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A Basic Compositional Requirement of Agents Having Heparin-Like Cell-Modulating Activities

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ABSTRACT. Heparin has been recognized as possessing a large variety of cell-modulating activities. Using compositionally simple, structurally rigid, and low molecular weight saccharide molecules (cyclodextrins), we demonstrated that these activities depend primarily on a single, gross compositional parameter: a minimum intramolecular density of neighboring anionic (sulfate) groups. This same critical parameter is shown to be involved in achieving cell-modulating behavior as diverse as angiogenesis, endothelial proliferation, inhibition of smooth muscle cell growth, and cell protection against virus invasion. Physical chemical evidence is presented that associates this property with multi-ionic complex formation between the clusters of anionic and cationic sites on the complexing partners. These observations revive early suggestions of the decisive role of electrostatic complexation capabilities of glycosaminoglycans like heparin; taken together with numerous observations on heparinoids and other agents reported in diverse specialized fields of cell biology and medicine, they provide evidence that molecular agents of critical anionic (sulfate) density (MACADs) represent a broad class of molecules that, in contrast to proteins, do not rely on structural detail for their cell biological activities, but function by ionic complexation with proteinic agents (e.g. growth factors), thereby modifying *their* structure-specific activities. BIOCHEM PHARMACOL **54**;1:149–157, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cyclodextrin sulfate; heparin; structural requirement; cell modulation activity

Heparin has long been known for its extraordinarily useful properties as an anticoagulant. It is a complex and heterogeneous polyanionic GAG†† [1]. Decades of research have shown that the anticoagulant activity of heparin depends on molecular size, degree of sulfation, and detailed structural composition [1, 2]. Starting some two decades ago [3], a recognition emerged that heparin also possesses a variety of cell-modulating capabilities [4, 5] that are relevant to many clinical pathologies [6]. Relatively little unifying knowledge has developed concerning the compositional requirements responsible for these various properties. We now report a summary multidisciplinary investigation concerning the critical compositional requirements for these activities and their applicability to a variety of cell-modulating capabilities, and we present physical chemical evidence for the role of multi-ionic binding in controlling these activities. Viewing these observations together with past reports on a variety of agents across diverse biomedical and pharmacological specialties suggests that MACADs need to be recognized as an important and general class of natural biological entities as well as potential pharmaceutical agents.

Research concerning the structural details responsible for the antithrombic activity of heparin generally has begun with heparin itself, generating smaller fragments or chemically modifying specific substituents of the complex and heterogeneous parent composition. In contrast, after the discovery that a small sulfated cyclodextrin was equally or more effective than heparin [7, 8] in modifying angiogenic cell behavior, we began to explore other biological properties of this relatively uncomplicated molecule [9]. Heparin, a polysaccharide with a molecular weight of 10,000–20,000, has sugar units substituted in irregular sequence with a number of different substituents. A cyclodextrin is a small, structurally simple molecule. β-Cyclodextrin is a cyclic chain of seven glucose units. The cyclic configuration makes it a rigid structure. The available substitutional positions, the glucose hydroxyl groups, are located in planes of seven (the 6-positions) and fourteen hydroxyl groups (the 2- and 3-positions) located at the "entrances" of a toroid formed by the sugar units. The no more than about 1.2-nm diameter assures a concentrated mutual density of

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†† *Abbreviations:* aFGF, acidic FGF; bFGF, basic FGF; CDS-14, cyclodextrin bearing an average of about 14 sulfate groups; CDS, highly sulfated cyclodextrin; CDSP, highly sulfated cyclodextrin in polymeric (solid) form; FGF, fibroblast growth factor; GAG, glycosaminoglycan; MACAD, molecular agents of critical anionic density.

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any substitutional groups, such as sulfates, around the ring. This stands in contrast to the long linear chains of GAGs such as heparin.

We have examined the dependence on sulfate substituents and other substituents of a variety of cell-biological activities: angiogenesis, endothelial cell proliferation, inhibition of smooth muscle cell proliferation, and cell protection against invasion by retrovirus. To explore the mechanistic aspects involved, we have used biochemical procedures involving molecular complexation: spectral shift in cationic dyes (dye staining, metachromasia) or electrical conductivity changes when complexation occurs with proteins such as protamine. We have also examined the ability to capture as well as to donate proteinic growth factors such as FGF when the cyclodextrin sulfate is presented as a surface to mimic heparin (or heparin sulfate proteoglycan) on a cell surface.

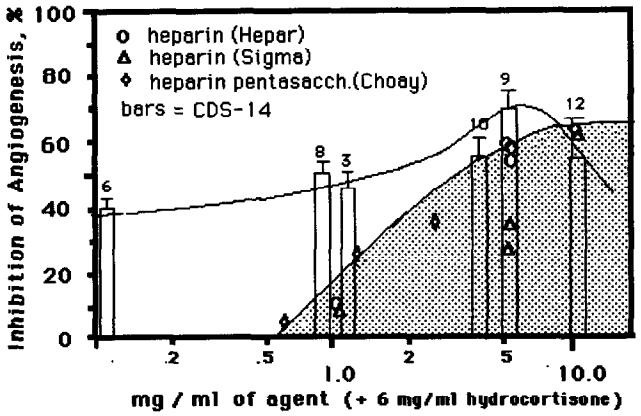

FIG. 1. Inhibition of angiogenesis in the CAM assay. Comparisons of various heparins and of CDS-14, for various weight concentrations of each, when combined with 6 mg/mL of hydrocortisone phosphate (from Refs. 7 and 8).

MATERIALS AND METHODS

Cyclodextrin Sulfate, Materials

β-Cyclodextrin (Cerestar, USA, formerly the American Maize-Products Co., Hammond, IN) was sulfated by standard synthetic methods, as described by Gilbert [10] using SO_3-complexes as sulfating agents. We have used SO_3-trimethyl amine as described in previous work [8], as well as products sulfated by chlorosulfonic acid in pyridine. Samples of varying average sulfate substitution were thus obtained by employing various methods and severities (time and temperature) of sulfation and separation into size fractions over Sephadex. Elemental analyses were performed to determine the degree of sulfate substitution for each fraction. Mass spectrometer analyses consistently indicated a distribution of sulfate number around the average given by analytical analysis. The numbers used in this work are those based on the average analytical value. The polymeric cyclodextrin sulfate (CDSP) was made as in Ref. 11. Larger quantities were obtained by similarly sulfating cyclodextrin-polymer by courtesy of Cerestar USA.

Angiogenesis Inhibition (Fig. 1)

Data were obtained by the chorioallantoic membrane (CAM) test, an accepted *in vivo* angiogenesis assay [12, 13] that observes the inhibition of the initial embryonic vascularization (angiogenesis) of a fertilized chicken egg. Data are taken from our previous work as noted above [8]. Numbers of replicate experiments are shown above the bars that represent the data obtained for CDS-14.

Endothelial Cell Promotion (see Figs. 2 and 3A)

The method employs an *in vitro* assay previously described [14]. Agents at indicated concentrations and 30 ng/mL of bovine recombinant aFGF were added to 24-well tissue culture plates previously seeded with a cell density of approximately 1×10^4 cells/cm^2. Four days later, the cell densities were measured electronically using a Coulter counter. Reported percent cell densities were calculated by subtracting the value for aFGF in the absence of other agents.

Inhibition of Smooth Muscle Cell Proliferation (see Figs. 3B and 4)

The methods employed have been described previously [15, 16]. Briefly, human umbilical vein and coronary artery smooth muscle cells were cultured using the explant technique. The ability of CDS-14 or heparin to inhibit proliferation induced by 10% fetal bovine serum (FBS) was then measured.

Inhibition of Virus Infection (see Fig. 3C and Tables 1 and 2)

The HIV-virus data were obtained by observing the dose requirement for inhibition of syncytia formation as described previously [17]. For the HIV-1 assay, H9 cells infected with the IIIb isolate were cocultured with uninfected Supt-1 cells (a human CD4+ T cell line) as targets. For the HIV-2 assay, HUT 78 cells infected with the ROD-2 isolate were cocultured with H9 cells. Infected cells were plated on 96-well plates (10^4 cells/well in RPMI + 10% FBS) and incubated without or with an agent at varying dilution for 30 min at 37°. Target cells were added at 5×10^5/well. The number of syncytia was determined after 24 hr, or as stated in Table 2. The HTLV assay observed plaque formation in 50 mL of a 2-day conditioned medium from an HTLV-1 chronically infected cell line placed on confluent monolayers of rat XC cells in a 6-well plate with and without various dilutions of the agents. Plaque numbers were counted after 48 hr. Each evaluation reported is based on twelve concentration point data based on successive dilutions.

Alcian Blue Complexation ("Staining") (see Table 3)

A 1-μL droplet of 0.8 mg/mL solution of the test substance was placed on an Alumina G coated TLC plate (Analtech, Newark, DE) and dried for 10 min at 60°. An aqueous solution of 0.5 mg/mL Alcian Blue (8GX, Aldrich), 0.25 M acetate buffer (5.6 pH), and $MgCl_2$ was allowed to rise past the impregnation spots. Complexing resulted in dark blue staining of the spot, leaving a clear trail of dyeless solvent behind the spot.

Azure A Complexation (Metachromasia) (see Fig. 3D)

This assay was performed as previously used and described [18]. It relies on spectrophotometric measurement of the change in optical density at 620 nm, due to the shift in the Azure A absorption spectrum upon complexation of the cationic dye with polyanionic centers.

"Titration" of MACAD Agent with Protamine

In the Azure A method (see Fig. 5A), a solution was prepared containing 16 mg/mL of heparin or CDS-14. Protamine was added by weight ratios, as indicated. After brief stirring, the remaining activity (as above) was determined and compared with a standard scale determined with premeasured standard dilutions of Azure A. The electrical conductivity method (see Fig. 5B) employed a standard conductivity probe. The relative concentration of protamine to the complexing agent was changed successively from complexing agent alone by removing measured amounts of fluid and adding measured amounts of protamine solution so as to attain a constant total concentration of (heparin or CDS-14 + protamine) of 4 mg/mL. There is little experimental error noted in these techniques.

Adsorption on Polymeric CDSP-14 (see Fig. 6)

Azure A dye adsorption studies on polymeric CDSP-14 particles were done typically with a 50- and 200-mL solution of 0.1 mg/mL of dye and 5 mg of CDSP-14 particles, constantly stirred. The Azure A remaining in the solution was determined photometrically at 620 nm. Equilibrated (saturated) solid was separated by filtration, and desorption was followed similarly after immersion in dye-free water. The studies of binding and release of bFGF were done with ^{125}I-radiolabeled recombinant growth factor (2.3×10^6 cpm/mg). ^{125}I-FGF (final concentration = 0.25 mg/mL) was added to 1.7 mL of a suspension of CDSP (10 mg/mL) in DME/F12 with 10% FBS. Desorption was studied on saturated CDSP materials that were washed and placed into FGF-free medium. Details are described in Ref. 19.

RESULTS

Inhibition of Angiogenesis

Heparin in conjunction with hydrocortisone has been shown to inhibit angiogenesis [7]. The small 7-glucose

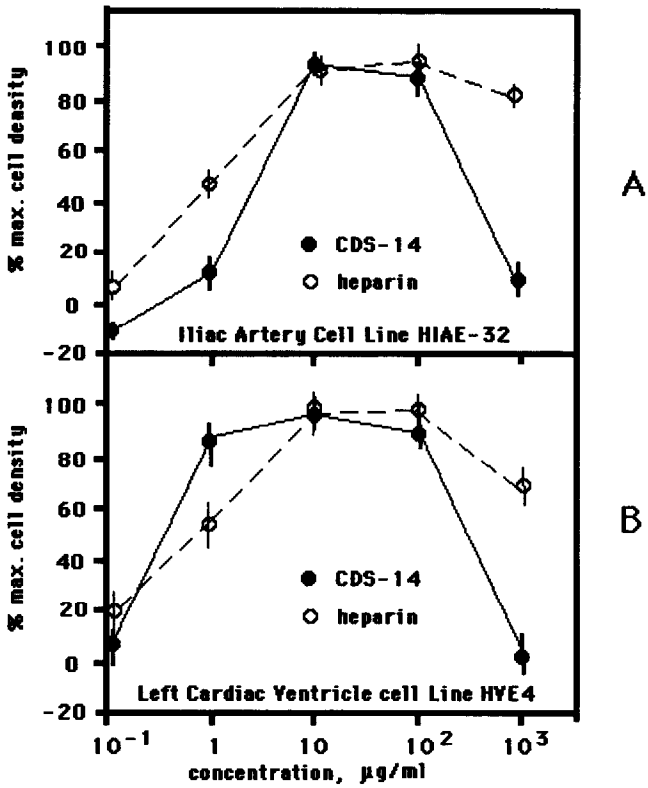

FIG. 2. Promotion of endothelial cell growth *in vitro*. Comparison of heparin and CDS-14 vs weight concentration. Each data point denotes the average, and the bar represents the spread of three experiments for each case.

saccharide β-cyclodextrin (CD) *per se* was inactive. However, addition of only sulfate substituents (approximately 14 per molecule) rendered it (CDS-14) equally or more effective than heparin for inhibiting neovascularization with steroidal or other angiostatic agents [8] in *in vivo* models such as in the chorioallantoic membrane (CAM) [12, 13], or in endotoxin-stimulated corneal vascularization [20]. Figure 1 shows a comparison of the antiangiogenic activity seen in the CAM assay for CDS-14 (closed symbols), two different heparin samples, and a synthetic pentasaccharide heparin (open symbols), when combined with 6 mg/mL of hydrocortisone phosphate [7, 8]. Unsulfated cyclodextrin was virtually inactive (4.4% inhibition at 2.5 mg/mL), and sulfation to about seven sulfate groups per molecule also had very low activity (8% at 2.5 mg/mL). We noted that maximum inhibitory activity of the best heparins and of CDS-14 occurred at a similar magnitude of weight, not molar, concentration, in spite of the great difference in molecular weight (heparin ≈ 15,000; CDS-14 ≈ 2600).

Promotion of Endothelial Cell Proliferation

Heparin will potentiate endothelial cell proliferation induced by FGF [14]. We have examined this activity for CDS-14 and compared it to heparin for two different human endothelial cell lines: iliac artery (HIAE-32; Fig.

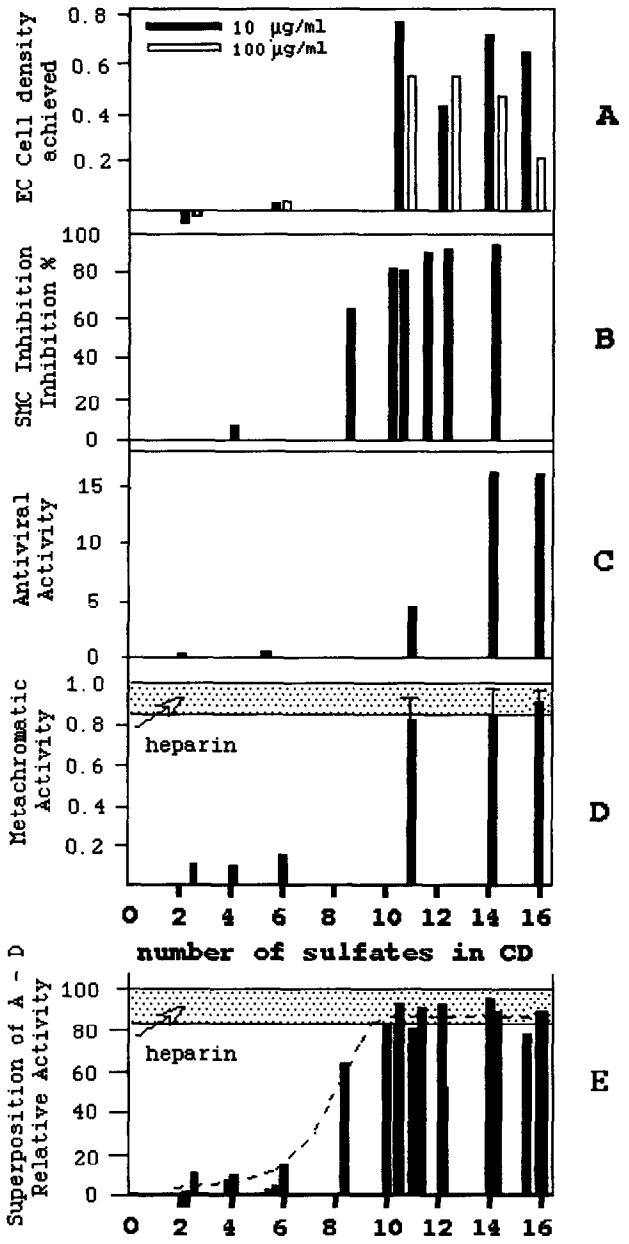

FIG. 3. Modification of cell behavior and metachromasia affected by cyclodextrin polysulfates as a function of the number of sulfate substituents per molecule. (A) Promotion of endothelial cell proliferation; (B) inhibition of smooth muscle cell proliferation; (C) antiviral activity in HIV-1 infection; (D) electrostatic (ionic) complex formation with cationic dye molecules (metachromatic activity of Azure A); and (E) superposition of all of the properties observed in panels A–D.

2A) and left ventricle (HVE-4; Fig. 2B). As in the case of angiogenesis inhibition, the maximum effect occurred at similar weight, not molar, concentration; also as in the former case, a saturation effect occurred at higher concentration levels.

Results with cyclodextrins of varying numbers of sulfate, at 10 and at 100 mg/mL, were obtained for the human endothelial cell line HIAE-32. In Fig. 3A, the relative degree of stimulation of proliferation is shown for each

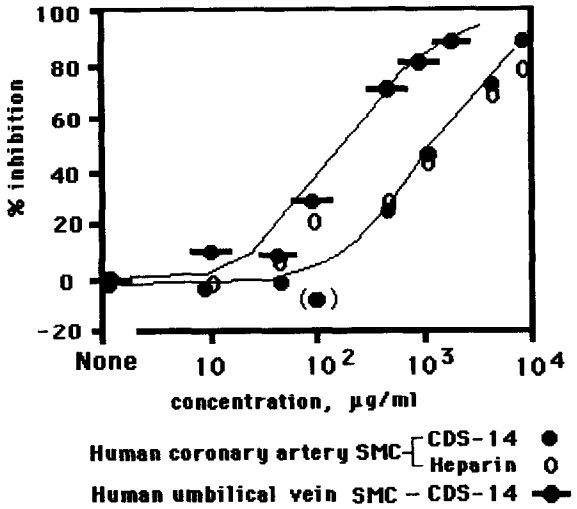

FIG. 4. Dependence of the inhibition of smooth muscle cell proliferation on weight concentration of heparin and of CDS-14.

sulfated cyclodextrin. The molecular sulfate densities are expressed as the average number of sulfate substitutions per molecule, calculated from the elemental sulfur analysis in each sample. Unsulfated cyclodextrin itself as well as hydroxypropyl-, hydroxyethyl- and maltosyl-β-cyclodextrin were also tested but showed no activity (data not shown). On the other hand, sulfated maltosyl-β-cyclodextrin showed activity comparable to that of CDS-14, with no substantial effect by the added maltosyl substituent.

The FGF-dependent endothelial cell proliferation activity was seen to require a minimum number of sulfate groups per cyclodextrin molecule, whereupon its activity for endothelial promotion was similar to that of heparin (Fig. 2).

Inhibition of Smooth Muscle Cell Proliferation

Heparin is known to inhibit proliferation of smooth muscle cells [21]. We examined the ability of sulfated cyclodextrins to inhibit proliferation of human coronary artery and umbilical vein smooth muscle cells and compared it with that of heparin. The inhibitory effects and their concentration dependence were similar for CDS-14 and for heparin, as shown in Fig. 4. The effective magnitudes of concentration required again were similar when expressed in weight rather than molar amounts. The results were similar for both cell lines. The dependence on intramolecular sulfate contents of the cyclodextrin saccharide is shown in Fig. 3B.

Inhibition of Viral Infection

Heparin and dextran sulfate have been observed to protect cells from viral infection [22]. We have reported [9, 17] on the anti-HIV activity of cyclodextrin sulfates. We have extended these studies to: (a) the dependence of anti-viral activity on molecular sulfate density; (b) the effectiveness

TABLE 1. CDS-14 concentrations for inhibition of three virus species

Virus	Target cells	Method	Concentration
HIV-1	Supt T1	Syncytia formation	0.4 ± 0.1 mg/mL
HIV-2	H9	Syncytia formation	0.4 ± 0.1 mg/mL
HTLV-1	Rat XC	Plaque formation	0.9 ± 0.1 mg/mL

for different human retroviruses; and (c) a comparison of the action of CDS-14 with that of dextran sulfate.

The relative effectiveness of the degree of sulfation of cyclodextrin polysulfates for inhibiting HIV-1 infection, obtained from measurements of the degree of syncytia formation (generation of multinucleated giant cells as a result of HIV-mediated infection) is shown in Fig. 3C. Based on ten measurements and a control, the ordinate corresponds to 1/concentration of inhibitory agent at which infection is not detectable.

Table 1 summarizes findings comparing the antiviral activity of CDS-14 for two human immunodeficiency viruses, HIV-1 and HIV-2, and for leukemia HTLV virus. The data indicate the concentrations beyond which no infection was observed: by syncytia formation for HIV-1 and HIV-2, and by plaque formation for infection of rat XC cells by human T cell leukemia virus. Similar inhibitory effects were observed for the three viruses.

A comparison was also made between CDS-14 and dextran sulfate in relation to inhibition of HIV-1 virus infection. Table 2 summarizes the minimum concentrations of the two agents at which viral infection (syncytia formation) was fully inhibited after 1, 2, and 6 days of incubation. Dextran sulfate DS-8000 exhibited a very rapid loss of anti-viral activity compared with CDS-14. This finding is of interest in view of the fact that dextran sulfate has been shown to be active *in vitro* but failed to be useful in human trials [23].

Heparin-Like Dye Complexation

Complexation with Alcian Blue (staining) is used in histology to identify heparinic cell surfaces on tissues. Differentiation from other anionic GAGs is successfully accomplished by determination of the critical concentration of a competing inorganic electrolyte beyond which successful complexation (staining) is inhibited [24, 25]. We

TABLE 2. Comparison (life-time) of CDS-14 and dextran sulfate in *in vitro* anti-HIV activity

Days without infection	CDS-14		DS-8000	
	C_{min} (mg/mL)	Act. loss (%)	C_{min} (mg/mL)	Act. loss (%)
1	0.4		0.16	
2	0.4	0	1.25	87
6	0.8	50	2.4+	94+

TABLE 3. Alcian Blue staining of CD-sulfates and GAGs

Compound (mg/mL)	No stain beyond $MgCl_2$ Molar concentration
This work:	
Heparin (0.8)	>1.0
CDS-14 (0.8)	>1.0
CDS-7 (0.8)	0
CDS-7 (2.4)	0
CD (0.8)	0
GAG staining in histology [24, 25]:	
Hyaluronic acid	0.2
Keratan, dermatan, chondroitan sulfates	>0.3–0.7
Heparin	>0.7–1.0

adopted this technique to compare the staining (complexing) property of CDS-14 with that of heparin. To create a "surface," we impregnated a spot on a thin-layer chromatography plate of alumina with a drop of solution of the agent and allowed it to dry. We then observed the "staining" of that spot as a solution of Alcian Blue dye with various strengths of electrolyte ($MgCl_2$) migrated through and past the spot. Table 3 summarizes the observations, and also compares them to the classical staining experience with various GAGs [24]. CDS-14 stained in the same manner as heparin. No staining was obtained when the cyclodextrin had only seven sulfate substituents (CDS-7). It is very significant that no staining occurred when the CDS-7 concentration was increased 3-fold so as to have the *extra*-molecular sulfate density concentration exceed the *intra*-molecular sulfate density of the active test surface spot.

Complex formation between heparin and another cationic dye, Azure A, is known to produce metachromasia, a shift of the dye's absorption spectrum [18, 26]. It is an accepted characteristic of heparinic material. We used this technique [26] to determine this property for the cyclodextrin-polysulfates with various sulfate contents and for heparin, as shown in Fig. 3D.

Interaction with Proteins: Protamine

In view of the well-known neutralizing interaction of heparin with protamine, we also examined and compared the ability of complex formation of protamine with heparin to that with CDS-14. The method employed was that of "titration" of a protamine solution with increasing amounts of heparin or of CDS-14. We determined the remaining active heparinic agent by spectrophotometric determination of the amount of Azure A that would still undergo metachromasia (see above) after contact with various amounts of protamine (Fig. 5A).

Since the interaction is expected to be a cation/anion complexation reaction, we also examined the "titration" by following the electrical conductivity of the solution in the manner done for other interactions of charged species [27].

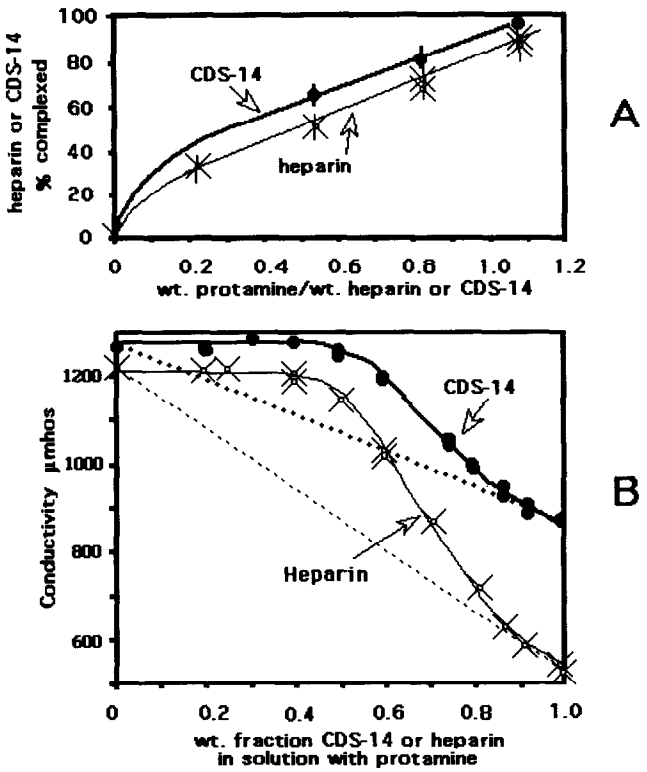

FIG. 5. Complexing between protamine and heparin or CDS-14. (A) The amounts of heparin or CDS-14 complexed upon the addition of protamine. (B) Course of electrical conductivity upon the addition of CDS-14 or heparin to the protamine solution. Straight lines indicate conductivities corresponding to no electrostatic interaction between protamine and the agent. For both measurements, the results of two independent experiments are shown.

Measurements were made using a pure water (conductivity water) solution of 4 mg/mL of protamine with heparin or CDS-14 added (Fig. 5B).

The analogous behavior of both heparin and of CDS-14 vis-à-vis protamine demonstrated the common physical biochemical principle of complex formation for both; it resulted in removal of the individual identity of the interacting species ("titration"); alteration of the free ionic species and their mobilities led to a similar course of electrical conductivity, deviating from the linearity that would result from a non-complexing additive combination of the two components in the solution.

In both demonstrations of similar complexation (Fig. 5, A and B), the concentrations are expressed in weight and not molar quantities. Similarity of action again occurred at the same weight magnitudes, analogous to the effects on cell modulation.

A Model of Cell Surface

We also examined an immobilized *surface* of critically sulfated cyclodextrin as a model of heparin-like species on cell surfaces or extracellular membrane. For this purpose, we used porous β-cyclodextrin polymer particles [28] and

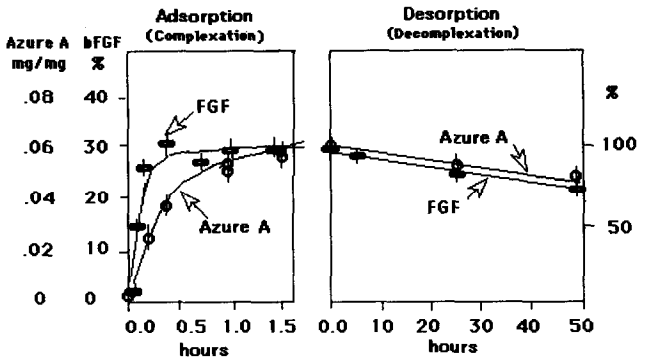

FIG. 6. Surface adsorption and release (reversible complexation) on porous particles of cyclodextrin sulfate polymer CDSP-14 as a model for a heparinic cell surface. Capture (left panel) and release (right panel) of the cationic dye Azure A and of FGF growth factor protein. Data represent the average of three independent series of measurements.

sulfated its internal and external surfaces to about 12–14 sulfate groups per constituent monomer cyclodextrin. This cyclodextrin sulfate polymer (CDSP) was described previously by us and shown to provide a substrate for heparin-affinity chromatography [11].

Just as CDS in solution, the immobilized CDS molecules in this cell model were capable of adsorption-complexation of the polycationic dye Azure A. At room temperature, the porous CDSP was found to adsorb (complex) 0.7 to 0.9 mg of the dye per mg of CDSP, i.e. a quantity close to its own weight. This equilibrium sorption was attained from a solution of 0.1 mg/mL, indicating a volume enrichment by a magnitude of almost 10,000. Figure 6 shows the kinetic behavior, i.e. the adsorption and desorption rates observed, of the dye Azure A. Desorption was observed after the equilibriated polymer suspension was filtered from solution and placed into aqueous solution free of dye.

Surprisingly similar behavior was observed for the ad- and desorption kinetics for the proteinic growth factor bFGF [19]. Both the cationic dye Azure A and the bFGF kinetics are compared side-by-side in Fig. 6. They demonstrated strikingly similar rapid complexation, and very slow desorption into fresh solvent as would be expected from a large but finite complexation equilibrium constant between the heparin/cell surface mimic CDS and the bFGF in the surrounding medium.

DISCUSSION

The designations of heparin, heparan sulfate, and heparan proteoglycans are influenced by historical usage and are not always based on strict compositional knowledge. Heparins proper have sulfate contents higher than "heparan sulfates" and highest among the GAGs [6, 29, 30]. Both heparin or heparan sulfate *per se* may exist on membranes [6, 31] or cause creation of heparan sulfate proteoglycans [6, 32, 33]. In this study, which is concerned with the structurally simplest mimics of heparinic properties, it appears most

useful to focus generally on "heparin-like" moieties or "heparinoids" [34].

The observations of the effect of addition of only sulfate groups in progressively increasing numbers to the small, structurally bare and rigid cyclodextrin sugar molecule (Fig. 3, A–D) led to the conclusion that the primary if not sufficient molecular property involved is a critical minimum *intramolecular anionic charge density*, i.e. the existence of polyanionic *centers*. Furthermore, the same property was indicated to be basic to a wide variety of cell modulation-related behavior. As best seen in the superposition of all the observations of Fig. 3A–D in Fig. 3E, the critical condition for sulfate density was achieved at somewhere near ten sulfate groups per molecule of the cyclodextrin, or *ca.* 1.4 sulfates/glucose unit. We note that for the chain-like glycosaminoglycan saccharides, the heparinic properties were achieved at higher average sulfate ratios, approaching 2. We also note that sulfate groups are indeed forced to closer grouping, i.e. to higher anionic density, in the rigid ring of the cyclodextrin structure. Recognition of multi-anionic *centers* rather than molecules ("electrolytes") as a whole as the activity controlling factors was supported by several related findings and our observations.

The glucose sequence in heparins is heterogeneously sulfated. Typically, the number of sulfate groups per each glucose unit along the chain of heparins is described [2] by sequences such as -1-2-0-1-0-3-1-2-. High density sulfate ("crowded") regions (3-1-2) occur at distances of some 6 to 7 glucose units. This corresponds to the molecular size and weight of the CDS-14 molecule (7 glucose units). Indeed, this is consistent with the observation that upon fragmenting heparin to various smaller sizes, fragment sizes of some six to ten glucose units are seen to be needed for activity in cell binding [35] and protein interaction [36, 37], as well as for their metachromatic activity [18]. (Clearly, some variability in the harvesting of the centers depends on where in the heterogeneous heparin chains the scission occurred in any particular chemical procedure.)

Our findings above of comparable activity at similar *weight* rather than molar concentrations (Figs. 1, 2, and 4) for heparin (mol. wt ≈10,000–20,000) and the small molecule CDS-14 (mol. wt ≈ 2500) is, again, consistent with dependence of the activity on the number of the high density sulfate *centers* rather than on the number of *molecules* (moles). The observed equivalents in weight thus follows from the equivalent moles (numbers) of high density sulfate *centers*. This recognition brings into harmony other historical observations such as the inverse relationship between binding constants versus molecular size of progressively smaller heparin fragments, since that constant is by convention expressed as units per mole of molecules; upon fragmentation, the moles (number of molecules) increase but the number of centers does not have to change.

The importance of viewing heparin and other glycosaminoglycans as "polyelectrolytes" has been proposed and taught for many years [3, 4, 38]. These observations advance the focus of that importance from the molecule as a whole as a polyelectrolyte to the *multi-anionic sites* or centers on the molecules in question. This appears most timely now that the heparin binding regions of proteins are also recognized to be characterized by centers of spatially closely located highly basic Arg and/or Lys residues, i.e. *multi-cation sites* [39]. As regards questions of affinity, reactivity, and selectivity, major attention is thereby drawn to the relevant and relative equilibria for interaction (binding) between multi-cationic and multi-anionic partners.

It is this interaction that is common to the complexing capability of the multi-anionic centers on heparin or the small cyclodextrin sulfates, as seen—again at similar weight concentration—with either the multi-cationic dyes (Alcian Blue, Table 3; Azure A, Fig. 3D); or the bonding with multi-cationic proteins both in media (FGF involved in modulating cell activities, Figs. 1, 2, and 4; protamine in Fig. 5) or FGF on cell surfaces (as modeled in Fig. 6). Activity determining behavior must be thought to reside in the kinetics and equilibria of complex formation between the specific multi-anionic and specific multi-cationic site-bearing partners. This, including the inherent reversibility of equilibria, is demonstrated by the CDS/dye as well as the CDS/FGF model surface behavior study presented above (Fig. 6). The reversibility of associations with "polyelectrolytes" has long been pointed out [40]. It has been the basis for the description of K_d values in many studies.

The multi-ionic center interaction is entirely analogous to ionic bonding leading to salt formation. The products (salts) may be soluble or insoluble. For example, it has already been demonstrated that in the interaction between growth factor proteins with heparin, the complex remains as long as the stoichiometric ratio of ionic centers leaves some non-neutralized ionic sites [41, 42], but precipitation occurs upon neutralization of all sites [41]. Biochemically speaking, we deal essentially with soluble salt formation, and insoluble salt generation in the case of complete charge neutralization.

The conclusion that cell-modulating action can depend on intramolecular anionic density sites such as sulfates alone, without major sensitivity to other compositional detail, is supported by observations in a broad spectrum of specialized cell biological and medical reports involving cell-modulating activities of a wide range of materials. These include greatly varying and sometimes undetermined chemical structures, but they consistently bear a large number of sulfate groups. Examples are suramin [43, 44], pentosan polysulfate [45, 46], ibisterol sulfate [47], sucralfate [48], agents derived from marine algae such as fucoidans [49] or carrageenans [50], and others. We also note the early observation that in the binding to plasma fibronectin "highly sulfated polysaccharides showed potent affinity irrespective of polysaccharide structure" [51].

We are thus led to recognize the importance of a basic and general class of MACADs; they include the glycosaminoglycan of high sulfate density, heparin, but define a broad

class of compositions from complex chain to simple rigid structures such as the cyclodextrin polysulfates. In stark contrast to the dominating experience with proteins of the critical selectivity and importance of great structural detail, the biological activities of this class of molecular agents do not depend on their own total structural detail. Instead, their modulating influence is exerted by ionic complex formation with proteins which for their part introduce the structural selectivity of action.

While the central cell-modulating property of this class appears to be insensitive to other structural elements, these can, of course, provide additional desirable or undesirable functions that may affect toxicity, absorption into the plasma, pharmacokinetics, and other biologically and medically relevant additional parameters. The observations on antiviral activity (Table 2) provide an example of an ancillary but not trivial effect of other structural elements: cyclodextrin sulfate is seen to have a multifold larger life in the biological system than the straight chain dextran sulfate. This is consistent with the chemical knowledge [52] that acid or enzymatic attack on saccharides is preferentially directed to the terminal glycosidic bond that is absent in the cyclic structure. This example of ancillary structural properties is noteworthy, in view of past unfavorable *in vivo* experience with dextran sulfate [23] following encouraging *in vitro* observations of HIV inhibition.

There appears to exist a universal role of the complexing reactions between MACADs, including heparinoids, and multi-cationic proteinic partners. It reminds one of the fact that, in any cell-biological system, the prevalence or suppression of various actions must be determined by the relative equilibrium constants and prevailing relative concentrations and locations of the existing species capable of seeking complexation, of proteinic moieties (cytokines, growth factors, enzymes) on one hand, and MACADs including glycosaminoglycans on the other hand, as well as any therapeutic agents that may be added. Therefore, there is need to focus on the basic biochemistry of binding of multi-anion/multi-cation sites, and on systematic exploration of the magnitudes of equilibrium constants among these species; many of these partners are currently known only by name and recognized effects. This would serve basic cell biochemical understanding and can become an important ingredient in rational design of pharmaceutical agents and appraisal of therapeutic potential.

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