatographic purification produced active preparations with the same major components at molecular weights of approximately 50,000 and 70,000. A case might be made for the possibility that the active retinal material could be a small molecule, trapped by or somehow linked to a larger carrier molecule.

However, neither overnight dialysis of the retinal extract against urea (4 M) or guanidine·HCl (2 M) nor increasing the ionic strength of the extract altered the nondialyzable nature of the activity. Furthermore, the active factor could not be extracted from lyophilized retinal extract into either 90% or absolute ethanol, again indicating that the active factor most probably is a macromolecule.

Our concern for demonstrating the macromolecular nature of the retina-derived angiogenic factor is due to the fact that various angiogenic factors reported to date are of a low molecular weight. The angiogenic factors isolated from tumors by a number of investigators (refs. 12 and 13; unpublished data), from synovial fluid (14), from BALB/c 3T3 cells (22), and from bovine parotid glands (22) appear to be small molecules with molecular weights of less than 500. Furthermore, McAuslan has claimed that the angiogenic factors that he is investigating contain copper that is essential for their activity (22). We have examined the copper content of our retinal extracts from various purification stages by atomic absorption spectroscopy. At 10 times the concentrations needed to display activity in the *in vitro* assay, these preparations do not reveal detectable amounts of copper. Thus, the bovine retina-derived angiogenic factor appears to differ in size from these other angiogenic factors and in copper content from those factors described by McAuslan.

On the basis of this report, it would appear that not all angiogenic factors are the same with regard to molecular structure. Whether the stimuli that function in various processes to induce angiogenesis act via a common mechanism remains to be seen. Completion of the purification of the angiogenic factor and determination of the source of the vasoproliferative activity in the retina, the mode of its liberation, and the mechanism of its action will aid in assessing the role of an angiogenic activity in the etiology of ocular neovascularization. Additionally, comparison of the retina-derived factor and its mode of action with angiogenic factors from other tissues may provide insight into the process of angiogenesis in general.

We acknowledge Donald Kaiser for his expertise in tissue culture techniques, Shirley Metzger for her excellent typing, Dr. Allyn Kimball for his assistance with statistical analyses, Dr. Ira Herman for his helpful discussion of the manuscript, and Dr. Arnall Patz for his continuing support. This work was supported by National Institutes of Health Grants CA 15381 and EY 02603 and a National Eye Institute Postdoctoral Fellowship to P.A.D'A.

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