Biochemistry

Volume 24, Number 19 September 10, 1985

Accelerated Publications

Induction of Angiogenesis by Bovine Brain Derived Class 1 Heparin-Binding Growth Factor†

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Received May 20, 1985

ABSTRACT: The angiogenic capacity of the class 1 heparin-binding growth factor from bovine brain, an anionic endothelial cell mitogen of M_r 16 000, has been evaluated. Its ability to induce the growth of new blood vessels has been assessed by means of two established assay systems. On the embryonic chick chorioallantoic membrane dose–response studies demonstrate that 160 ng (10 pmol) of mitogen is required to induce angiogenesis in >50% of the eggs within 72 h. In the presence of 1 unit of exogenous heparin only 40 ng of mitogen (2.5 pmol) is needed to induce a similar response. Moreover, this occurs within 48 h, indicating that heparin also augments the angiogenic response by enhancing the rate of induction of angiogenesis. Eighty nanograms (5 pmol) of mitogen also induces the ingrowth of new blood vessels into the rabbit cornea, both in the presence and in the absence of heparin. These results establish that the class 1 heparin-binding growth factor from bovine brain is an angiogenesis factor. Importantly, the neovascularization induced by this angiogenesis factor is enhanced by heparin. The mechanistic implications for neovascularization under certain normal and pathological conditions are discussed.

A new class of growth factors has been defined on the basis of its affinity for heparin (Shing et al., 1984; Lobb & Fett, 1984; Klagsbrun & Shing, 1985; Lobb et al., 1985). These heparin-binding growth factors (HBGF's)[1] can be subdivided into two distinct classes on the basis of a series of criteria including differential heparin affinity, isoelectric point, and amino acid composition (Lobb et al., 1985). Class 1 HBGF's are anionic mitogens of M_r 15 000–17 000 found in neural tissue, including bovine and human brain (Lobb & Fett, 1984; Maciag et al., 1984; Conn & Hatcher, 1984; Klagsbrun & Shing, 1985; Lobb et al., 1985), bovine hypothalamus (D'Amore & Klagsbrun, 1984; Lobb & Fett, 1984), and bovine retina (D'Amore & Klagsbrun, 1984). Class 2 HBGF's are cationic mitogens of M_r 18 000–20 000 that have a wide tissue distribution, including neural tissue (Gospodarowicz et al., 1984; Lobb & Fett, 1984; Klagsbrun & Shing, 1985), cartilage (Sullivan & Klagsbrun, 1985), macrophages (Baird et al., 1985), and tumors (Shing et al., 1984; Lobb et al., 1985).

The in vivo role of these mitogens is unclear, but they may play a role in neovascularization in a variety of normal and pathological states since they induce both migration and proliferation of endothelial cells in vitro (Gospodarowicz et al., 1978, 1984; Azizkhan et al., 1983; Shing et al., 1984). Moreover, partially purified pituitary fibroblast growth factor (FGF), which can be classified as a class 2 HBGF (Gospodarowicz et al., 1984; Lobb et al., 1985), and partially purified retina-derived growth factor (RDGF), which can be classified as a class 1 HBGF (D'Amore & Klagsbrun, 1984; Lobb et al., 1985), have been shown to induce new blood vessel growth in vivo (Gospodarowicz et al., 1978, 1979; D'Amore et al., 1981).

We have demonstrated that heparin affinity chromatography rapidly and efficiently purifies both classes of mitogen from bovine brain (Lobb & Fett, 1984). This report dem-

† This work was supported by The Endowment for Research in Human Biology, Inc.

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[1] Abbreviations: CDGF, cartilage-derived growth factor; HBGF, heparin-binding growth factor; RDGF, retina-derived growth factor; FGF, fibroblast growth factor; IdU, iododeoxyuridine; CAM, chorioallantoic membrane; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/85/0424-4969$01.50/0

onstrates that the class 1 HBGF from bovine brain [formerly designated HGFα (Lobb & Fett, 1984)] induces angiogenesis as demonstrated by two established assay systems, the embryonic chick chorioallantoic membrane (CAM) and the avascular rabbit cornea.

Experimental Procedures

Materials. Bovine brain was obtained from Pel-Freez Biologicals (Rogers, AR). CM-Sephadex C50 and heparin–Sepharose were from Pharmacia (Piscataway, NJ). Hen egg white lysozyme and porcine heparin (endotoxin free) were from Sigma (St. Louis, MO). [^{125}I]Iododeoxyuridine was from New England Nuclear (Boston, MA). Methylcellulose (4000 cP) was from Fisher Chemical Co. (Pittsburgh, PA).

Growth Factor Preparation. The class 1 HBGF from bovine brain was purified to homogeneity by ammonium sulfate precipitation and CM-Sephadex C50 ion-exchange and heparin–Sepharose affinity chromatography exactly as described (Lobb & Fett, 1984). Purity was established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, in which the mitogen migrates as a doublet of M_r 16000, and by amino acid composition (Lobb & Fett, 1984).

Mitogenesis Assays. Growth stimulation of quiescent, confluent monolayers of Balb/c 3T3 cells was measured as described elsewhere (Lobb & Fett, 1984). Twenty percent bovine serum was used as a positive control, resulting in a 15–30-fold increase in iododeoxyuridine (IdU) uptake. One unit of activity is defined as that concentration of mitogen required to stimulate half-maximal IdU incorporation.

Angiogenesis Assays. Samples were assessed for angiogenic activity by implantation on the chick embryo CAM and in the avascular rabbit cornea. The standard CAM assay used in our laboratory has been described in detail elsewhere (Fett et al., 1985). Briefly, 5-μL volumes of aqueous, salt-free samples were applied to sterile Thermanox 15-mm disks (Flow Laboratories Inc., Rockville, MD) and allowed to dry under laminar flow conditions. The loaded disks were inverted and applied to the CAM surface of 9-day-old chick embryos (Spafas Inc., Norwich, CT) through 1–2-cm "windows" cut through the shell on day 4. Eggs were viewed through a Nikon stereoscope at 20 ± 2, 44 ± 2, and 68 ± 2 h (designated days 1, 2, and 3, respectively) and scored qualitatively for infiltration of blood vessels into the sample area. Results were made semiquantitative by recording the ratio of positive to total eggs surviving each day.

A second CAM method was used in which samples were implanted in methylcellulose pellets (Taylor & Folkman, 1982). Methylcellulose was autoclaved and then dissolved (1% w/v) in sterile water by stirring overnight at 4 °C. Lyophilized, salt-free samples were dissolved by gentle stirring in 1% methylcellulose for 2 h at 4 °C. Ten-microliter volumes were placed on a clean, dry mylar sheet (LKB, Gaithersburg, MD) and air-dried under laminar flow conditions to form a clear pellet. The sheet containing the pellets was placed in a sterile square Petri dish, placed in a desiccator, and lyophilized for a further 60 min to ensure that the pellets were completely dry. The intact pellets were gently peeled from the sheet and placed on the day 9 CAM, where they instantly rehydrated and formed a clear dense gel. Eggs were then viewed and scored as indicated above.

The statistical significance of the CAM results on the second and third days after implantation was assessed by methods detailed elsewhere (Fett et al., 1985). Briefly, since the only designations are as positive or negative, CAM assays constitute Bernoulli trials and can be analyzed as binomial distributions (Kendall & Stuart, 1969). The frequency of positive responses in a series of 1834 controls, which includes the controls presented in this paper, is 0.0676 (124 positive and 1710 negative), with a standard deviation of 0.0059 (Kendall & Stuart, 1973), yielding upper and lower 0.1% confidence limits of 0.0857 and 0.0495. We have employed 0.0857, the upper limit, as the probability of obtaining a positive result with a test group of N eggs. Tables of the cumulative probabilities of positive results were prepared with 0.0857 as the probability of a positive result, and test results are interpreted in terms of these tables, yielding the range over which, and the lower limit at which, a positive response is significant. A significance level of $\leq$5% has to be attained for a sample to be considered active.

The rabbit corneal implant assay was carried out by using a modification of established procedures (Langer & Folkman, 1976). New Zealand White rabbits were anesthetized by intravenous administration of sodium nembutal (40 mg/kg) and local anesthesia in the area of the eyelids attained by subcutaneous injection of 0.5–1.0 mL of 2% lidocaine hydrochloride. A 3–5-mm slit was made at the apex of the cornea and a "pocket" produced with an iris spatula extending to within 2 mm of the corneal limbus. Mitogen-containing and control methylcellulose pellets, prepared as described above, were placed between two 40-μL methylcellulose blank pellets, and the resulting "sandwich" was implanted into the rabbit cornea. The methylcellulose sandwich was touched against the corneal slit where it immediately hydrated and could be conveniently folded and inserted into the pocket. Chloromycetin ophthalmic ointment (1%) was applied to each eye, and the eyes were taped shut loosely to avoid drying and infection prior to the effects of anesthesia wearing off. Stereomicroscopic observations were made on days 6 and 13 for the presence of infiltrating vessels extending from the corneal limbus toward the gel implant.

Sample Preparation. Purified growth factor was eluted from heparin–Sepharose with 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.0, containing 1 M NaCl, and its protein concentration was determined by amino acid analysis. Lysozyme was added as a protein additive to an aliquot of the mitogen. The same amount of lysozyme was added to an equal volume of buffer as control. Samples were dialyzed extensively in the same container against sterile water in M_r 6000–8000 cutoff dialysis tubing, filtered through 0.8-μm filters (Centrex; Schleicher & Schuell, Keane, NH), and then lyophilized in sterile polystyrene tubes (Fisher).

For CAM assays employing Thermanox disks the mitogen sample was reconstituted in sterile water such that 5 μL contained 2 μg of lysozyme and $\sim$200 ng of mitogen. The same volume of water was added to the control tube, such that 5 μL contained 2 μg of lysozyme alone. The mitogen was serially diluted into lysozyme in water such that 5-μL volumes always contained 2 μg of lysozyme. One unit of sterile heparin in water was added to appropriate samples from a concentrated stock solution.

For CAM assays employing methylcellulose pellets, lyophilized samples were reconstituted with 1% methylcellulose in water at 4 °C, such that 10 μL contained 2 μg of lysozyme and $\sim$100 ng of mitogen.

For both disk and pellet CAM assays aliquots from samples to be tested were removed immediately prior to implantation, diluted 100-fold into Dulbecco's calcium- and magnesium-free phosphate-buffered saline containing 0.1% bovine serum albumin, and assessed for mitogenic activity. The final concentration of mitogen implanted was estimated from a dose response on 3T3 cells, assuming 1 unit of activity to be 400

Table I: CAM Assay of Bovine Brain HBGF: Disk Method[a]

sample	(A) no heparin			(B) 1 unit of heparin		
	day 1	day 2	day 3	day 1	day 2	day 3
160 ng of HBGF	3 (1/36)	19 (5/26, 6)	56 (14/25, ≪0.1)	6 (1/18)	44 (7/16, ≪0.1)	62 (8/13, ≪0.1)
80 ng of HBGF	3 (1/36)	21 (7/34, 2)	29 (10/34, <0.1)	6 (1/17)	57 (8/14, ≪0.1)	64 (9/14, ≪0.1)
40 ng of HBGF	0 (0/28)	8 (2/26, 65)	17 (4/24, 13)	0 (0/20)	55 (11/20, ≪0.1)	56 (10/18, ≪0.1)
20 ng of HBGF	0 (0/34)	3 (1/29, 92)	15 (4/26, 16)	0 (0/15)	7 (1/14, 70)	18 (2/11, 23)
control A[b]	0 (0/31)	4 (1/26)	9 (2/23)	0 (0/17)	0 (0/16)	7 (1/15)
control B[c]	0 (0/39)	0 (0/38)	9 (3/33)	0 (0/32)	0 (0/32)	8 (2/25)

[a] Samples were implanted on Thermanox disks as described under Experimental Procedures. Results are expressed as the percentage of positive eggs. The ratio of positive to total surviving eggs and the percent significance are given in parentheses. [b] Buffer control containing 2 μg of lysozyme prepared in the same manner as the mitogen sample. [c] Control containing 2 μg of lysozyme in water.

Table II: CAM Assay of Bovine Brain HBGF: Pellet Method[a]

sample	day 1	day 2	day 3
80 ng of HBGF	25 (4/16)	25 (4/16, 4)	50 (8/16, ≪0.1)
80 ng of HBGF with 0.2 unit of heparin	18 (4/22)	43 (9/21, ≪0.1)	89 (16/18, ≪0.1)
control[b]	0 (0/22)	0 (0/17)	6 (1/17)
control with 0.2 unit of heparin	6 (1/18)	6 (1/17)	6 (1/17)

[a] Samples were implanted in methylcellulose pellets as described under Experimental Procedures. Results are expressed as the percentage of positive eggs. The ratio of positive to total surviving eggs and the percent significance are given in parentheses. [b] Control pellets contained 2 μg of lysozyme.

pg/mL (Lobb & Fett, 1984).

Results

CAM Assay. (1) Thermanox Disk Method. Serial 2-fold dilutions of homogeneous mitogen, in the presence and absence of 1 unit of heparin, were implanted onto the CAM on Thermanox disks. At least 20 eggs were used per each diluted sample. Induction of angiogenesis was observed in >50% of the eggs at 160 ng of mitogen (10 pmol), but not until the third day after implantation. Statistically significant angiogenesis (i.e., ≤5% significance) was also induced by 80 ng of mitogen, but not by 40 or 20 ng of mitogen (Table I, A). In the presence of 1 unit of heparin, which of itself is inactive, 160, 80, and also 40 ng of mitogen induced statistically significant angiogenesis (Table I, B). As little as 40 ng of mitogen (2.5 pmol) was required to induce neovascularization in >50% of the eggs. Moreover, highly significant angiogenesis (significance ≪0.1%) was observed for 160, 80, and also 40 ng of mitogen by the second day after implantation. In the absence of heparin, highly significant angiogenesis is not observed on the second day even with 160 ng of mitogen (Table I, A). Thus, heparin enhanced both the rate of mitogen-induced angiogenesis and the minimum dose of mitogen required to induce a given level of activity.

(2) Methylcellulose Pellet Method. The angiogenic capacity of the mitogen on the chick CAM was also examined with methylcellulose pellets (Table II). The results were in good agreement with those obtained by the disk method. In the absence of heparin 80 ng of mitogen induced angiogenesis in 50% of the implanted eggs by the third day after implantation. In the presence of 0.2 unit of heparin 80 ng of mitogen induced angiogenesis in 43% of the eggs by the second day and 89% of the eggs by the third day. Highly significant angiogenesis was observed on the second day after implantation only in the presence of heparin. Thus the growth factor itself is angiogenic, and added heparin greatly enhances its activity. The responses induced by mitogen-containing and control methylcellulose pellets on the CAM are illustrated in Figure 1.

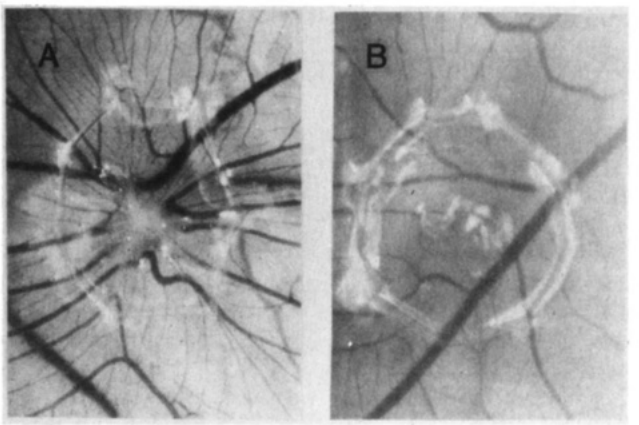

FIGURE 1: CAM assay of bovine brain class 1 HBGF. The angiogenesis induced in 48 h by a methylcellulose pellet containing 80 ng of HBGF is shown in panel A. A control pellet has no effect on the CAM vasculature (panel B). Both pellets also contained 2 μg of lysozyme and 0.2 unit of heparin.

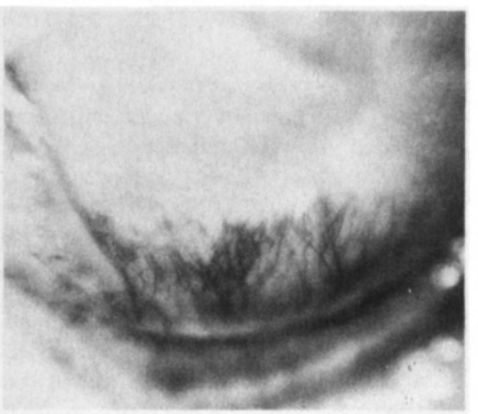

FIGURE 2: Pellets from the same batch as used in Figure 1 were implanted into the rabbit cornea. The neovascularization induced by 80 ng of HBGF and 0.2 unit of heparin is illustrated (day 13 after implantation).

Rabbit Corneal Assay. Methylcellulose pellets from the same batch as those implanted onto the chick CAM as described above were also implanted into the avascular corneal stroma of the rabbit eye. Two pellets were implanted per eye into pockets made opposite each other from a single slit at the apex of the cornea. Eighty nanograms of mitogen was implanted into each eye, as was a lysozyme control pellet. In addition, in one eye both samples contained 0.2 unit of heparin. Two rabbits were used.

The rabbit corneas were examined at days 6 and 13 post-implantation. By day 13 strong neovascularization could be detected in both eyes, but only in the pockets containing mitogen. Negligible neovascularization was observed in control pockets. No attempt was made to quantify the rates of vessel ingrowth and no qualitative differences were apparent in the

intensity of angiogenesis induced by HBGF in the presence or absence of heparin. Figure 2 shows a typical positive response.

Discussion

The endothelial cell proliferation that accompanies neovascularization in both normal and pathological processes (Folkman & Cotran, 1976) has led to the suggestion that the induction of some forms of angiogenesis may be linked to the local production of endothelial cell growth factors (Folkman, 1974; Suddith et al., 1975; Fenselau & Mello, 1976; Gospodarowicz, 1976; Atherton, 1977; Birdwell et al., 1977; Gospodarowicz et al., 1978). Because of the difficulties inherent in the in vitro growth of endothelial cells, particularly from microvessels (Zetter, 1981), and in growth factor isolation in general (Gospodarowicz & Moran, 1976), a detailed examination of this hypothesis has been difficult. However, the finding that virtually all the endothelial cell mitogens that have been described in the last decade have a strong affinity for heparin [for review, see Lobb et al. (1985)] has greatly simplified their purification and characterization.

These heparin-binding growth factors (HBGF's) fall into two classes that are structurally distinct but functionally similar (Lobb et al., 1985). Class 1 HBGF's are anionic mitogens found in neural tissues, typified by acidic brain FGF (Thomas et al., 1984) and RDGF (D'Amore & Klagsbrun, 1984). Class 2 HBGF's are cationic mitogens typified by pituitary FGF (Gospodarowicz, 1975) and cartilage-derived growth factor (CDGF) (Sullivan & Klagsbrun, 1985).

There is already evidence that class 2 HBGF's induce neovascularization. The angiogenic capacity of pituitary FGF upon implantation into the rabbit cornea was first demonstrated several years ago (Gospodarowicz et al., 1978, 1979). It is now clear that the mitogen was not purified to homogeneity (Gospodarowicz et al., 1984), and its angiogenic capacity has been recently reevaluated. Slow-release forms of homogeneous pituitary FGF containing 1 μg of mitogen stimulate new blood vessel growth in both the chick CAM and the hamster cheek pouch assays (Gospodarowicz et al., 1984). A tumor-derived HBGF from a rat chondrosarcoma also stimulates neovascularization (Shing et al., 1984, 1985). In the presence of two units of heparin 60–120 ng of mitogen stimulates angiogenesis in the chick CAM assay within 24 h. Slow-release pellets containing 6–12 ng of mitogen induce neovascularization in the rat cornea. In addition, CDGF increases the levels of granulation tissue in experimental wound chambers that, upon histological examination, showed a marked increase in vascular density in the absence of an inflammatory response (Davidson et al., 1985). Thus, three homogeneous endothelial cell mitogens, isolated from different tissues, but all class 2 HBGF's stimulate neovascularization in vivo.

The angiogenic capacity of the other group of mitogens, class 1 HBGF's, has not been studied extensively. Partially purified RDGF, an endothelial mitogen found in bovine retinal tissue, was reported to be angiogenic (D'Amore et al., 1981). Recent work shows that RDGF can also be purified by heparin affinity chromatography (D'Amore & Klagsbrun, 1984) and that it is a class 1 HBGF (Lobb et al., 1985).

We report here that the class 1 HBGF purified to homogeneity from bovine brain is a potent angiogenesis factor. Neovascularization was assessed in two established assays, using the rabbit cornea and the chick CAM. In the rabbit cornea 80 ng of mitogen induces neovascularization, in the presence and absence of heparin. Accurate dose–response studies were performed with the chick CAM assay. The results show that in the absence of heparin 160 ng of mitogen (10 pmol) is required to induce angiogenesis in >50% of the implanted eggs. Importantly, with added heparin only 40 ng of mitogen (2.5 pmol) induces a similar response, and the rate of angiogenesis induction is increased (Table I). Similar results are obtained with an alternative method of implantation (Table II).

The observation that mast cells accumulate around tumors [for review, see Kessler et al. (1976)] led to the finding that heparin enhances endothelial cell migration in vitro (Azizkhan et al., 1980). Subsequent studies showed that tumor-induced angiogenesis was enhanced by heparin in vivo, although heparin itself was not angiogenic (Taylor & Folkman, 1982). This heparin-enhancing effect has also been observed with other partially purified angiogenesis factors (Castellot et al., 1982; Weiss et al., 1983). Our results provide further evidence for an involvement of heparin in the enhancement of neovascularization and constitute the first demonstration of such enhancement using a pure angiogenesis factor.

Our data, in conjunction with the results of others, establish that HBGF's of both classes are potent inducers of neovascularization in vivo. Thus a clear link has now been established between endothelial mitogens and induction of angiogenesis. However, while it is tempting to assume that the effect of these mitogens occurs at the level of the endothelium, in fact there is no direct evidence that this is the case. Paradoxically, pituitary FGF, a potent mitogen for subconfluent endothelial cells, has no effect on confluent endothelial cell monolayers in vitro (Gospodarowicz et al., 1978), and indeed, confluent endothelial cells are refractory to all growth stimuli (Haudenschild et al., 1976). Either the confluent endothelial monolayer in vitro is an inappropriate model for the vascular wall in vivo or HBGF's induce angiogenesis by an indirect mechanism. Of relevance is the fact that HBGF's are mitogens for a wide variety of mesoderm-derived cells (Gospodarowicz et al., 1978). It is possible that the local proliferation of other cell types, such as fibroblasts, might trigger new blood vessel growth indirectly. For example, both the generation of a hypoxic environment (Knighton et al., 1983) and the local production of proteases (Gross et al., 1983) are believed to play an important role in neovascularization.

The angiogenic capacity of class 1 HBGF's has a number of implications. To date class 1 HBGF's have been found only in neural tissue (Lobb et al., 1985). The role of a normal angiogenesis factor in such tissues is unknown but may involve maintenance of the cerebral vasculature. However, it is clear that the release of these mitogens in pathological situations could have profound consequences. RDGF has been implicated as one of the mediators of abnormal retinal neovascularization (D'Amore et al., 1981). Moreover, since most brain tumors are highly vascularized (Brem, 1976), it is possible that expression of class 1 HBGF's may play a significant role in the angiogenesis induced by brain tumors. This could occur by secretion of the mitogen by the tumors themselves, since a variety of brain tumors have been shown to secrete both angiogenesis factors (Matsuno, 1981) and endothelial cell mitogens (Suddith et al., 1975; Kelly et al., 1976; Olander et al., 1982) in vitro. Alternatively, local release of HBGF's from neural tissue could be induced by tumor-associated or tumor-induced proteases. It is of interest that the cerebrospinal fluid of patients with brain tumors not only induces angiogenesis (Lopez-Pousa et al., 1981) but also induces migration of 3T3 cells (Brem et al., 1983). This may be due to the presence of a class 1 HBGF. If confirmed, such a finding would have significant diagnostic implications.

The availability of homogeneous HBGF's in large quantities should allow detailed mechanistic studies at the molecular level of the neovascularization induced by this class of normal angiogenesis factors. Moreover, more detailed investigations into the role of heparin and its subfragments in the process should be possible.

ACKNOWLEDGMENTS

We thank Dr. Bert L. Vallee for continued advice and support. We are grateful to Drs. Michael Klagsbrun and Patricia D'Amore for helpful discussions and to Susan Kane and Judith LaLonde for excellent technical assistance.

Registry No. Heparin, 9005-49-6.

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