the other two varieties include the Kunitz-insert at nucleotide 865. βAPP is now known to occur physiologically as a group of 110- to 135-kD membrane-associated proteins in brain and other tissues (*3*). Prediction of its amino acid sequence based on cDNA clones indicates the molecule possesses a large extracellular domain, a single membrane-spanning region, and a small cytoplasmic moiety (*8*). βAPP is not homologous to any known protein and its post-translational modifications or processing, if any, as well as its function in vivo are unknown (*14*).

On the basis of immunohistochemical elucidation of binding sites for a portion of β-amyloid and on structural similarities between βAPP and the epidermal growth factor precursor, it has been suggested that the βAPP may be processed to release an active peptide ligand (*15*) having regulatory properties, that is, a neurotrophic factor or hormone. According to this model, abnormal processing of βAPP may result in the formation of amyloid fibrils or in the production of an altered inactive ligand. Our results suggest a possible physiological trophic function for the βAPP ligand in, but not necessarily limited to (*15*), the central nervous system. The concentrations of β1-28 that produced neurotrophic effects here are higher than those required for well-characterized trophic factors such as nerve growth factor, which is typically added to cultured neurons at low concentrations (nanograms per milliliter). However, because β1-28 may be expected to represent only a fragment of any active physiological ligand formed from βAPP, it may lack both primary sequences and tertiary structures that enhance binding. It is also possible that high local concentrations of the physiological ligand occur normally in brain tissue, thus showing similarity to the in vitro situation described here.

A working hypothesis is that amyloid deposition and neuritic plaque formation are linked to a regenerative response of the brain mounted in an attempt to ameliorate the denervation or some cellular abnormality associated with AD. Evidence for such a regenerative response is found in the recent demonstration that AD brain tissue extract exhibits more trophic activity in cortical cultures than normal brain extract (*16*). Specifically, we suggest that the physiological product of βAPP may perform a trophic function supportive of normal brain plasticity and, when altered by aberrant processing, may be precipitated as amyloid in an inactivated or inaccessible form. If the βAPP product is normally retrogradely transported from target cells to the cell bodies of neurons specifically dependent on it (as is the case with nerve growth factor), aberrant processing could result in the transport of a product lacking full trophic activity. Thus, cells dependent on βAPP product for trophic support would die. Alternatively, β-amyloid and the proteins known to be associated with it in AD (for example, α1-antichymotrypsin and others) could serve as the initial nucleus for a neuritic trophic response, yielding plaques. The demonstration of biological trophic activity for the β1-28 portion of β-amyloid points to new avenues of research and may open the door to new therapeutic approaches.

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Control of Angiogenesis with Synthetic Heparin Substitutes

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Many diseases are dominated by persistent growth of capillary blood vessels. Tumor growth is also angiogenesis-dependent. Safe and effective angiogenesis inhibitors are needed to determine whether control of angiogenesis would be therapeutic. Heparin and certain steroids, administered together, can inhibit angiogenesis in a synergistic manner. This "pair" effect suggested that specific hydrophilic cycloamyloses may be suitable heparin substitutes. β-Cyclodextrin tetradecasulfate administered with a steroid inhibits angiogenesis at 100 to 1000 times the effectiveness of heparin in the chick embryo bioassay. This cyclic oligosaccharide also augments the anti-angiogenic effect of angiostatic steroids against corneal neovascularization in rabbits when β-cyclodextrin tetradecasulfate and a steroid are inserted into the cornea or applied topically as eyedrops.

PERSISTENT CAPILLARY BLOOD VESSEL growth is often associated with disease, such as diabetic retinopathy, neovascular glaucoma, rheumatoid arthritis, and hemangiomas (*1*). Progressive tumor growth and metastases also appear to depend on angiogenesis (*2, 3*). In contrast, angiogenesis is not usually active in the normal adult except during wound repair, ovulation, menstruation, and the formation of the placenta. Therefore, the potential therapeutic benefit of controlling pathologic angiogenesis has led to a search for reliable and effective angiogenesis inhibitors.

Angiogenesis is inhibited when heparin, or one of its non-anticoagulant fragments, is administered simultaneously with a steroid of specific structure (for example, an angio-

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static steroid) (*4–9*). Heparin alone can promote angiogenesis in vivo (*10*) and can potentiate endothelial locomotion (*11*) and proliferation (*12*) in vitro. The angiostatic steroids by themselves inhibit angiogenesis weakly or not at all. Potent inhibition of angiogenesis, while requiring the "pair" effect of the two components, is also subject to the variable activity of different heparin preparations (*4, 5, 9*).

Heparin preparations are nonuniform and heterogeneous in composition, molecular size, structure, position of substituents (*N*-sulfate, *O*-sulfate, and glucuronic acid), and sequence (*13*). This heterogeneity probably accounts for the differences between those investigators who report that heparin effectively augments angiostatic steroids (*14*) and those who find that it does not (*15*). We now demonstrate that comparatively simpler molecules may replace the anti-angiogenic properties of heparin when administered with steroids.

In previous investigations the parent heparin was modified or heparin fragments were synthesized (*16*). In contrast, we have examined cyclodextrins with highly hydrophilic and anionic substituents, which have the ability to interact with a hydrophobic steroid and to adsorb to endothelial cells, respectively (*17*).

Cyclodextrins are naturally occurring, cyclic nonreducing, water-soluble oligosaccharides built up from six to eight glucopyranose units (*18, 19*). The internal doughnut-shaped molecule provides a hydrophobic cavity at the center and a hydrophilic outer surface. The diameter of the cavity is determined by the number of glucose units that make up the ring (6, 7, or 8 units for α-, β-, or γ-cyclodextrins, respectively). Steroids and many other hydrophobic molecules with appropriate structures can form complexes with cyclodextrins (*20*); an inclusion complex between hydrocortisone and β-cyclodextrin has been demonstrated (*21*). Cyclodextrins have 18 to 24 hydroxyl units exchangeable for substituents that could increase the hydrophilic and cell-binding activity of the carrier molecule. We examined α-, β-, and γ-cyclodextrin derivatives for their angiostatic activity in combination with hydrocortisone on the chick embryo chorioallantoic membrane (*4*) and with cortexolone in the rabbit cornea (*4, 22*). Cortexolone is a derivative of hydrocortisone with little or no glucocorticoid or mineralocorticoid activity (*4*). Various β-cyclodextrin derivatives were prepared to modify the total hydrophilic activity, which was characterized by the compound's water solubility at 0°C (Table 1).

On the chorioallantoic membrane, in the presence of hydrocortisone, the most soluble variants of β-cyclodextrin had the highest anti-angiogenic activity. Unsubstituted β-cyclodextrin had virtually no effect, whereas β-cyclodextrin tetradecasulfate (Fig. 1), which has two sulfate groups per glucose unit, displayed the most anti-angiogenic activity. Anti-angiogenic activity increased with the number of sulfate substitutions. Some activity was also found with *O*-alkyl–substituted β-cyclodextrins (*23*), which derive their hydrophilic activity from the formation of hydrogen bonds between water and the remaining hydroxyl groups (*24*). When the cyclodextrins with two sulfate groups per glucose unit were compared, γ-cyclodextrin was less active than the β form, and the α form was the least active. This effect is probably due to the "looser fit" of the γ cavity and the inability of the α cavity to fully accept the steroid structure (*20*).

The anti-angiogenic effect of β-cyclodextrin tetradecasulfate was then compared to various heparins on the chick chorioallantoic

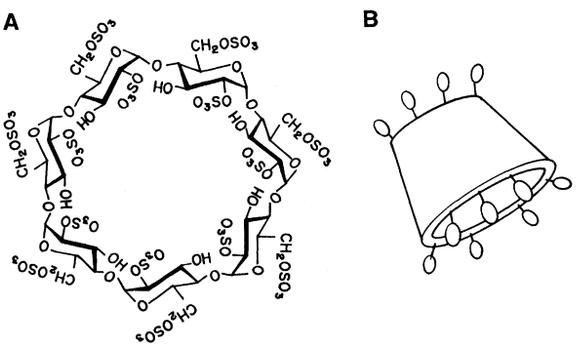

Fig. 1. (**A**) Chemical structure of β-cyclodextrin tetradecasulfate. (**B**) Three-dimensional approximation of the shape of the β-cyclodextrin molecule with oval appendices representing sulfate groups.

Table 1. Anti-angiogenic activity on the chick chorioallantoic membrane of various cyclodextrins (CDs) in the presence of hydrocortisone. Hydrophilic activity was measured by solubility in water at 0°C. The type of substituents are S, sulfate; Me, methoxy; and Pr, propoxy. The approximate number of substituents per CD molecule was determined from anionic conductivity in water solution and confirmed by analysis for β-cyclodextrin tetradecasulfate (β-CD-14S). Heparin was always from the same lot (Hepar). Sulfated CDs were synthesized by reaction of CDs with trimethylamine sulfate in dimethylformamide at 70°C for 24 hours. The solid product was dissolved in aqueous 6% sodium acetate, recrystallized in ethanol, washed in diethyl ether, and dried under vacuum over P_2O_5. Cyclodextrins or heparin were dissolved in 10 μl of 0.45% methylcellulose with hydrocortisone-21-phosphate (60 μg, Sigma) and air-dried on a Teflon mold, and the resulting 2-mm disk was implanted. Listed are the mean percentages and standard errors of avascular zones (diameter of 2 mm or greater) that developed on the chorioallantoic membrane of the 6-day-old chick embryo (*5, 8*) 48 hours after implantation of the test material. Each experiment used 8 to 16 eggs per concentration of CD or heparin; *n* indicates the number of replicate experiments conducted for each concentration over a period of 1 year. The number of replicate experiments for β-CD-14S is greater than for the other cyclodextrins because different batches were tested to determine the reproducibility of the synthesis. The combination of heparin (50 μg) and hydrocortisone (60 μg) was used as an internal control to test the response of each new batch of chick embryos. Heparin alone (without hydrocortisone) at 5 to 500 μg, β-cyclodextrin tetradecasulfate alone at 25 to 100 μg, and hydrocortisone alone at 60 μg did not inhibit angiogenesis in the chick embryo. β-Cyclodextrin tetradecasulfate (25 to 50 μg) inhibited angiogenesis in the presence of cortexolone (50 μg), a steroid of similar structure to hydrocortisone but without an 11-hydroxyl group, resulting in zones that were 53% ± 5% to 64% ± 15% avascular (9 to 15 eggs, two replicate experiments for each concentration).

Compound	Concentration (μg per 10 μl)	Number of embryos	Avascular zones (%)	*n*	Hydrophilic activity (g per 100 ml)
β-CD	25	23	4.5	2	0.7
	100	19	0	2	
β-CD-4Pr	25	50	31 ± 9.7	4	20
	100	52	29 ± 5.7	6	
β-CD-14Me	25	37	20 ± 4.2	2	32+
	100	57	22 ± 5.7	3	
β-CD-7S	25	27	8 ± 3.1	3	13
	100	25	20 ± 9.6	2	
β-CD-14S	25	107	58 ± 7.0	10	42
	50	75	70 ± 5.8	9	
	100	101	55 ± 7.5	12	
α-CD-12S	25	25	4 ± 2.7	2	36
	100	40	17 ± 4.7	3	
α-CD	25	20	0		6
	100	37	5 ± 0.3	2	
γ-CD-16S	25	20	19 ± 1.7	2	38
	100	19	32 ± 5.3	2	
Heparin	50	172	58 ± 3.5	21	>40

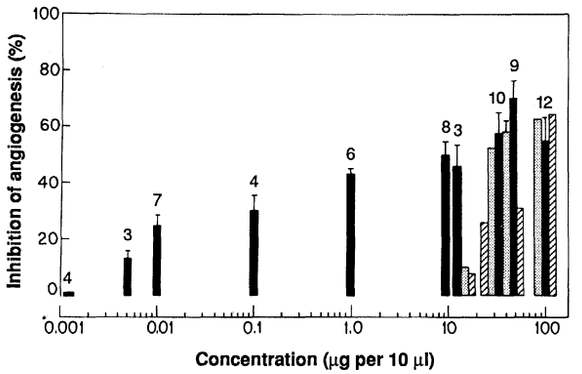

Fig. 2. Concentration dependence of the anti-angiogenic effect of hydrocortisone with β-cyclodextrin tetradecasulfate. Solid bars, β-cyclodextrin tetradecasulfate; number of replicate experiments is shown above the bar. Stippled bars, heparin (Hepar), n = 21 for 50 μg; 100-μg value is from (7). Hatched bars, heparin (Sigma) [from (7)]. The percentage of inhibition of angiogenesis represents the mean and standard error of the percentages of avascular zones 48 hours after the test compound was implanted on the chick embryo chorioallantoic membrane (legend to Table 1). Hydrocortisone (60 μg) was included in all implants; 8 to 16 chick embryos were used in each experiment. For comparison, a synthetic heparin pentasaccharide (Institute Choay, Paris, France) produced 33, 24, and 8% avascular zones at 25, 12, and 6 μg, respectively (*5*).

membrane when β-cyclodextrin tetradecasulfate or heparin was administered with hydrocortisone (Fig. 2). The anti-angiogenic activity of β-cyclodextrin tetradecasulfate was 100 to 1000 times that of commercially available heparins (on a per weight basis). The synthesis and anti-angiogenic effectiveness were fully reproducible with several preparations of β-cyclodextrin tetradecasulfate.

We tested the capacity of cyclodextrin-steroid combinations to inhibit corneal neovascularization. β-Cyclodextrin tetradecasulfate (final concentration, 15 μg/mm^3) and cortexolone (30 μg/mm^3) were incorporated into sustained-release polymer pellets of ethylene–vinyl acetate copolymer (Elvax, Alza) (*25*). These pellets were implanted into rabbit corneas between the vascular limbal edge of the cornea and a second Elvax implant containing endotoxin (17 μg/mm^3) (*26, 27*) (Fig. 3). Endotoxin induces consistent, corneal neovascularization (*26, 27*) by mobilizing mononuclear cells and macrophages into the cornea (*26*). These cells release angiogenic factors, including tumor necrosis factor (*28*). The largest differences between treated and untreated eyes occurred 13 days after implantation of the Elvax pellets. When mean maximal vessel lengths were measured, cortexolone alone inhibited linear capillary blood vessel growth to 49% of the value in the untreated eyes, whereas cortexolone together with β-cyclodextrin tetradecasulfate decreased vessel growth to 18% of that in the untreated control eyes. Vessel growth was stimulated by β-cyclodextrin tetradecasulfate alone to 164% of the growth in the untreated controls. Since quantitation of vessel length underestimates the effect of treatment on neovascularization because vessel density is not measured (for example, number of new vessels that have entered the cornea), we also measured vessel density (legend to Fig. 3). Cortexolone alone inhibited vessel density to 61% of that in untreated controls, cortexolone with β-cyclodextrin tetradecasulfate suppressed vessel density to 8% of the density in untreated eyes, and β-cyclodextrin tetradecasulfate alone stimulated vessel density to 303% above the control level. β-Cyclodextrin tetradecasulfate (1.0 mg/ml) was also administered together with hydrocortisone-21-phosphate (0.5 mg/ml) to the cornea as eyedrops (*29*) (an optimum ratio). Capillary growth (induced by an endotoxin pellet) was not only inhibited, but the new capillaries that had already developed underwent regression.

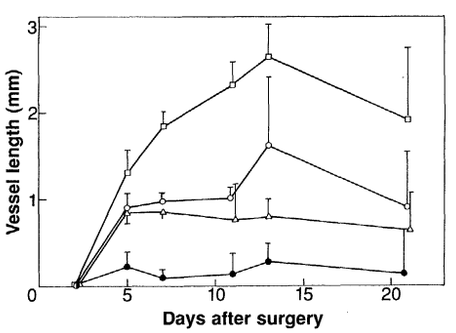

Fig. 3. Implantation of sustained-release polymer pellets in the rabbit cornea. Neovascularization was stimulated by implantation of a 1-mm^3 Elvax pellet (*25*) of endotoxin (from *Escherichia coli*, Sigma, 17 μg/mm^3) into all eyes by the method described in (*22*). Pellets (1 mm^3) of steroid or cyclodextrin were implanted at the same time and positioned between the endotoxin and the limbal edge of the cornea. Symbols: (□) β-cyclodextrin tetradecasulfate alone (15 μg/mm^3); (○) no drug (endotoxin only); (△) cortexolone alone (30 μg/mm^3); and (●) β-cyclodextrin (15 μg/mm^3) and cortexolone (30 μg/mm^3). Vessel length was measured every 2 days with a slit-lamp stereoscope at ×10, on an ocular grid calibrated to ±0.1 mm. The mean maximal length and standard error of new capillary blood vessels in the rabbit corneas (four eyes per experimental group) are shown. Vessel density was also measured and was graded from 0 to 4, where 0 denotes no vessels, 1 denotes 1 to 4 vessels per cornea, 2 denotes 5 to 20 vessels per cornea, 3 denotes 20 to 50 vessels per cornea, and 4 denotes more than 50 vessels per cornea. When this grade was multiplied by mean maximal length, a semiquantitative estimate of vessel density (length-density index) was obtained for each cornea (see text).

The pronounced difference in effectiveness of the β-, γ-, and α-forms of otherwise similar polysulfate derivatives is consistent with the assumed need for complex formation with the steroid. The increased effectiveness of β-cyclodextrin tetradecasulfate (two sulfate groups per glucose unit) is consistent with the suggested role of sulfate groups for the adhesion of glycosaminoglycans to endothelial (*17*) and other (*30*) cell surfaces.

In conclusion, several saccharide carriers mimicked heparin and, in combination with a steroid, acted as effective angiostatic agents. Their angiostatic effectiveness was dependent only on overall physicochemical properties. These findings are consistent with a model in which the saccharide carries the steroid to the endothelial cell surface where it is adsorbed by strong hydrophilic bonding. A single species, such as β-cyclodextrin tetradecasulfate, can potentiate capillary growth when administered alone yet inhibit angiogenesis when administered with an angiostatic steroid, suggesting that the similar dual action of heparin does not require that the heparin be heterogeneous or molecularly complex. Thus, β-cyclodextrin tetradecasulfate has therapeutic potential as an angiogenesis inhibitor when administered with angiostatic steroids or other anti-angiogenic compounds, or as a promoter of angiogenesis when administered with angiogenic molecules.

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Prevention of Rapid Intracellular Degradation of ODC by a Carboxyl-Terminal Truncation

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Ornithine decarboxylase (ODC) was converted from a protein with a short intracellular half-life in mammalian cells to a stable protein by truncating 37 residues at its carboxyl terminus. Cells expressing wild-type protein lost ODC activity with a half-life of approximately 1 hour. Cells expressing the truncated protein, however, retained full activity for at least 4 hours. Pulse-chase experiments in which immunoprecipitation and gel electrophoresis were used confirmed the stabilizing effect of the truncation. Thus, a carboxyl-terminal domain is responsible for the rapid intracellular degradation of murine ODC.

WE REPORT THAT TRUNCATION OF the carboxyl terminus of ornithine decarboxylase (ODC), a cytosolic enzyme with a short intracellular half-life in mammalian cells, converts it into a stable protein. Three lines of evidence suggested a role for the carboxyl terminus in the turnover of ODC. First, although ODC is rapidly degraded in mammalian cells (*1–8*), the equivalent enzyme is stable in trypanosomes (*9*). The mouse and parasite forms of ODC are highly homologous in structure and similar in enzymatic properties, but the former has a carboxyl-terminal portion not present in the latter (*9*). Second, previous studies on mouse ODC expressed in *Escherichia coli* showed that a truncated protein containing the first 423 amino acids of the 461 present in the native protein is enzymatically active (*10*). We thought the carboxyl terminus could mediate a biologically important nonenzymatic function because the sequence in this region is conserved among mammalian ODCs but dispensable for catalytic activity. Third, the "PEST" hypothesis postulates that stretches of amino acids comprised predominantly of proline (P), glutamatic acid (E), aspartic acid, serine (S), and threonine (T) are found in proteins that are rapidly degraded (*11–14*). Murine ODC has two regions that score high on the PEST test. Amino acids 423 through 449, which correspond to one of the PEST regions, lie within the carboxyl terminus of the murine enzyme (*11*).

ODC catalyzes the first committed step in polyamine synthesis, the decarboxylation of ornithine to putrescine (*15, 16*). Intracellular levels of ODC are rapidly modulated under different physiological conditions. Its rate of degradation has been examined by measuring the decay of enzymatic activity or immunoreactive protein after inhibition of protein synthesis by agents such as cycloheximide, and by pulse-chase labeling of ODC followed by immunoprecipitation or two-dimensional gel analysis (*1–8*). It appears that the decay in enzymatic activity is due to the instability of the protein itself rather than an inactivation process that preserves the gross

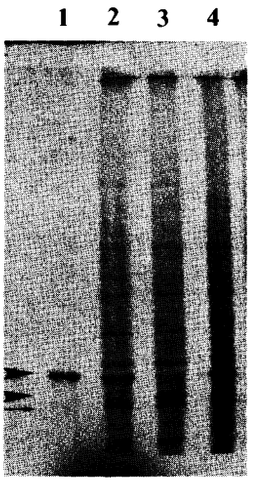

Fig. 1. Immunoprecipitation of ODC proteins expressed from pODg461 and pODg424. ODC⁻ C55.7 cells were cotransfected with pSV2neo (*28*) and either pODg461, a plasmid containing DNA coding for the full-length ODC, or pODg424, a plasmid containing DNA coding for the truncated ODC. After selection for G418 resistance, pooled transfectants were grown in the presence of 10 μ*M* α-difluoromethylornithine for 3 weeks to allow increased expression of the ODC protein (*5*). Cells were labeled for 10 min in methionine- and cysteine-free medium supplemented with 5% fetal calf serum and Tran[^{35}S]-Met (250 μCi/ml) (ICN). Lysates from transfectants carrying pODg461 and pODg424 contained 1.6×10^8 and 2.1×10^8 acid precipitable counts, respectively. These were immunoprecipitated with rabbit antibodies to murine ODC as described in (*5*) and were analyzed by 10% SDS-PAGE and autoradiography. Lanes 1 and lane 4, cytoplasmic lysate from D4.1 ODC overproducing cells (*29*), immunoprecipitated and unprecipitated, respectively; lane 2, immunoprecipitate of pODg461-transfected cells; lane 3, immunoprecipitate of pODg424-transfected cells. Arrowheads mark the positions of wild-type (top) or mutant (bottom) ODC; small arrow indicates the position of actin, whose mass is 42 kD.

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