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Band, Philip A.

**THE CATALYTIC INFLUENCE OF HEPARIN ON AURAMINE O
HYDROLYSIS: A COMPARATIVE STUDY OF THE POLYELECTROLYTE
PROPERTIES OF HEPARIN AND OTHER GLYCOSAMINOGLYCANS**

City University of New York

PH.D. 1982

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HEPARIN AND OTHER GLYCOSAMINOGLYCANS.

by

PHILIP BAND

A dissertation submitted to the Graduate Faculty
in Biochemistry in partial fulfillment of the
requirements for the degree of Doctor of Philosophy,
The City University of New York.

1982

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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

THE CATALYTIC INFLUENCE OF HEPARIN ON AURAMINE O HYDROLYSIS:
A COMPARATIVE STUDY OF THE POLYELECTROLYTE PROPERTIES OF
HEPARIN AND OTHER GLYCOSAMINOGLYCANS.

by

Philip Band

Adviser: Professor Aaron Lukton

A new approach has been developed to investigate the microenvironment of glycosaminoglycans, and in particular, heparin. It is based on the use of the cationic dye Auramine O as an extrinsic probe; but rather than deriving information from changes in spectral or chiroptical properties, the catalytic influence of heparin on the dye's hydrolysis is exploited. In this regard, heparin is unique among the glycosaminoglycans of animal connective tissue, and therefore the reaction can serve as the basis for a specific heparin assay. The variation of reaction rate with polymer, dye and hydronium ion concentration, ionic strength, and various ratios of these parameters are all consistent with an interpretation of the catalysis in terms of the well known catalytic properties of polyelectrolytes. It is concluded that the catalysis is a consequence of counterion condensation, as strictly defined in Manning's limiting laws of polyelectrolyte solutions. Most significantly, the data demonstrate the participation of hydronium ion in this process, and in the differentiation of heparin from other glycosaminoglycans. Though the rate enhancements cannot be

dealt with quantitatively by a Bronsted-Bjerrum-Manning type approach, the data can be straightforwardly explained based on the concept of a critical charge density, as described in Manning's limiting laws. The explanation is offered in terms of Manning's linear charge density parameter, ξ , and the all or none aspect of counterion condensation. Based on the relative catalytic activity of different heparin preparations and subfractions, the implications of this work with regard to the unique biochemical properties of heparin are examined.

Acknowledgements

It is with great affection that I would here like to thank my wife and parents for the many ways in which they helped me in this endeavor. Without their love, faith and understanding this work might never have been completed.

I would also like to acknowledge the help and cooperation which I have always received from my sponsor, Professor Aaron Lukton. Throughout the years I spent in his laboratory, his advice and suggestions enhanced my personal and scientific development.

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List of Abbreviations

A -	Absorbance
AuO -	Auramine O (monoprotonated form)
AuO ²⁺ -	diprotonated AuO
AuO ³⁺ -	triprotonated AuO
ξ -	linear charge density parameter
Σ -	extinction coefficient
Σ_{app} -	apparent extinction coefficient
f -	The fraction of a polyelectrolytes structural charge not compensated for by bound counterions
GAG -	glycosaminoglycan
MK -	Michler's Ketone
MPS -	mucopolysaccharide
P/D -	polymer site to dye concentration ratio
PE -	polyelectrolyte
PG -	proteoglycan
Rc/Ro -	catalytic rate enhancement - ratio of the rates of the catalyzed and non-catalyzed reactions.
Zi -	valence of counterions
Zp -	valence of charged groups on polyelectrolyte backbone.

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I. Background

A. Introduction

In recent years, interest in the biochemical properties of mucopolysaccharides (MPS) has been steadily increasing. Originally considered to be merely the amorphous constituents of connective tissue ground substance, they have now been implicated as functionally important in a wide range of physiological processes, e.g. wound healing (1), tissue remodeling (2), filtration properties of basement membranes (3), and cellular developmental phenomena (4,5). Alterations in MPS distribution patterns accompany a number of pathological conditions. The progression of rheumatoid arthritis can be quantitated by measuring MPS levels in the inflamed tissue (6). Correlations between malignancy and tissue MPS content have been observed by several investigators (7,8). In mucopolysaccharidoses, hereditary disorders of MPS catabolism, MPS accumulation parallels the presence of mental retardation (9). It is thus no longer tenable to relegate these biopolymers to a simple structural role.

The unifying chemical characteristic of the MPS is that they are all linear anionic polyelectrolytes. As such, the voluminous literature regarding polyelectrolyte influences on physiochemical phenomena in solution should represent a warehouse of information upon which to establish a mechanistic basis for understanding the function of their biological counterparts. It is towards this end that this dissertation is addressed.

More specifically, attention has been focused on the ability of polyelectrolytes to catalyze ionic reactions in solution (10). This has been an important area of polymer chemistry for many years, and a large body of experimental data is available for comparison to new systems. The various mechanisms involved in such catalyses have been elucidated amply enough to enable straightforward application of the underlying principles. Thus development of a model system demonstrating catalysis by MPS will provide a new tool for studying the physicochemical properties of these biopolymers.

The use of extrinsic probes to deduce biochemical information is a well established approach in molecular biology. Fluorescent probes of proteins and polynucleotides have long been a valuable source of specific and detailed information of macromolecular microenvironment (11,12). In the case of MPS, extrinsic chromophores have yielded biochemical information via the observation of effects such as metachromasia and induced circular dichroism (13). Yet these methods have limitations with respect to the details of the structural information they yield. The study of MPS catalytic influences represents a new tool aimed toward characterizing the microenvironment of these macromolecules. The type of information extracted is different and complementary to that obtained by spectroscopic means. Dynamic, rather than static equilibrium factors, influence the catalytic properties observed in a particular system. Thus studies in this area represent an

untapped source of data regarding the chemical influences of MPS on species brought within their electrostatic domain.

The structural variations among the MPS must provide the chemical source for their specific abilities in biological systems. As always, the essential chemical link to be established is that between function and structure. In the case of MPS, this link has proven elusive (15). The small differences between the classes of MPS are overshadowed by their sharp delineation from other biopolymers. Yet this fine structural variation must be the source of their specific biological activities. There is a pressing need to understand this relationship, both for its theoretical interest, and its potential for clinical application. Heparin represents a prime example. This substance is an extremely potent mediator of biological phenomena in a highly specific fashion- no other MPS can duplicate its effects. Yet much evidence implies that heparin's biological influences are a physical consequence of its polyelectrolyte nature (15). The study of catalysis by MPS should provide information regarding how small structural variations within a biological polyelectrolyte can modulate these general polyelectrolyte properties, to the point where the specificity so characteristic of biochemical systems emerges.

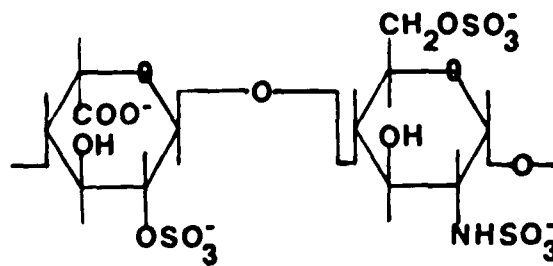
B. Mucopolysaccharide Chemistry and Biology

1. Structure

The nomenclature of this important class of biopolymers has become somewhat muddled over the last several years, owing to the usage of different terminology by different investigators. I shall use the nomenclature recently suggested by Jaques (16). The term mucopolysaccharide (MPS) is taken as an all encompassing term, denoting any anionic polyelectrolyte of biological or medical interest, occurring in or influencing the properties of connective tissue. The term glycosaminoglycan (GAG) is thus reserved for structures consisting only of copolymers of an amino sugar with another monosaccharide moiety; they may or may not be poly-sulfated. The term proteoglycan (PG) retains its original meaning, namely describing the covalent adducts formed between GAG and protein.

The GAG are linear chain polysaccharides essentially composed of repeating disaccharide units, to form an alternating copolymer of molecular weight generally ranging from 10,000 to 50,000 (14). The disaccharide units are composed of an amino sugar and a hexuronic acid moiety, except in the case of keratin sulfate, which is made up of an amino sugar and the simple sugar galactose. The predominant disaccharides of the various GAG's are shown in Figure 1. All are polyelectrolyte in nature, due to the presence of sulfate and/or carboxylate groups. The sulfate groups are added on to the polymer in the course of the extensive post-synthetic modifications (14).

Heparin



Dermatan sulfate

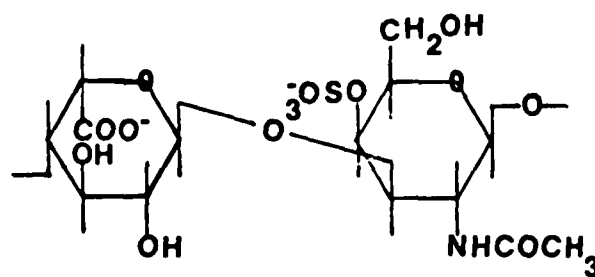
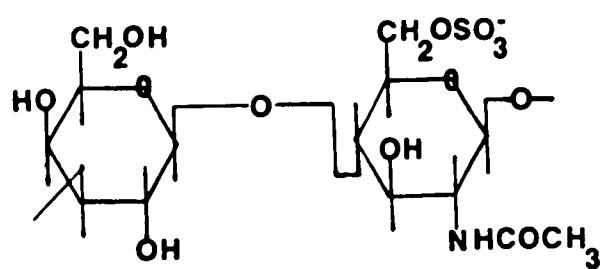


Figure 1: The predominant disaccharide repeating unit of connective tissue glycosaminoglycans.

Keratan sulfate



Heparan sulfate

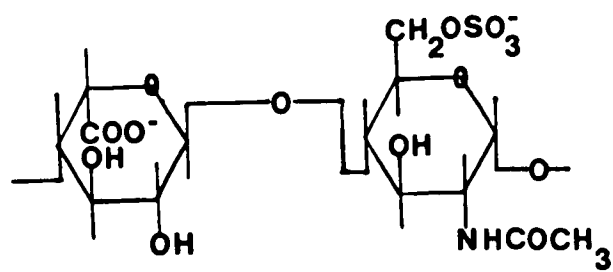
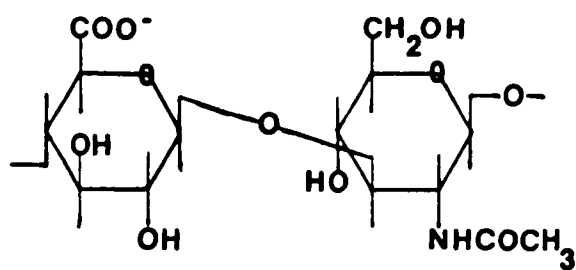
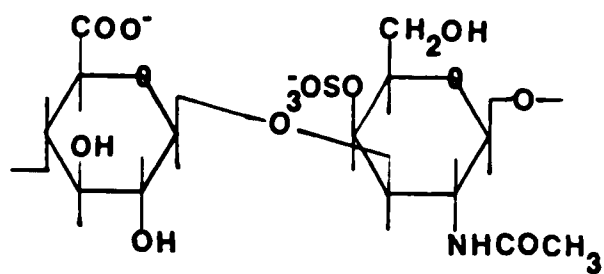


Figure 1 Cont.

Hyaluronate



Chondroitin-4-sulfate



Chondroitin-6-sulfate

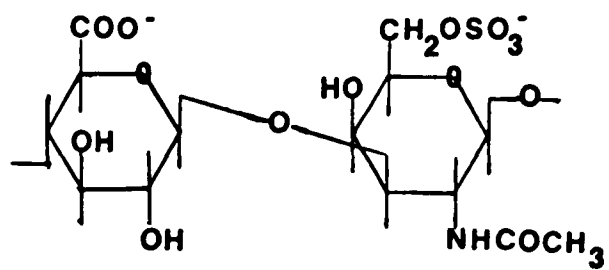


Figure 1 Cont.

The above statements are an oversimplification of the true structures of GAG. Hyaluronic acid appears to truly be a simple repeating copolymer along the entire length of the polymer chain. On the other hand, the dermatan and heparin species contain several different disaccharide units, the arrangement of which are still poorly defined. This leads to an important point which must be brought up in any discussion of GAG structure. As isolated from the tissue, the GAG are not homogeneous in nature (17). That is, unlike the situation with respect to proteins, a given preparation does not consist of one unique molecular species, but rather what appears to be a continuum of closely related species. This phenomena, which has been termed microheterogeneity, largely results from the variability in the extent of post-synthetic modification and breakdown of the basic GAG structure (14). Although early studies implied that this heterogeneity was random in nature, recent demonstrations of specific saccharide binding sequences with distinct functional roles (18) have led to an increased interest in the non-random aspects of the post-synthetic modification reactions, and to the development of procedures enabling the sequencing of these polymers (19,20).

Generally GAG do not occur in tissue as the free polysaccharide, but are rather covalently bound to protein to form PG. The general structure of PG have recently been worked out and reviewed (21). They typically consist of core proteins of molecular weight about

200,000, to which 100-200 GAG chains are covalently linked. The GAG are attached to the protein core in clusters, with an average of four chains per cluster. The resulting structure has the appearance of a bottle brush, with the GAG chains representing the bristles. GAG are linked to the protein core via their reducing termini, which contain a sugar sequence atypical of their copolymeric nature. Most commonly the sugar sequence -Gal-(1-3)-Gal-(1-4)-Xyl-O-Protein is observed. The amino acids serine, threonine and glutamic acid have been demonstrated in the protein half of this linkage. The resulting GAG-core protein adduct, which has been termed the PG subunit, has a molecular weight of about 4 million. Such PG subunits have been isolated from cartilage, and shown to consist of a disperse population of molecules in terms of molecular weight and GAG content, again stressing the heterogeneous nature of this class of biopolymer. The core protein from cartilage PG has been extensively studied, and is believed to contain three functionally distinct regions, differing markedly in amino acid composition (22). These are - 1) a main GAG attachment region, rich in chondroitin sulfate; 2) a region containing only keratin sulfate bound by alkalai labile bonds; and 3) a globular region containing a noncovalent binding site for hyaluronic acid. Binding of hyaluronic acid to core protein is stabilized by two low molecular weight link proteins, to generate a higher order of PG structure- termed the PG aggregate. This PG aggregate, consisting of a long extended

molecule of hyaluronic acid from which PG subunits extend laterally at fairly regular intervals, has been demonstrated in an impressive electron micrograph (22).

2. Conformation

The conformational properties of connective tissue polysaccharides have been studied by several techniques. An important source of data has been X-ray diffraction studies of MPS dry spun into fibers, and cast onto films (23). These studies clearly demonstrate that helical symmetry can exist for all these macromolecules. The exact nature of the helices is more in question, in that each polysaccharide may take up different symmetries depending on the experimental conditions. A 2_1 helix seems to be most prevalent. The glycosidic linkage is apparently the major conformational determinant, though substituents also play a role. When the diffraction pattern of rat chondrosarcoma PG was examined, an important point was demonstrated. It was found to be indistinguishable from that of chondroitin-4-sulfate, its major constituent, indicating a conservation of GAG conformation in PG structures.

The applicability of these studies to the conformations of MPS in physiological solutions, is questionable. Early investigations demonstrated these macromolecules to behave as typical polyelectrolytes in that they assumed extended random coil

configurations in aqueous solution, occupying a large solvent filled domain (21). Electrolyte influences on the solution properties of GAG are also typical of random coil polyelectrolytes (25).

Decreasing the ionic strength results in a molecular expansion which can be demonstrated by either viscosity or light scattering techniques. However recent NMR evidence has confirmed the existence of local ordering in GAG solutions (26,27). This does not totally invalidate the earlier concept of GAG conformation in solution, since a polyelectrolyte with local order can still behave as a random coil to a first approximation, and the X-ray diffraction results may just be a manifestation of such localized ordering.

All of the GAG display circular dichroism (CD) spectra in the far ultraviolet (below 230 nm) region of the spectrum. These spectra can be classed into two groups of optical conformation, which correlate with a GAG class distinction based on primary chain structure (28). The first class consists of heparin, heparan sulfate and keratin sulfate, all of which have their amino-sugar glycosidically linked at the 4 position, and display CD spectra with a negative band at 210nm and a positive band at 190nm. The second class consists of dermatan sulfate, hyaluronate and the chondroitin sulfates, which have their amino-sugar linked at the 3 position, and display a CD spectrum characterized by two negative bands, the first at 208 nm, and the second poorly resolved, but below 190 nm. The idea that these two optical classes relate to conformational classes

of the MPS is supported by X-ray data- the crystalline salt forms of heparin, heparan sulfate and keratin sulfate (the 1-3 linked group) show preference for two-fold helices in the solid state, whereas the chondroitins, dermatan and hyaluronate show a preference for 3-fold helices in the crystalline state (23). The observation that GAG can induce an extrinsic cotton effect in several cationic dyes is further evidence that the helical structure deduced from diffraction studies may pertain to the conformation of these molecules in solution (28) (see section I C₃). Unfortunately a clear consensus as to the solution conformation of connective tissue polysaccharides has not yet emerged.

3. Biological Functions

The best known, and most clearly understood, functions of MPS relate to their role in generating both the macro- and microscopic properties of connective tissue. Similar compounds occur as extracellular materials for plants, algae, fungi, and even bacteria to an extent. From an evolutionary viewpoint, it has even been suggested that the emergence of these type polysaccharides corresponded to the emergence of tissue organized life forms (8).

As the major filling material in extracellular matrix, MPS are important determinants of connective tissue functions. These include water and ion retention, viscoelastic shock absorption, lubrication, exclusion and filtration effects, among others (24).

To a large extent these properties are a general consequence of their flexible polyanionic nature. The electrostatic repulsion that forces these molecules into extended coils also favors their occupation of large solvent filled domains. Counterion retention further reinforces this maximal extent of solvation. The resulting osmotic pressure creates turgor, which is necessary to keep the tissue in a hydrated state. The hydrodynamic volumes of hyaluronate and cartilage PG are respectively, 1000 and 100 times their dry volume (21).

This gel-like network provides the substratum necessary for the livelihood of the individual cells within a multicellular organism. The entangled polymer chains act as a selective filter to other molecules. Physiologically significant exclusion effects have been demonstrated. A 5% hyaluronate solution excludes 75% of the solution space for albumin, and 85% for a larger protein like IgG (29). This contributes to the observed steady state distribution for plasma proteins between the blood and extracellular fluid. Further consequences of these properties are apparent in MPS influences on thermodynamic functions, such as activity coefficients, equilibrium constants and solubilities. The physiological and pathological manifestations of these effects are seen in processes such as fiber formation, immune complex precipitation and lipid deposition (14).

Particular attributes of different connective tissues may also be traced to their MPS content. Variations in MPS distribution demonstrate a tissue, rather than a species, specificity (8). This implies that the physical requirements of tissues with different functions (e.g. heart and liver) are at least partly determined via modulation of their MPS content. Influences on cell differentiation and developmental phenomena have also been reported (30,31). Research in this area will ultimately lead to an understanding of how the mutual influences of cells and matrix on each other are involved in maintaining the dynamic steady state that characterizes normal healthy tissue, and is broken down in pathological conditions such as fibrosis and malignancy.

In addition to the generalized tissue functions of MPS, there are several examples of physiological MPS influences on specific biological systems. A cervical GAG accelerates the activation of boar sperm proacrosin (32). MPS in the follicular fluid have been postulated to play a role in oocyte maturation (33). GAG influences on pepsinogen activation have been postulated to play a role in the etiology of peptic ulcers (34). In the adenylyl cyclase system, enzyme-receptor coupling is subject to GAG modulation (35). This is especially interesting in light of the ubiquitous occurrence of GAG in the cell coat of mammalian cells in culture (36), and implies a possible involvement of GAG in c-AMP mediated hormonal or neurotransmitter responses.

Heparin is perhaps the best example of a GAG with specific biochemical properties that extend beyond a structural role in the extracellular matrix. The best known of these properties relate to the pharmacological applications of this compound as a therapeutic agent. It is the drug of choice for the prophylactic treatment of thromboembolic disorders (37), being the most potent of the anticoagulants known to medical science, and possessing a very low level of toxicity. The anticoagulant action is always accompanied by an antilipemic effect, caused by a heparin induced release of lipoprotein lipase from liver and vascular endothelium (38). As such heparin influences the lipoprotein distribution of plasma (39), which may in turn control lipid deposition and formation of atherosclerotic lesions (40). Direct interactions between serum lipoproteins and MPS, with precipitation occurring in the presence of divalent metals, have also been implicated as etiological factors in atherosclerosis (40,41).

In addition to these two best known functions of heparin, several lesser known properties will serve to demonstrate how truly far-reaching the clinical possibilities for MPS extend. Heparin administration stimulates the release of the enzyme diamine oxidase in a number of species, including man (42). This enzyme plays a critical role in the response of the organism to shock, and the therapeutic application of heparin for the shock syndrome following thermal injury has been suggested (43). This application of heparin

is further complemented by its strong tendency to bind biogenic amines, such as histamine, thereby acting as a detoxifying agent (44). The inhibitory influence of heparin on leukocyte chemotactic factors (45); coupled with its ability to cause a suspension of leukocytes to form a gel (46), suggest a possible role in localization of inflammatory responses. This viewpoint is further supported by reports of heparin's antimicrobial activity (47), its influences on the complement system (48), and its conspicuous presence in highest concentration in lymphoid tissue (49).

In summary, MPS in general, and heparin in particular, have been rapidly emerging as important modulators of physiological phenomena, extending far beyond the original viewpoint that they are merely structural components of tissue. Though the literature cited is far from a complete review of the tremendous activity in this area of biomedical research, it demonstrates the far ranging functional potential of MPS. The hope here has been to generate in the reader the feeling that the diverse biological properties of MPS merit a strong research effort to more fully understand their actions in physicochemical terms.

C. Physicochemical Properties of Polyelectrolytes in solution.

Polyelectrolytes (PE) are macromolecules which have ionized groups covalently bonded along a more or less flexible polymeric backbone. Unlike neutral polymers, they are generally quite soluble in aqueous solution. From a biological standpoint this fact is reflected in the PE nature of the major functional biopolymers- the nucleic acids, proteins and MPS. The interchain repulsion due to the presence of like ionic charges along the polymeric backbone keeps the macromolecule in a fluid enough state to participate in life processes. Complexes between polyions of opposite charge do not generally form ordinary solid precipitates, but rather complex coacervates, which can be thought of as liquid precipitates (50). This concept is significant when one realizes the prevalence of such semi-solid structures as functional units of cells- e.g. the protein-RNA complexes of ribosomes, or the protein-DNA complexes in chromatin.

1. Counterion Condensation and the Manning Limiting Laws

The most striking property of polyelectrolytes is their tendency to condense counterions into their electrostatic domain. The forces underlying this phenomena are the same electrostatic forces which in the Debye-Huckel treatment of simple electrolyte solutions leads to the statistical ionic atmosphere around any ion; however in the case

of PE, this tendency is so strong that PE salts behave as weak rather than strong electrolytes in aqueous solution. The experimental evidence for this degree of counterion binding is overwhelmingly strong. Diffusion measurements using radioactive tracers demonstrate that the counterions in a PE solution have a diffusion coefficient approaching that of the macromolecule with which they are associated (51). The magnetic spin resonance signals of ions in polyelectrolyte solutions differ from that of the ion in free aqueous solution (52). Colligative properties (53) and electrical conductivities (54) of PE solutions are also indicative of a high degree of ion-polyion association.

The behavior of simple electrolyte solutions can be quantitatively described by the Debye-Huckel limiting laws. These are derived from the Poisson-Boltzman equation for a spherically symmetrical system, which can only be solved by making the linear approximation that $Z_i\psi/kT \ll 1$, which is valid for simple salts. However, in the case of PE solutions, the approximation is not valid, because the electrostatic potential (ψ) for polyions is large (compared to kT , the thermal energy). Furthermore, there is the additional complication that polyion expansion and counterion condensation are interdependent (55). Early attempts to quantitatively describe PE solutions were largely based on solving the Poisson-Boltzman equation for the cylindrically symmetrical case, which can be solved without a linear approximation. Despite

the limited applicability of this description (it could not be applied to PE solutions containing added simple salts), it still led to the striking conclusion that polyions could not sustain a charge density exceeding a critical value. When the structural charge on the polyion exceeded this critical value, counterion condensation inevitably resulted. In fact this tendency is so strong, that even at infinite dilution in pure aqueous solution, there is enough of a reservoir of hydronium and hydroxyl ions from the autoionization of water, to promote charge neutralization by counterion condensation (55). Qualitatively, this phenomena can be thought of as arising from the need of a minimal electrostatic potential to overcome the normal randomization of ionic distribution arising from thermal and entropic forces, and to generate a microenvironment within which counterion condensation can occur. It should be noted here that the term counterion condensation is meant to imply a much firmer type of ionic association than the diffuse statistical ionic atmosphere referred to in Debye-Huckel theory.

Based on this principle, Manning has presented a theoretical description of counterion condensation which gives good quantitative agreement with much of the experimental data regarding these phenomena (56,57). The equations derived can be considered as limiting laws, in the sense that they are derived based on a Debye-Huckel expression for the electrostatic potential affecting small ions, and thus are valid only at ionic strength values

sufficiently low for the Debye-Huckel approximation to be valid (with respect to the mobile counterions). The major innovation in Manning's treatment is that the PE is conceived of as an infinite line of uniform charge density. Though the assumptions and methodology of the derivation are still somewhat controversial (55), the final simple statements that the theory is reduced to merit review, as they provide an accurate empirical representation of the properties of PE solutions. In two brief statements the theory enables calculation of the fraction of a polyion's structural charge not compensated for by bound counterion (designated f). This calculation is based on definition of the parameter ξ ; a dimensionless measure of linear charge density:

$$\xi = \frac{q^2}{\epsilon k T b}$$

where q is the protonic charge, ϵ the bulk dielectric constant, k the Boltzman constant, T the absolute temperature, and b the average spacing between charged groups. In a solution containing a PE of charge density ξ and counterions of valence Z_1 :

$$\begin{aligned} f &= |Z_1 \xi|^{-1} & \text{when } \xi > |Z_1 Z_p|^{-1} \\ f &= 1 & \text{when } \xi < |Z_1 Z_p|^{-1} \end{aligned}$$

Thus counterion condensation will only occur in a polyion-counterion system when the ξ parameter of the PE is greater than 1 for a system with univalent counterions, greater than 0.5 for a system with divalent counterions, etc. This amounts to the statement that in a solution containing PE and added counterions, the minimal polyion charge density promoting counterion condensation corresponds to approximately one ionic charge per $7\text{\AA}$ of the rodlike polyion. Below this value, the process now defined as counterion condensation does not occur ($f = 1$). When the polyions charge density is greater than this value condensation will occur, and always to the extent necessary to reduce ξ to the same net value ($\xi_{\text{net}} = 1/Z_1$), independent of the structural charge density on the PE, and dependent only on the valence of the counterions in the system. Furthermore, the theory predicts that the extent of counterion condensation is independent of ionic strength, and thus cannot be treated by a conventional mass action formulation (58). Rather the situation is such that counterion condensation behaves as though it were an all or none, concentration independent phenomenon.

Within the context of this theory, only long range electrostatic interactions are taken into account, and no provisions are made for specific polyion-counterion interactions. The counterion condensation effect is not a physical binding in the ordinary sense of site-ligand interaction; rather, it is a "statistical thermodynamic binding", somewhat akin to the diffuse atmospheric

type of binding referred to in the classical Debye-Huckel theory. Karenzi et al have described two distinct modes of ion-polyion association- site binding and territorial binding (59). Site bound counterions are those which are in direct contact with charged groups on the polyelectrolyte, with no intervening water molecules; they are thus truly physically bound in the usual sense of the word. Territorially bound counterions are those which are merely localized in the ionic atmosphere of the polyelectrolyte, but still retain their inner hydration spheres and freedom of motion; they are statistically localized within the polyion's domain by electrostatic forces, but have almost complete freedom of motion. Though the Manning theory does not explicitly differentiate between these two modes of binding, it is implicit from the derivation that the territorial mode of binding is that which the theory is primarily meant to account for. Site bound counterions are dealt with in terms of their contribution to the PE's structural charge density (i.e. before counterion condensation takes place) (58).

2. Polyelectrolyte influences on ionic reactions in solution.

The ability of PE to influence the rates of ionic reactions in solution has been known for almost thirty years (55). From the wide variety of systems studied, the following generalizations can be made (55,57). Reactions between positively charged ions are accelerated by polyanions; reactions between negatively charged ions are accelerated by polycations; reactions between oppositely charged ions are hindered by both anionic and cationic PE. These rules are not steadfast, and the systems wherein exceptions occur reflect the fact that many types of forces may contribute to the observed kinetic influences. Localized concentration of reactants, kinetic salt effects, hydrophobic interactions, medium effects and altered solvation structures have all been shown to play a role in the phenomenon (60-64). No one of these forces can be claimed as foremost in all systems, and the relative contribution of each must be assessed for individual cases.

Though the use of the term catalytic when discussing PE rate enhancements has gained widespread acceptance, it is not strictly correct (60). The classical Ostwald definition of a catalyst stipulates that both the forward and reverse rate constants must be equally influenced, so that there is no overall change in the equilibrium constant. This is generally not the case for polyelectrolyte catalysts, as the reverse reaction usually involves ions of different charges than the forward reaction, and hence the

PE influence will be different. Despite the demonstration of PE induced equilibrium changes in several systems (57,60), the term catalysis has remained the predominant descriptive term used by investigators in this field, and will therefore be thus loosely used here.

Morowetz and his collaborators were among the first to extensively study this phenomena, and the "accumulation effect model" (55) upon which their data is interpreted represents the simplest qualitative context within which to understand the aforementioned generalizations. They found that polyanions inhibited the hydroxyl ion catalyzed hydrolysis of cationic esters (61), while conversely accelerating by as much as 10^5 -fold the reduction of $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$ by Fe^{2+} (62), and the Hg^{2+} induced aquation of $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$ (63). From the viewpoint of counterion condensation, this is precisely the result to be expected. Reactant ions of the same sign will be concentrated into the electrostatic domain of an oppositely charged polyion, wherein their collision frequency will increase and hence their reaction become accelerated. When the reactant ions are of opposite sign, only one will be concentrated into the PE domain, thereby effecting a spatial separation of the reacting species, and thus a reduced reaction rate.

This simplistic interpretation, based only on the accumulation effect, was found by Morowetz et al. to be consistent with many of

their observations on how experimental conditions influenced the polyvinylsulfonate enhancement of $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$ reduction and aquation (62,63). Insofar as counterion condensation is a ubiquitous property of PE, these observations may serve as suitable criteria for identifying PE catalysis, even in systems where other forces have a pronounced kinetic influence. Furthermore, these principles provide a satisfying heuristic model upon which experimental data can be evaluated. They are: 1) Addition of non-reacting counterions will cause a precipitous drop in the rate acceleration. Though this effect was originally interpreted as due to increased shielding of the polyion's fixed charges resulting in less of a tendency to concentrate reactant ions, in more recent terminology it is simply an example of competition between the various counterions in solution for the condensation effect; according to Manning's limiting laws, condensation only proceeds until the limiting charge density, $\xi_{\text{net}} = |Z_i Z_p|^{-1}$ is reached (56). 2) Plotting the catalytic rate enhancement vs the PE concentration gives a curve with a distinct maximum, rather than the hyperbolic curve displayed in other types of catalytic systems. This dependence can be accounted for in terms of how the ratio of PE sites to reactant ions influences local reactant concentration. When the reactant ions are in large excess, the distribution of reactants within the PE domain is independent of PE concentration, and the rate increases in proportion to the fraction of reactant

ions localized within this domain. But as PE is added beyond the point where essentially all of the reactant ions are thus localized the rate decreases, due to reactant ions becoming "diluted", as they distribute within a more extensive polymer domain. 3) The influence of reactant concentration will also follow a biphasic pattern. As the concentration of either of the reactants is singly increased, the rate at first increases as is typical for any reaction, but then a maximum is reached, followed by a sharp decrease in rate with further elevation of the reactant concentration. This is in sharp contrast to the influence of reactant concentration on reactions occurring free in solution; and the steepness of the rate decline at high reactant concentration is much greater than that observed in other catalytic systems where it is due to product inhibition. It is however consistent with an interpretation based on local concentration, since as one of the cationic reactants increases relative to the other, it will tend to displace that reactant from the polymer domain, thereby preventing the simultaneous increase in the local concentration of both reacting ions, which is necessary for the rate enhancement to occur.

Okubo and Ise have attempted to quantitatively deal with PE catalysis as an extension of the theoretical treatment of simple salt influences on ionic reactions by applying Manning's limiting laws to the Bronsted-Bjerrum treatment of kinetic salt effects (57,60,64). In the classical primary salt effect, a reaction

between ions of the same sign is accelerated by increasing the ionic strength, whereas a reaction between oppositely charged ions is decreased by increasing the ionic strength (65). These rate influences can be both qualitatively and quantitatively accounted for in terms of activated complex theory (65). Qualitatively, for a reaction between ions of the same sign, the transition state can be expected to be of higher electrostatic potential than the reactants, and will thus be stabilized by increasing the ionic strength; the reverse argument would hold for a reaction between ions of opposite sign. Quantitatively, the ionic strength influence on the rate can be calculated by substituting the Debye-Huckel equation for the activity coefficient term in the Bronsted-Bjerrum equation. In order to quantitatively evaluate the PE influence on reaction rate in terms of activated complex theory, rather than using the Debye-Huckel expression for activity coefficients, Okubo and Ise used an expression based on Manning's limiting laws (56,59). The kinetic equation resulting depends on the charge type of the reaction, and requires assumptions to be made regarding the nature (i.e. free or condensed) of the ionic species involved in the reaction (57,60,64). Though several systems have displayed impressive agreement between the calculated and observed values for the rate enhancement, the applicability of this approach is far from universal, as the Manning theory upon which it is based takes only electrostatic effects into account.

Though both of the above explanations of PE catalysis have served quite well as heuristic models, there are no doubt other factors important to the catalytic activities of PE. Even the major proponents of the "accumulation effect" and "primary salt effect" theories acknowledge that neither viewpoint is adequate in most cases to fully account for the observed rate enhancements. Morowetz et al (62) calculated the extent of local concentration from equilibrium dialysis data and concluded that other factors must also be operative in the activation process. Strong candidates for these other forces include: influences on reactant solvation, hydrophobic forces and steric factors (66-69). Polystyrene sulfonate can even catalyze the acid hydrolysis of neutral acetate esters, if the chain length of the ester is long enough to promote hydrophobic association between the polyion and the ester (67). In fact hydrophobic forces can sometimes be so important that they can result in PE catalysis of a reaction between ions of opposite charge. An example of this is the acceleration of the alkaline hydrolysis of cationic triphenylmethane dyes by cationic polyelectrolytes, such as poly(4-vinylpyridine) quaternized with bulky alkyl or benzyl halides (68). Hydrophobic forces are evidently strong enough to overcome the electrostatic repulsion between the organic cation and the polycation. Thus localized the dyes are subject to the elevated hydroxide ion concentration in the PE domain, and thus their rate of hydrolysis is accelerated. Other

more specific forces, such as direct nucleophilic attack by groups on the polyelectrolyte have also been demonstrated (69), such as in the influence of polyethylene imine on the decomposition of aspirin. However diverting toward systems demonstrating specificity of this sort only tends to distract from a discussion of the generalized forces always operative in systems displaying PE catalysis.

The implicit aim generally set forth by biochemists studying PE catalysis is to mimic and understand the ways in which enzymes, the quintessential biological catalysts, function. The aim of this dissertation comes from a different angle. It is not meant to assert that MPS will displace proteins from their role as the primary biological catalysts. It is, however, desired to express the idea that some of the ways in which PE influence ionic reactions bear relevance to the mechanism by which MPS exert their biological functions. In the course of biochemical evolution, a phenomenon which results from so fundamental a property of PE as counterion condensation, must have developed functional manifestations.

3. Polyelectrolyte influences on the spectral properties of dyes.

The color changes occurring upon the binding of biological stains to the different molecular components of cells and tissues have been known for over a century. These color changes, termed metachromasia, served as early tools for the microscopic

identification of tissue constituents. A great deal of progress has been made in understanding the early histological observations based on systematic studies of dye binding to biopolymers in solution. Yet the complicated forces underlying this binding process and the resultant spectral perturbation still remain a subject of continuing research. Despite the complexities, many valuable insights have been drawn from these studies regarding the generalized aspects of macromolecule-ligand interactions.

The discussion here will be limited to the metachromatic shifts induced in cationic dyes by anionic polysaccharides. Bradley and Stone (70,71) have conducted an extensive study of this phenomenon using cationic phenazine, phenothiazine and acridine dyes, with a wide range of synthetic and biological polyanions. In all of these systems, upon dye binding to the PE (termed a chromotrope in this context), there is a decrease in absorptivity at the free dye's absorption maximum (hypochromism), with a concurrent blue shift in the spectrum of the bound chromophore (bathochromic shift). Similar spectral changes had been observed in simple dye solutions at very high concentrations, where aggregation was known to occur (72). A likely parallel being drawn at the time was that metachromasia simply resulted from aggregation of the cationic dyes onto a polyanionic template.

To examine this possibility, Bradley and Stone compared the relative metachromacy of the different dye-polyanion pairs in

quantitative terms (71). They looked for correlations between structural features of the polyanion and the resultant metachromatic spectra. The polyanions were compared in terms of R , their linear mean interchange distance (analogous to Manning's linear charge parameter), as calculated from chemical analysis and X-ray data. The metachromatic changes in the dyes' visible absorption spectra were quantitated in terms of the change in oscillator strength (ΔF), transition moment (Δq), center of gravity (ΔCg), and λ_{max} of the absorption band. Their data showed no consistent pattern, and thus did not support the notion that metachromasia was simply a consequence of polyanion promoted aggregation. For example, when comparing the metachromasia induced in methylene blue by different polyanions, ΔF , Δq , ΔCg and λ_{max} varied widely and in a pattern which showed no consistent correlation with R . Conversely, in the case of acridine orange the spectral parameters of its metachromatic complexes are essentially constant, and independent of which particular polyanion is inducing the spectral change. Evidently in some systems dye-dye (aggregation type) interactions were predominant in producing the metachromatic changes, whereas in other cases dye-polyanion interactions played a more critical role.

Stone went further toward elucidating the nature of metachromasia by analyzing induced Cotton effects in the metachromatic absorption bands (73). Extrinsic circular dichroism (CD) had already been demonstrated by Stryer and Blout for dyes

bound to helical polymers (74). From examination of CD spectra of the various dye-polyanion complexes, a cohesive pattern began to emerge. Dyes could be broadly classified into two categories- those like Acridine orange, wherein both the extrinsic CD and the metachromatic spectra are relatively independent of the polymeric chromotrope, and those like methylene blue, wherein the spectral and chiroptical changes are very dependent on the chromotrope. For the chromotrope independent class of dyes, a theoretical model based on the formation of a new absorbing unit via dye-dye interactions gives good agreement with much of the spectral data. This model, developed by Tinocco (75), is based on a quantum mechanical treatment of the aggregated dyes as a coupled oscillator, termed an exciton. The theory predicts: 1) that the metachromatic band should be asymmetric, with most of its intensity shifted to lower wavelengths; 2) a hypochromism due to non-resonance interactions should occur over the entire absorption spectra; 3) a doublet CD band should emerge, with zero crossover point at the maxima of the metachromatic absorption band; 4) that these changes should be relatively independent of the chromotrope producing them, since they are founded in dye-dye, rather than dye-polyanion interactions. This is in fact the pattern of spectral changes observed for all of the acridine orange complexes with GAG, at a polymer site to dye (P/D) ratio of about 2. But beyond this generalization, the spectral data become extremely complex. As the P/D ratio is varied

over a wider range, CD bands shift, change sign and emerge in a fashion not amenable to treatment from any single theoretical viewpoint, and is in fact indicative of multiple forces contributing to the dye-polyanion interaction. Thus only for the limited instances where dye-dye interactions predominate the metachromatic phenomena can the spectral data be interpreted based on a simple exciton model. In the majority of cases, contributions to the spectra from electrostatic, ion pairing, charge transfer, solvation, and steric forces complicate the spectral changes, making it impossible to characterize the data in a theoretical sense. It should be noted here that the complex variation of the acridine orange CD spectrum with P/D is not observed when only metachromasia is examined, illustrating the greater sensitivity of chiroptical analyses as probes of polyanion structure. Furthermore, despite its complexity, the chiroptical data clearly demonstrated that the GAG-Acridine complex exists in solution as a helical array (28), confirming that the helical symmetry observed in the X-ray patterns of crystalline GAG films, could also exist in solution (23,73).

Though studies of GAG induced Cotton effects in methylene blue also proved too complex to treat from a theoretical viewpoint, they did display a consistent pattern of spectral changes, enabling an interesting empirical observation. The GAG induced Cotton effects could be grouped into two classes which corresponded to the conformational classes of the GAG themselves, based on their

intrinsic ultraviolet CD spectra (28) and the X-ray diffraction patterns of their crystalline salts (23). For the 4-amino linked class of GAG (heparin, heparan sulfate, keratin sulfate), a doublet CD band was induced, with a positive peak at 590-600 nm, a negative peak at 550-560 nm, and crossover at about 570 nm. For the 3-amino linked class (dermatan sulfate, hyaluronate and chondroitin sulfates), the extrinsic methylene blue CD displayed only a positive band at about 575 nm. Apparently only the 4-amino linked GAG can adopt a helical conformation capable of incorporating methylene blue in a manner leading to exciton formation. Thus despite the absence of a complete theoretical framework for the metachromatic phenomena, it has proven a formidable tool in establishing our physicochemical understanding of MPS.

In addition to the theoretical importance of metachromasia, it has also led to important practical developments in histochemistry and biochemistry. Several dyes have proven useful for quantitative MPS assay. Stone's original purpose in examining the metachromasia of a large number of GAG-dye pairs was to compare their applicability for quantitative analysis (71). For such analysis, either one of two possible characteristics is desirable. If a dye could be found which reacted metachromatically with only specific GAG it would be useful as a specific chemical assay. However such a dye has yet to be demonstrated. Conversely, since metachromasia is a general property of polyanions, it would be useful to have a dye which bound in the most non-specific fashion possible; this would

enable development of two important biochemical tools- 1) a system for comparing the relative density of anionic sites on different GAG, and 2) an assay for detecting the total concentration of anionic sites in a particular sample. One of the conclusions of Stone's study was that acridine orange reacts in an identical fashion with the different GAG, giving very similar metachromatic spectra, and an equivalent weight per anionic site in good agreement with the weight per anionic site expected from analytical data (71). When plotting the drop in absorbance vs mass GAG added, acridine orange titrations give a sharp endpoint, further demonstrating this dye's suitability for assay procedures.

The utilization of acridine orange titrations as a tool for determining the anionic site density of MPS, and total MPS anionic site concentration in biological samples, has been more fully developed by Cundal et al (76,77) using fluorescence rather than absorbance measurements, thereby adding a new dimension in selectivity and sensitivity. Furthermore fluorimetric assays with acridine orange give a sharper endpoint, and are much less subject to interference from salt, a real problem in metachromatic dye binding assays. The method is based on the loss of the 530 nm emission band characteristic of the dye monomer, and its replacement by an emission band of much lower intensity at 640 nm, presumably due to the dimeric form of the dye favored non-specifically by the elevated local dye concentration within the electrostatic domain of polyanions. The mode of complexation of acridine orange to polyanions is thus always the same, with polyanions differing only

quantitatively in their abilities to induce condensation of this dimeric form of the dye in their electrostatic domain (77). This concurs with Stone's conclusions regarding the use of acridine orange as a tool for comparing the anionic site charge density among the MPS; and the experimental anionic site equivalent weights determined from the fluorimetric procedure give even better agreement with calculated values (based on analytical data) than those determined from metachromatic procedures. These investigators have even used such equivalent weights to calculate ρ , Manning's linear charge density parameter (78). In fact, for heparin subfractions with varying anticoagulant activity, good correlation was observed between this experimentally calculated value of ρ , and the anticoagulant activity of the heparin subfractions (78).

Menter et al. have taken the interaction between polyanions and acridine orange a step further. They observed that the red fluorescence of the bound dye dimer was photolabile (79), and that the rate of the photooxidation responsible for this fluorescence fading depends on the charge density of a polyanion. In particular, when comparing subpopulations of a single GAG class like heparin, linear correlation was found between the charge density of heparin fractions, their anticoagulant activity, and their ability to accelerate this fluorescence fading (80). The concept that there is a relationship between anticoagulant activity and charge density dates back many years (15,44), and the use of cationic dyes has proven to be a quite valuable tool towards the elucidation of this relationship (77,78,80).

II. Materials and Methods.

A. Materials

1. Organic reagents.

Auramine O (AuO), (bis[p-dimethylaminophenyl] methylene imine, Color Index No. 41000) was obtained as the certified biological stain (dye content 86%) from the Aldrich Chemical Co., lot 112787. The material used throughout the study was prepared by successive recrystallization from 0.02M aqueous NaCl as described by Conrad et al., (81), until a constant extinction coefficient and melting point were obtained. The material thus prepared decomposed at 260-265°C, rather than giving a clean melting point of 267°C, as stated in the literature (82). This behavior is most likely due to a persistent impurity, which could be demonstrated by thin layer chromatography on Brinkman silica gel plates. The preparation obeyed Beer's law up to a concentration of 5×10^{-4} M, giving an extinction coefficient of $4.45 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, in 0.1M pH 7.4 phosphate buffer, (as determined by linear regression analysis, correlation coefficient = 0.989), in excellent agreement with the properties reported by others for this dye (81,83). IR spectra of a sample in a KBr pellet coincided exactly in all its major peaks with the spectra presented in the literature (84). Attempts to prepare a more purified material by adsorption chromatography on dry silica

were unsuccessful because the purified dye tended to be unstable, and within two days of preparation impurities were again evident by TLC, even when stored in a dry vacuum dessicator. Since it could be demonstrated that heparin's influence on freshly purified material was not different from its actions on the recrystallized material, the latter was chosen as the substrate of choice for this study because of its stability and ease of preparation.

The Michler's ketone (MK)(4,4'-bis-[dimethylamino]-benzophenone) used for comparison to the product of the fading reaction was obtained from Aldrich Chemical Co., lot 102547. As originally obtained, the material was rather impure, having the appearance of blue crystals, rather than the pale green leaflets described in the handbook (82). The blue impurity was effectively removed by three successive recrystallizations from methanol, finally yielding a material with the properties expected for MK. These properties are described in detail in section B of results, since they are critical to the definitive identification of the fading reaction product as MK.

The reagents necessary for the m-hydroxydiphenyl uronic acid assay were obtained as follows, and used as such. m-hydroxydiphenyl was a product of Eastman Kodak Co., lot A6G. The glucuronolactone used as standard was obtained from Sigma Chemical Co., lot 54c-0341 and dried over P_2O_5 . Sodium tetraborate was certified A.C.S. grade, obtained from Fisher Scientific, lot 721374. All other

reagents necessary were of the highest quality commercially available.

The standardized HCl solutions used throughout the work were prepared by dilution of concentrated A.C.S. reagent grade HCl with Millipore Type I water. (This is the type of water used throughout the work). Dried potassium biphthalate (Baker analyzed reagent grade, lot 36695) was used as the primary standard for preparation of standardized NaOH solutions. These were then used to determine the normality of the HCl dilutions by titration, using a buret which could be read with an accuracy of 0.01ml.

2. PE preparations.

The heparin preparation used for all experiments, except where differently specified, was a Sigma Chemical Co. product, the Grade I sodium salt, obtained from porcine intestinal mucosa. It had the highest commercially available anticoagulant activity - lot 49c-0496, 169 USP anticoagulant units/mg; lot 96C-0093, 170 USP units/mg; lot 60F-0621, 159 USP units/mg. Further purification of this material by cetylpyridinium chloride precipitation, solubilization, and repeated precipitation from 80% ethanol containing 0.1M NaCl, as described by Roden et al. (85), did not result in any change of the heparin's catalytic activity with respect to AuO hydrolysis.

The average residue molecular weight for these Sigma heparin preparations was calculated based on the structure recently reported by Jaques for the typical heparin hexasaccharide (44). The weight of the hexasaccharide and its associated counterions is divided by six to obtain a mean monomer residue weight of 342. This is the value used to determine the molarity of gravimetrically prepared heparin solutions.

The NIH standard heparin preparation used in section L of results, was obtained from Dr. Martin B. Mathews, University of Chicago, Department of Pediatrics. It was prepared from a commercial beef lung heparin sodium salt (Upjohn, lot 501DF) by using gel-filtration to remove low molecular weight contaminants, and showed an anticoagulant activity of 183 USP units/mg. Further detailed analytical data on the preparations are presented in Table I, along with the analytical data for the other NIH standard GAG.

The heparin oligosaccharide fragments used for section J of results were the kind gift of Dr. Robert Rosenberg, Sidney Farber Cancer Institute of the Harvard Medical School. They were prepared by chemical depolymerization of porcine intestinal mucosa heparin, utilizing a mild nitrous acid procedure (86), wherein deaminative cleavage will only occur at unsubstituted glucosamine residues. The resulting oligosaccharide products were fractionated on a Biogel P-10 column (Biorad), with 0.5M ammonium carbonate as the mobile phase. The analysis of the eluted fractions for glucuronic acid and

anticoagulant activity is presented in Figure 2, a chromatogram of the run provided by Dr. Rosenberg. The fractions were pooled as indicated, lyophilized, and provided as such. Before used, the samples were redissolved and subjected to a second lyophilization to ensure removal of all of the ammonium carbonate. The pH of the solutions used to determine catalytic activity was determined potentiometrically, and in all cases found to be at pH 5.1, ± 0.2 . This value is about 2 pH units lower than that typically observed when dissolving commercial heparin in water (pH 6.9 ± 0.1), and thus some contamination with ammonium ion probably persists. However, since the contamination should be constant in all the samples, it should not interfere with the relative catalytic activity determined for the different oligosaccharide chain lengths.

The undegraded heparin fractions of defined molecular weight ranges, also used in section J, were the gift of Dr. Sanford Gitel, New York University School of Medicine. They were prepared by gel-filtration chromatography of commercial heparin (Cal-Biochem., anticoagulant activity 167 units/mg) on Sephadex G-100 (Pharmacia) in 0.01M Tris, pH 7.5, containing 0.15M NaCl. The broad peak of eluted heparin was arbitrarily divided into four sets of pooled fractions, which were dialyzed extensively against distilled water, and provided as such. Before use, they were further purified by ethanol precipitation (86) and dried over P_2O_5 , so as to ensure uniformity and enable comparison to other preparations similarly treated. In aqueous solution all gave a pH of 7.0 ± 0.2 .

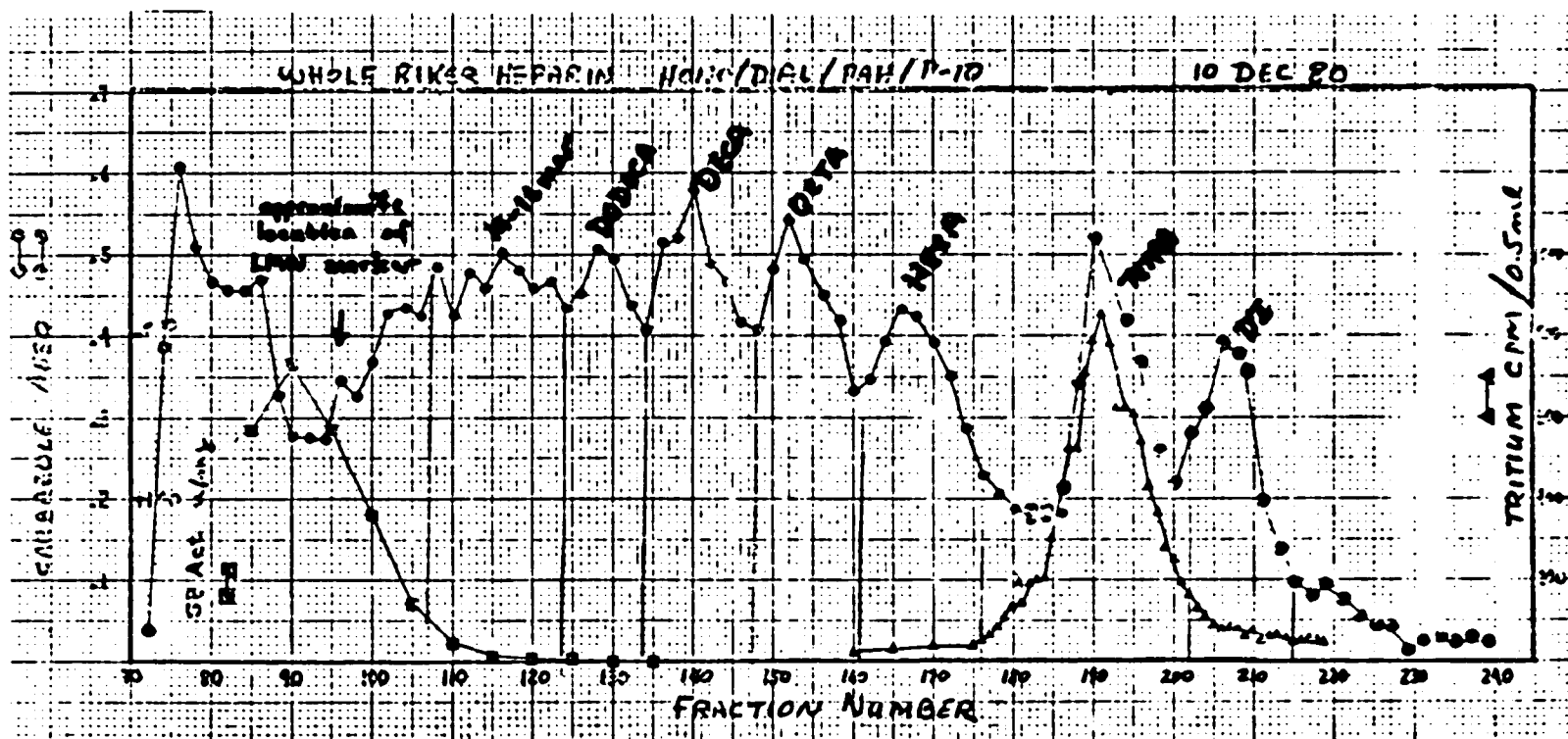


Figure 2: Chromatogram of heparin oligosaccharides
provided by Dr. R. Rosenberg.

Heparin subfractions of varying charge density were obtained from Dr. Milton Smith, technical director of Hepar Industries Inc., Franklin, Ohio. They were prepared by fractionation with the quaternary ammonium salt trimethyl-hexadecylammonium chloride, using a procedure similar to that described by Scott (87), and then precipitated from 80% ethanol as sodium salts. The anticoagulant activities listed for the fractions in section K of results were those quoted by Dr. Smith. Samples were directly dissolved in water and assayed for specific activity as such.

The NIH GAG reference standards were provided by Dr. M.B. Mathews of the University of Chicago, Department of Pediatrics. All were supplied as the solid sodium salts. Aspects of the analytical data provided with the samples relevant to their use here are presented in Table I. The monomer residue molecular weights listed in the table were calculated based on the idealized disaccharide structures for the GAG (Figure 1) and the analytical sulfate/hexoseamine ratio. The weight of Na^+ ions necessary for charge neutralization in the salt is included in the calculations. These average monomer molecular weights were used to prepare GAG solutions of equivalent molarities, for comparison of relative catalytic activities (section L, results).

TABLE IAnalytical Data for NIH Reference Glycosaminoglycans

	<u>Sulfate/hexosamine molar ratio</u>	<u>Serine/hexosamine molar ratio</u>	<u>M.W.</u>	<u>Average monomer residue wt.</u>
Heparin	2.77	0.012	14,000	354
Hyaluronate	0.00	0.001	230,000	270
Chondroitin-4- sulfate	0.98	0.060	15,000	268
Chondroitin-6- sulfate	0.90	0.021	29,000	264
Heparan sulfate	0.97	0.020	not determined	247
Dermatan sulfate	1.13	.001	45,000	278
Keratin sulfate	1.26	0.088	25,000	267

Assorted other commercial PE preparations were used throughout the study. From Sigma Chemical we obtained: chondroitin sulfate type A, sodium salt, lot 77C-0187; chondroitin sulfate Type B, (predominantly dermatan sulfate) sodium salt, lot 66C-3975; chondroitin sulfate Type C, sodium salt, lot 56C-3881; hyaluronate, Grade I, lot 115C-009; dextran sulfate, sodium salt, lot 59C-0362; polyvinyl sulfate, potassium salt, lot 109C-5000; polyanethole sulfonate, sodium salt, lot 77C-0221; protamine hydrochloride, lot 107C-0352; polylysine hydrobromide type V, lot 28C-5058; ribonucleic acid from yeast, Type III sodium salt, lot 68C-8025; deoxyribonucleic acid from calf thymus, Type I, sodium salt, lot 126C-9600.

B. Methods

1. Kinetic experiments.

The rate of the fading reaction, which will be shown to correspond to the hydrolysis of AuO to MK and ammonium ion (illustrated in figure 3), was determined by monitoring the decrease in absorbance at 430nm, the visible absorption maximum of AuO. MK has almost no absorptivity at this wavelength. Generally the kinetic tracings were obtained on a Gilford 2400-S recording spectrophotometer. Under conditions where the hydrolysis rate was so slow that the inherent instability of this instrument resulted in an intolerable error level, a Carey 17 spectrophotometer was used. Both these instruments were calibrated against National Bureau of Standards liquid absorbance standards (lot NBS-SRM 931B), so that measurements made on the two instruments were directly comparable. Temperature was maintained with a circulating water bath at 20°C, except where otherwise indicated. All rates reported are initial rates, determined from the initial linear portion of the recorder tracings. Measurements were performed in triplicate, and in all cases the maximal deviations observed were 5% or less. Reaction mixtures were prepared by first adding heparin to the standardized acid solution, and then initiating the reaction by delivering AuO from a stock solution with a calibrated silanized pipet.

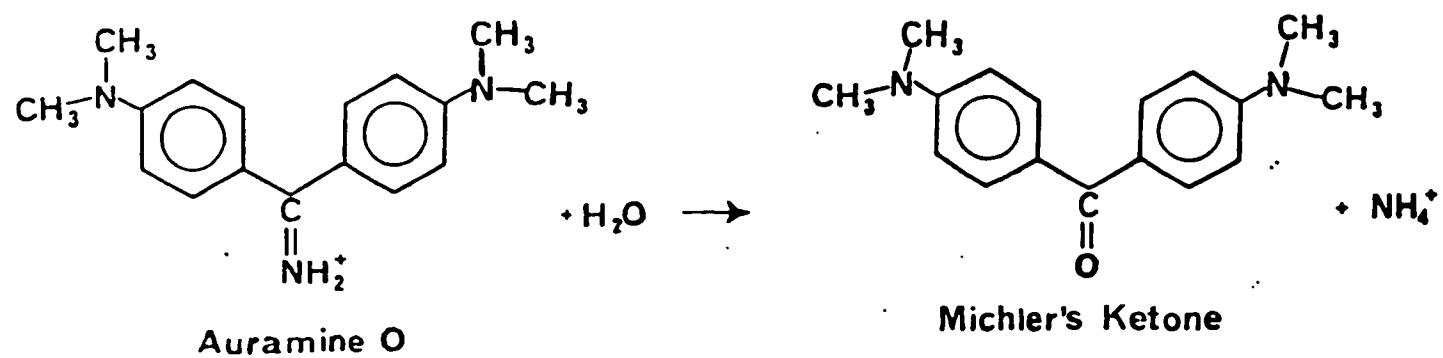


Figure 3: The acidic hydrolysis of AuO

2. Uronic acid assay procedure

Comparison of the various heparin preparations with respect to catalytic activity could not be done on a weight basis alone because of the microheterogeneity problem in all preparations of natural origin (17). It was therefore necessary to use a reliable analytical procedure for comparing polymer concentrations in order to perform specific activity calculations. The technique chosen was the m-hydroxydiphenyl assay for uronic acid (88), which does not require hydrolytic breakdown of the polymer prior to analysis. When the procedure described in the literature was slightly modified so as to improve the uniformity in the rate of furfural formation, heparin-uronic acid concentrations could be determined within a precision level of 1%. This was accomplished by cooling the tube containing the sulfuric acid-tetraborate reagent in a dry ice-propanol bath, prior to the careful layering of sample over this reagent, and then immediately vortexing vigorously and placing the assay mixture in a boiling water bath. The precision of the assay is critically dependent on maintaining conditions so that the amount of furfural formed in this step of the procedure, which is extremely exothermic, depends only on the mass of uronic acid present; it is the furfural concentration which determines the final color yield (A_{595}) of the assay, since its reaction with the m-hydroxydiphenyl

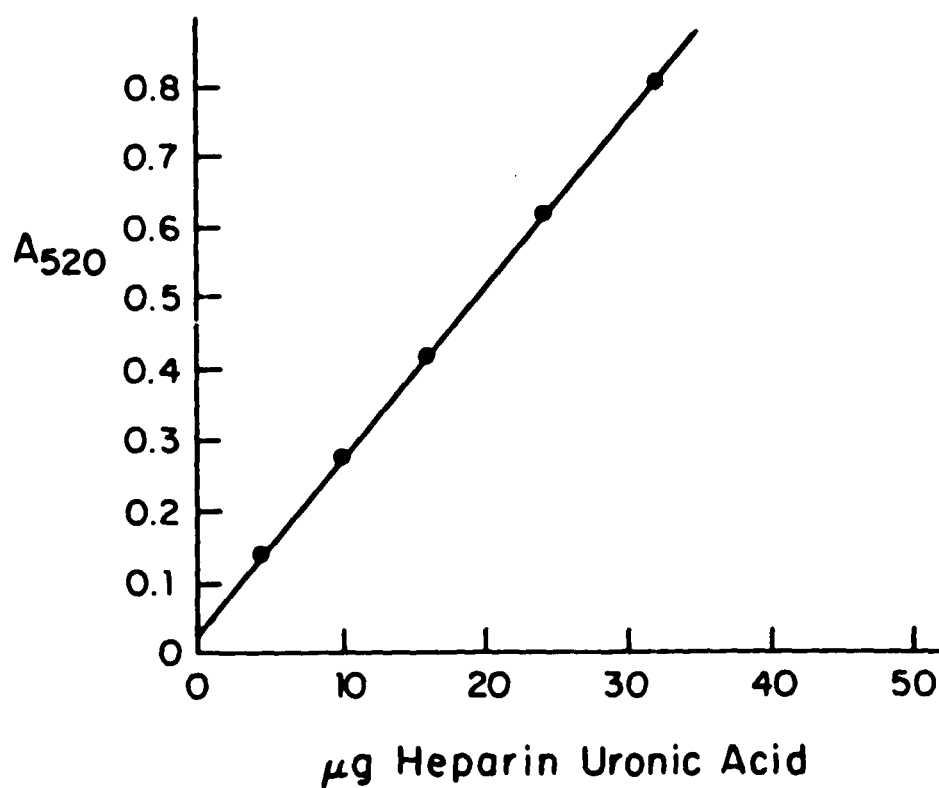


Figure 4: Heparin-uronate standard curve for the m-hydroxydiphenyl procedure.

is what forms the chromogen being measured. The standard curve presented in Figure 4 was generated using Sigma Grade I heparin, lot 96C-0093, which was dried over P_2O_5 in a vacuum dessicator for at least two weeks prior to use.

3. Specific activity definition and method for its determination

The specific activity of a heparin preparation with respect to its catalytic influence on AuO hydrolysis, can be defined in terms of the rate of the catalyzed reaction per ug heparin uronic acid. The conditions described in Appendix I were used to determine the hydrolysis rates and uronic acid concentration was assayed as described in section IIB2. For specific activity calculations, samples were appropriately diluted so as to be within the linear and most accurate range for both measurement procedures. Hydrolysis rate and heparin-glucuronic acid content were then simultaneously determined in triplicate, and reported in units of $\text{min}^{-1}/\text{ug}$ uronic acid. The specific activities thus calculated for a sample are precise to within 5% when in the mid-linear range of the assay.

4. Procedures and instrumentation for spectral measurements.

Visible absorption spectra were taken on a Cary 17 spectrophotometer at 20°C with the slitwidth and recorder speed adjusted to give a pen-fidelity of approximately 1. Recordings were typically traced at a rate of 30nm/minute, with a slitwidth of

0.295mm, giving a spectral bandwidth of 1nm. To take spectra at pH values wherein hydrolysis of AuO was fast enough to occur significantly during the time it takes place to record a spectrum (e.g. Section III E), the temperature was lowered to 8°C and the speed increased to 60nm/minute. This only resulted in a decrease in pen fidelity to 0.5.

Infra-red spectra were recorded on a Beckman model IR-18A infra-red spectrophotometer, at a gain setting of 4, and a scan rate of 300 cm⁻¹/min. KBr pellets were prepared by mixing the sample 1:100 with IR-grade KBr using an amalgamator (Crescent Dental Mfg., model 5A), and pressed into pellets using a Barnes model 129 KBr die and a Wilks 30-ton press.

Σ_{app} was determined under various experimental conditions from initial absorbance measurements at 430nm taken as follows (except when determined by a stopped flow procedure). The Carey 17 spectrophotometer was used with spectral parameters set as described above. Samples were set up with all reagents except the AuO and equilibrated in the same water bath circulating through the spectrophotometer water jacket. AuO equilibrated to the same temperature is then added from a silanized pipet, the sample is rapidly vortexed, and its absorbance measured. This operation could be accomplished within 30 seconds. Measurements were performed in triplicate and showed a standard deviation of 2% from the mean.

4. Stopped-flow spectrophotometry.

It was impossible to accurately determine the extinction coefficient of AuO at low pH by ordinary mixing procedures, due to the rapid non-linear rate of hydrolysis under these conditions. Therefore, it became necessary to obtain these values by stopped-flow spectrophotometry. The instrument employed consisted of an Aminco-Morrow stopped-flow apparatus (catalogue No. 4-8409) mounted on a Beckman DU spectrophotometer, set to 430nm. The photomultiplier output was placed on line with a Tektronix type 549 storage oscilloscope, which was calibrated so as to correspond to 10 volts between 0-100% transmission. Transmittance measurements were then converted into absorbances using the Beer-Lambert definition. The tracings were recorded at a sweep rate of 0.2 second/cm.

Sample measurements were taken as follows: In one reservoir of the stopped-flow, a stock solution of $4.1 \times 10^{-5} \text{M}$ AuO in Millipore water was maintained. The standardized HCl solutions used to determine the pH dependence of the extinction coefficient were placed in the second reservoir. Both reservoirs were preequilibrated at 20°C, and the spectrophotometer was maintained at the same temperature by a circulating water bath. For the set of samples containing heparin, solid sodium heparinate was added to the HCl solution immediately prior to use at a concentration of 200 ug/ml and maintained at 0°C until placement in the sample

reservoir, so as to minimize any hydrolytic cleavage of the heparin between the time of dissolution and measurement. The effectiveness of this procedure was evidenced by the lack of any systematic variation in the successive voltage measurements. Except at the highest acid concentrations, the oscilloscope tracings were essentially horizontal, indicating that the hydrolysis rates were effectively distinguished from the changes in initial absorbance by this procedure. Even when there was a slight slope to the tracing, it was straightforward to read the initial voltage reading right off the scope. All determinations were performed at least in triplicate, and never displayed more than a 3% standard deviation from the mean.

Using the initial absorbance values measured in this way, and the known concentration of AuO in the mixing chamber (based on a 1:2 dilution of the analytically determined AuO concentration in the stock solution) the apparent extinction coefficient at 430nm (ϵ_{app}) could be calculated as a function of the experimental conditions. The acidities of the samples are reported as $-\log [HCl]$ analytical, as based on a 1:2 dilution of the standardized HCl solutions. No corrections are made for activity coefficients because of the acid concentration range being considered (i.e. between the limits of the pH and H_0 acidity function scales), and therefore the abscissa is simply reported as the $-\log [HCl]$.

6. Methods of pH determination and control

Because of the difficulty in using buffers to investigate this system (see section IIID, IIIG), the problem of pH determination and control needs to be addressed. This need is accentuated by the importance of acidity to the rate of the hydrolysis reaction, and the mechanism of heparin's influence thereon. Therefore the general procedures followed for dealing with this problem will be here outlined. Where different methods were necessitated for specific experimental purposes, they are separately specified.

The basic assumption involved throughout the work, is that the HCl concentrations determined by titration give a reasonable approximation of the pH under the various experimental conditions. The validity of this assumption and its limitations was assessed by comparing calculated pH values with those potentiometrically measured using a combination glass microelectrode (Beckman futura model 39030), connected to a Beckman model 3500 digital pH meter. The electrode was standardized against a pH 2.00 Beckman "Green Seal" buffer solution, and was quoted by the manufacturer as being accurate over the pH 0-11 range. The measured pH recorded was the value obtained after allowing 1 minute for equilibration of the electrode with the solution in question. This last point is important because after the pH meter appeared to reach an equilibrium value (defined as the 1 minute reading), it continued to

TABLE IIComparison of measured and calculated pH values

- a. Under the conditions used to determine the pH dependence of AuO's extinction coefficient and hydrolysis rate.

<u>pH calculated</u>	<u>pH measured in the presence of 100 ug/ml heparin</u>	<u>pH measured in absence of heparin</u>
0.39	0.42	0.45
0.72	0.79	0.70
1.04	1.09	0.97
1.27	1.24	1.23
1.61	1.68	1.59
2.33	2.41	2.31
2.59	2.68	2.61
2.81	2.95	2.79
3.03	3.24	2.98

- b. Under the conditions used to compare the catalytic efficiency of the various GAG. pH calculated = 2.57 in all cases.

<u>GAG added</u>	<u>pH measured</u>
none	2.55
heparin	2.65
C-6S	2.59
C-4S	2.58
HA	2.58
4S	2.60
DS	2.61
KS	2.59

undergo a slow, but steady upward drift, which was especially consistent in the presence of heparin. Despite this limitation, there was still excellent agreement between the measured and calculated pH values at acidities greater than 0.003M HCl as illustrated in Table II.

The conditions under which the measured and calculated pH did not agree are also notable for their illustrative value. This was the case when the heparin and HCl concentrations were of comparable magnitude. For example, when determining Σ_{app} of AuO in the presence of 3×10^{-4} M heparin over the pH 3-6 range (section IIIE) the measured pH was significantly higher than the calculated value (e.g. pH measured = 5.79 when pH calculated = 4.10, pH measured = 3.75 when pH calculated = 3.39). Another instance wherein the difference between measured and calculated pH values became significant was in determining the influence of high heparin concentrations on the hydrolysis rate at a constant pH (section IIIG). The reason for the problem under these circumstances, and the specific ways it was dealt with depending upon the goals of the particular experiment, are discussed in the aforementioned sections (IIIE and IIIG).

III. Results

A. Demonstration of the influence of heparin on AuO fading.

In the course of experiments designed to develop Auramine O (AuO) as a fluorescent probe for MPS, it was noticed that under conditions of pH and temperature wherein AuO free in solution was stable, the addition of small quantities (ug range) of heparin dramatically accelerated the fading of the dye to a pale green (almost colorless) solution. The spectral changes accompanying this fading are illustrated in Figures 5a and 5b, for 2×10^{-5} M AuO in 0.01 M glycine buffer, pH 3.2. Figure 5a shows the zero time visible absorption spectra of the dye in the absence (solid line) and presence (dotted line) of 100 ug/ml heparin. The interaction between heparin and AuO is already apparent at this point, by the heparin induced metachromatic changes in the dye's spectra. Figure 5b illustrates the corresponding spectra for the same samples after being kept in the dark for five hours at 20°C. Though little change has occurred in the spectra of the free dye (solid line), the sample containing heparin (dotted line) has undergone pronounced spectral changes. The 420nm absorption band responsible for the original yellow color of the sample is completely lost, indicating that the original AuO has been destroyed. It is towards the explanation of this observation, that this thesis is addressed.

Figure 5: Visible absorption spectra for $2.0 \times 10^{-5} \text{ M AuO}$ in 0.01M glycine buffer, pH 3.2.

(a) zero time spectra.

(b) spectra after being kept in the dark for five hours.

Solid line - no further addition;
dashed line - heparin added to a final concentration of 100ug/ml.

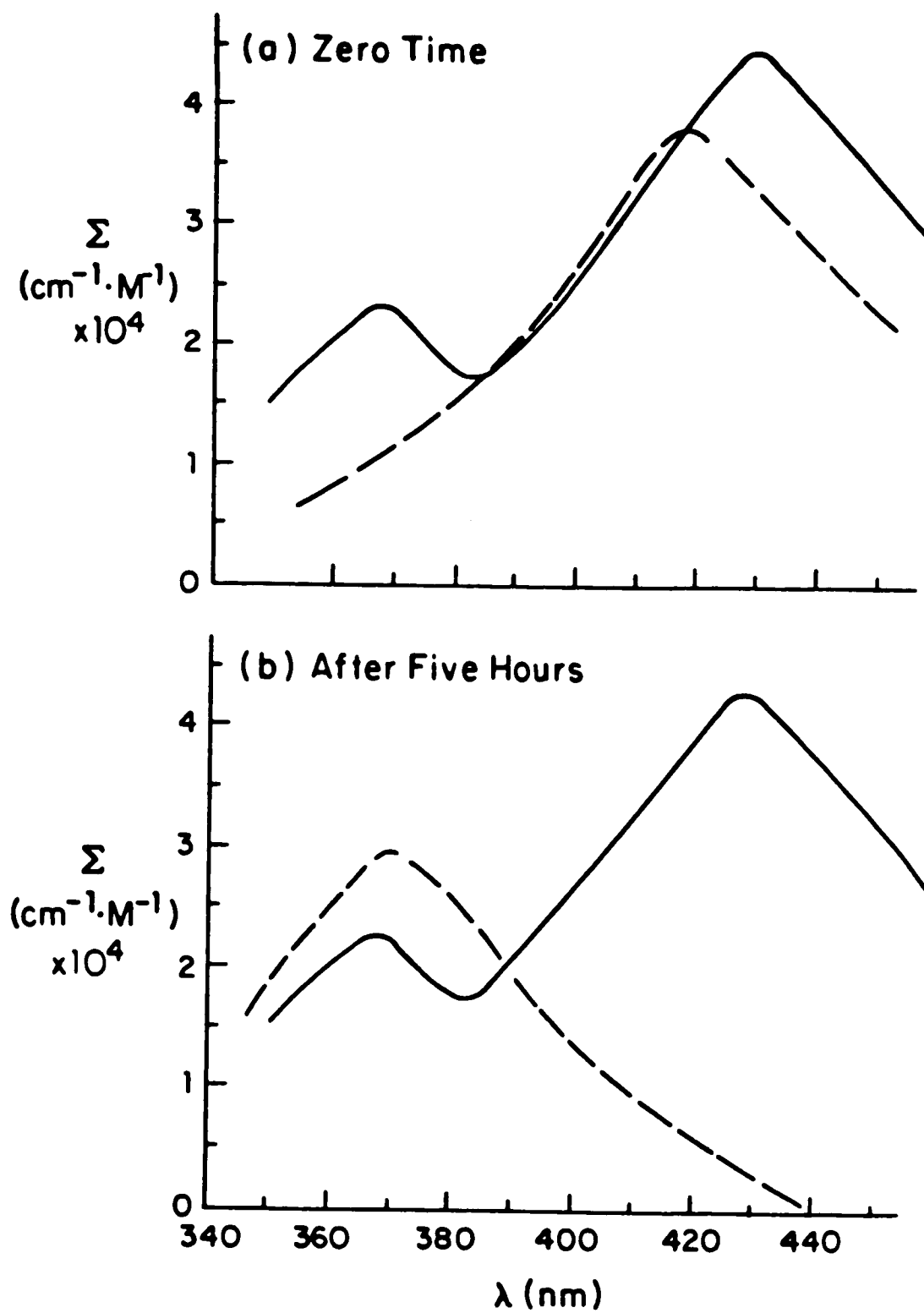


Figure 5

Based on these spectra it is apparent that the rate of the chemical reaction responsible for the fading is readily determined by photometric monitoring of light absorption at 430 nm, $\lambda_{\max}$ for AuO. Quantitative rate measurements revealed that the rate enhancement due to heparin was most pronounced in dilute unbuffered acid solutions. As will be later described, this is due to an opposite influence of ionic strength on the heparin enhanced and non-enhanced fading rate. Further characterization of this reaction was therefore carried out in dilute HCl solutions, standardized by the titration procedures described previously. Recorded kinetic plots of the reaction in 10^{-3} M HCl are shown in figure 6a. The rate of fading for AuO free in solution is essentially zero within the timescale of a typical kinetic recording. AuO hydrolysis under these conditions must be measured over days, the half-life of the reaction being about 12 days (Figure 6b). The addition of the sodium salt of heparin to this reaction mixture at a final concentration of 48 ug/ml (Figure 6a, dashed line) increases the rate to 0.010 min^{-1} , the half-life being reduced to 90 minutes. This amounts to a striking 200-fold rate acceleration. The addition of 100 ug of other commercial glycosaminoglycan (GAG) preparations caused little if any increase in the rate of AuO fading, as illustrated in figure 6a for chondroitin 4- and 6- sulfate, dermatan sulfate and hyaluronate. Thus it seemed that heparin accelerated the fading of AuO, and that it was distinguished from other GAG in

this property, in parallel to its distinctive biological and pharmacological actions (see section IB3,). Though not all rate enhancements are correctly termed catalyses, we shall henceforth use the term catalysis when referring to heparin's acceleration of this fading reaction. Justification of the term, and qualifications as to its meaning under these circumstances, will be fully dealt with in the discussion after the reaction responsible for the fading has been identified and the nature of heparin's influence described.

Figure 6: Kinetic plots of the fading reaction for $3.0 \times 10^{-5} \text{M}$ AuO in $7. \times 10^{-4} \text{M}$ HCl.

(a) on a minute time scale:

solid line - in the absence of any addition, or with 100ug chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate or hyaluronate added; dotted line - with 100ug heparin added.

(b) with no addition, on a day time scale.

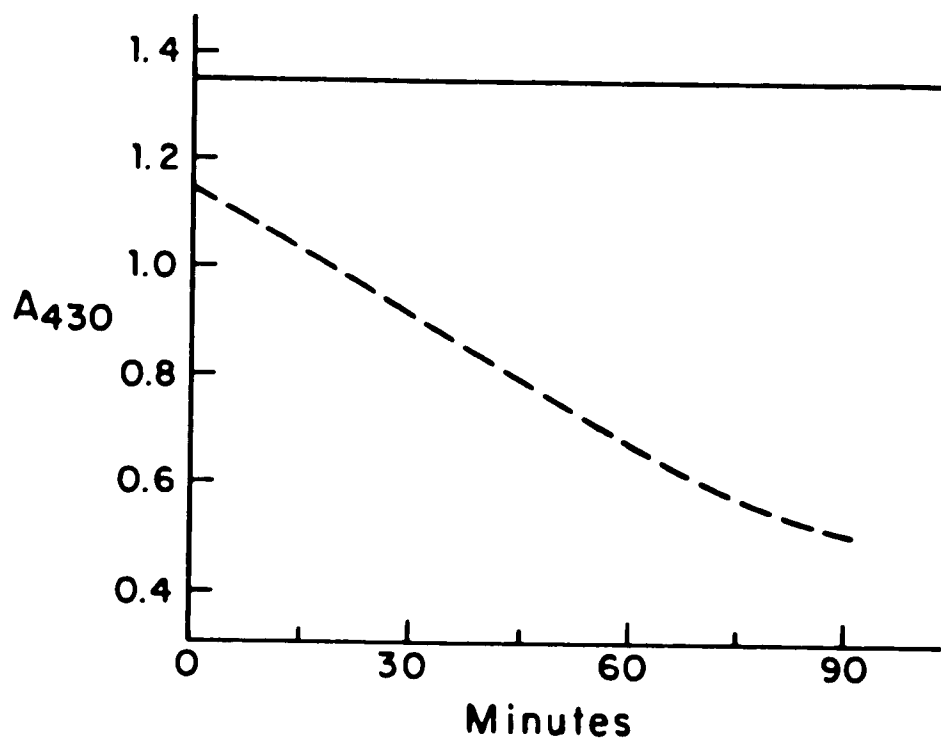


Figure 6a

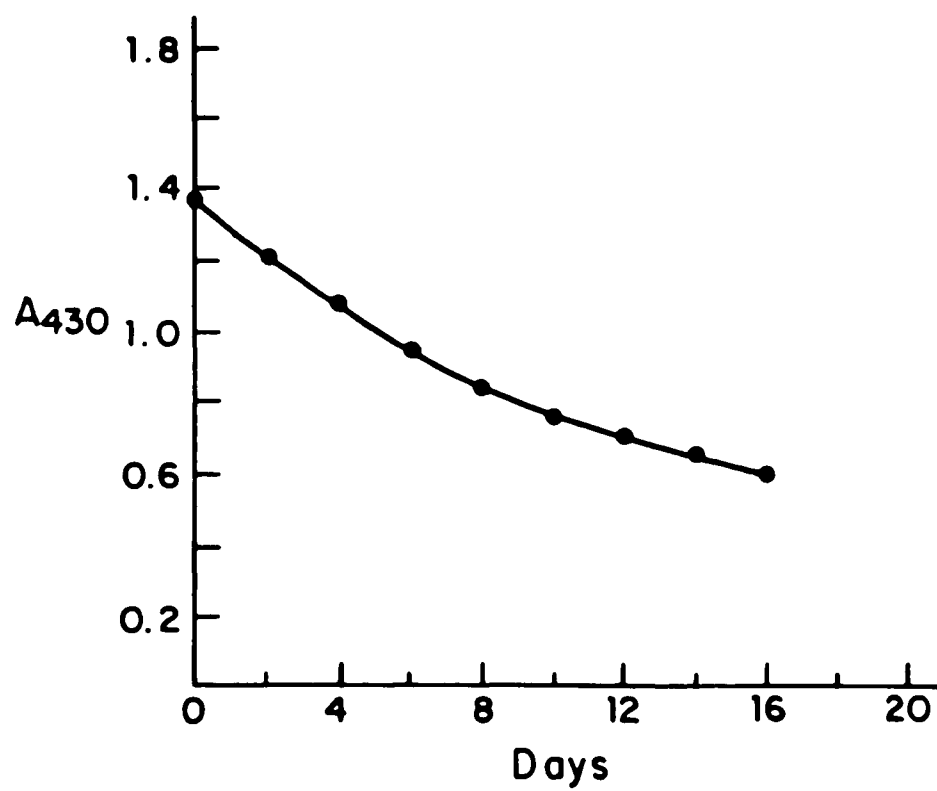


Figure 6b

B. Isolation and Identification of the Reaction Product.

To demonstrate that the chemical reaction responsible for AuO fading was its hydrolysis to Michlers Ketone (MK) (Figure 3), and that the same product was formed both in the presence and absence of heparin, product isolation was carried out from two identical reaction mixtures, differing only in that one contained added heparin. The products thus obtained were compared by their spectral and physical characteristics to a known sample of MK, and to the literature values for these parameters.

The reactions were set up as follows: In 1 liter of 0.010 M HCl, AuO was dissolved at a concentration of $1.0 \times 10^{-3} \text{ M}$, which is just under its solubility limit. This high concentration of AuO enabled quantitative product isolation without further chemical manipulation; MK is negligibly soluble in water, and thus would be expected to precipitate out directly as it is formed in the reaction. An acid concentration of 0.01 M HCl was employed for this experiment, despite the catalytic influence of heparin being more pronounced at lower HCl concentrations (see section IIID), in order that AuO hydrolysis in the absence of heparin would proceed at a reasonable rate. To one of two such identical reaction mixtures, 100mg of solid Na-heparin was added (final heparin concentration of 100 ug/ml, $3 \times 10^{-4} \text{ M}$). Temperature was maintained at 8°C, to minimize any slow cleavage of the N- and O- sulfate groups on heparin (89). Previous studies have demonstrated that the

biological activity of heparin in pH 2.0 dextrose-HCl solution is stable for 24 hours at room temperature (90). The reaction is allowed to proceed with mechanical stirring for 24 hours.

In Table IIIa, the final and initial absorbance at 430 nm for these reaction mixtures are compared as an index of the extent of reaction. The initial absorbance was determined for 1:100 dilutions due to the high A_{430} expected for $10^{-3}M$ AuO. Note the lower initial absorbance of the sample containing heparin; this is due to a metachromatic interaction between heparin and AuO rather than appreciable hydrolysis between the time the reaction is initiated and absorbance readings are taken. After 24 hrs, visible differences between the two reaction mixtures are readily apparent. The heparin containing sample has completely faded to a pale green ($A_{430} = .062$) and contains a voluminous precipitate. The reaction run without heparin is still quite yellow ($A_{430} = .328$ for a 1:100 dilution) and contains only a small amount of precipitate. Based on the decrease in A_{430} during the course of reaction, it can be calculated that 86% of the initial AuO remains for the non-catalyzed reaction, whereas less than 1% of the initial AuO remains unchanged for the catalyzed reaction. Despite the fact that the non-catalyzed reaction is still quite far from completion, (A_{430} is still measurably decreasing with time, whereas for the catalyzed reaction A_{430} is now stable), the precipitates formed in the respective reaction mixtures are both isolated at this point for direct comparison. The precipitates are collected on dry, tared, Whatman

Grade 1 cellulose filter papers, washed with cold distilled water, dried in vacuo at 60°C, and weighed on an analytical balance. For the catalyzed reaction 243 mg of solid are thus collected, while for the non-catalyzed reaction, only 26 mg of solid are collected. These solids are directly dissolved into a minimal volume of hot methanol, from which they are allowed to slowly recrystallize. The crystals, both samples of which have the appearance of pale green leaflets at this point, are collected and dried in vacuo at 60°C. A final yield of 74 mg is obtained as the product of the catalyzed reaction, and 10 mg as the product of the non-catalyzed reaction.

The physical and spectral characteristics of the reaction products thus isolated are given in Table IIb. Comparisons were also made to a known sample of MK, and the respective literature values. There is excellent agreement throughout between the experimentally observed values for the two products, the known sample of MK, and the literature. Infra-red spectra for KBr pellets of the two products and the known sample of MK are presented in Fig. 7. The precise coincidence of all the major peaks in these three spectra, and their correspondence to the literature spectra for MK (84,92), provides definitive evidence that the originally observed fading of AuO is indeed due to its acidic hydrolysis, and that MK is the product of both the accelerated and non-accelerated reactions. Furthermore, the identity of the product under these two sets of conditions provides the first criterion justifying use of the term catalytic in describing heparin's influence on this reaction.

TABLE 3

Comparison of the reaction conditions used to identify the reaction product. $1.0 \times 10^{-3}M$ AuO in 0.098M HCl, containing: A - no addition; B - heparin added to a final concentration of 100 ug/ml.

(a) Comparison of reaction rates under these experimental conditions.

	<u>A</u>	<u>B</u>
Initial A ₄₃₀ (1:100 dilution)	0.387	0.348
A ₄₃₀ after 24 hours	.328 (1:100 dilution)	0.062 (no dilution)
% of reactant hydrolyzed	15%	99%

(b) Comparison of the physical and spectral properties of the isolated reaction products, a known sample of PK, and the respective literature values.

	<u>A</u>	<u>B</u>	<u>PK</u>	<u>literature ()</u>
melting point (°C)	171.2	171.2	171.2	171.2 (91)
λ_{max} in ethanol (nm)	370	370	370	370 (82)
$\Sigma 370$ in ethanol (cm ⁻¹ M ⁻¹)	1.9×10^4	1.9×10^4	1.9×10^4	1.9×10^4 (82)
λ_{max} in glacial acetic acid (nm)	374	374	374	374 (82)
$\Sigma 374$ in acetic (cm ⁻¹ M ⁻¹)	3.2×10^4	3.2×10^4	3.2×10^4	3.2×10^4 (82)

Figure 7 Infra red spectra of the reaction products and
the known sample of Michler's ketone.

- (a) Spectrum of product isolated from the non-catalyzed reaction.
- (b) Spectrum of product isolated from the catalyzed reaction.
- (c) Spectrum of known sample of Michler's ketone.

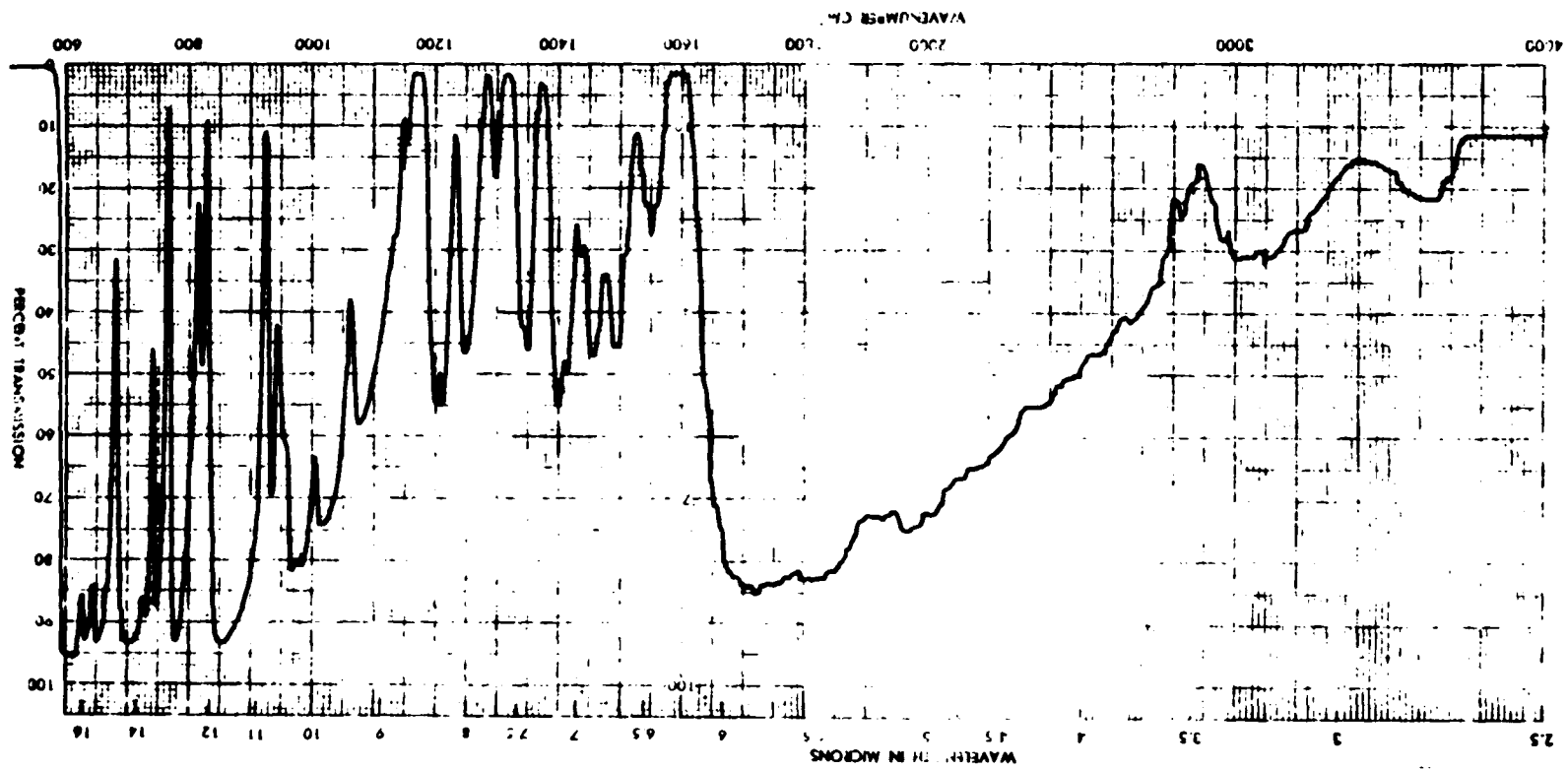


Figure 7a

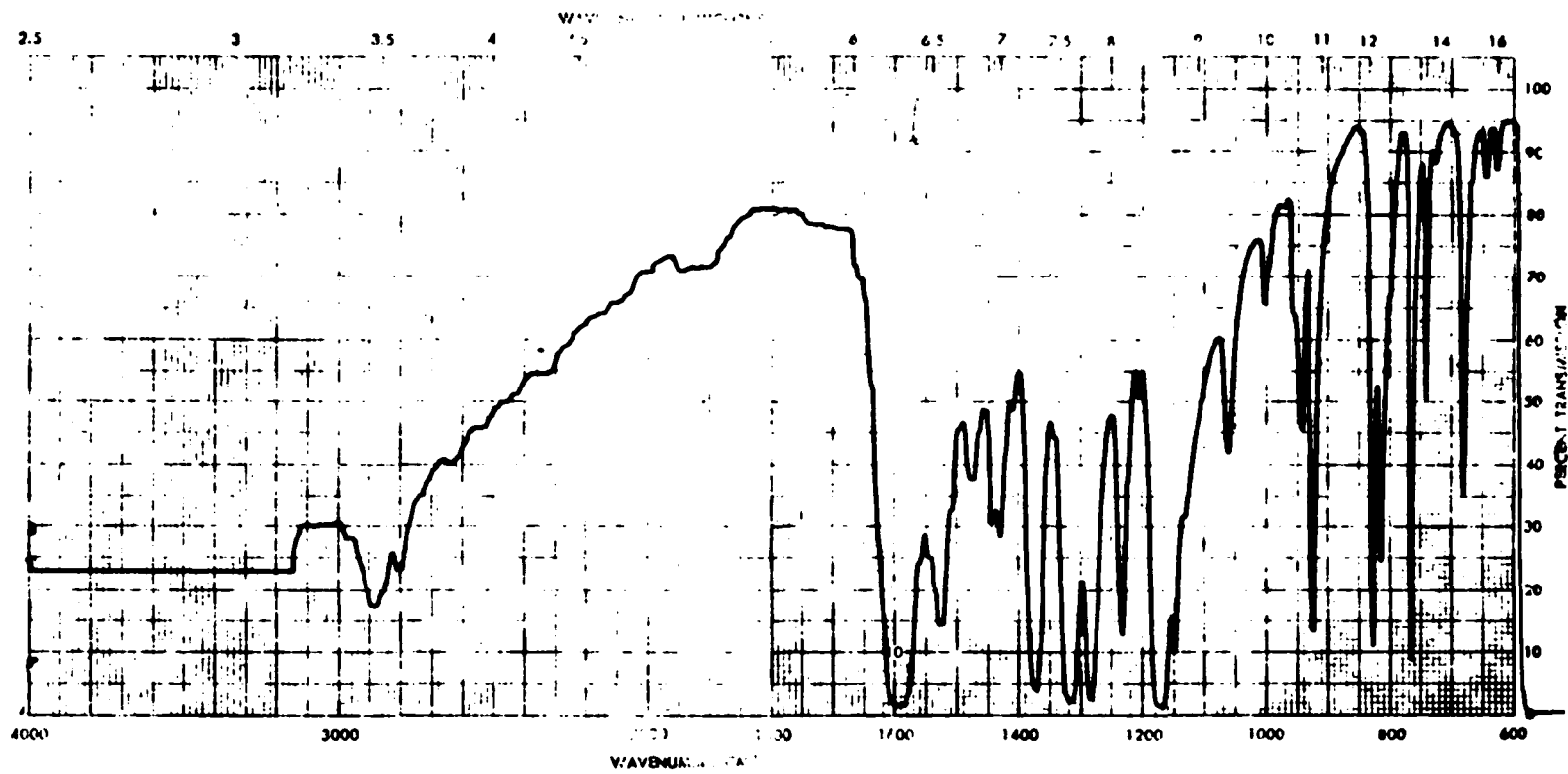


Figure 7b

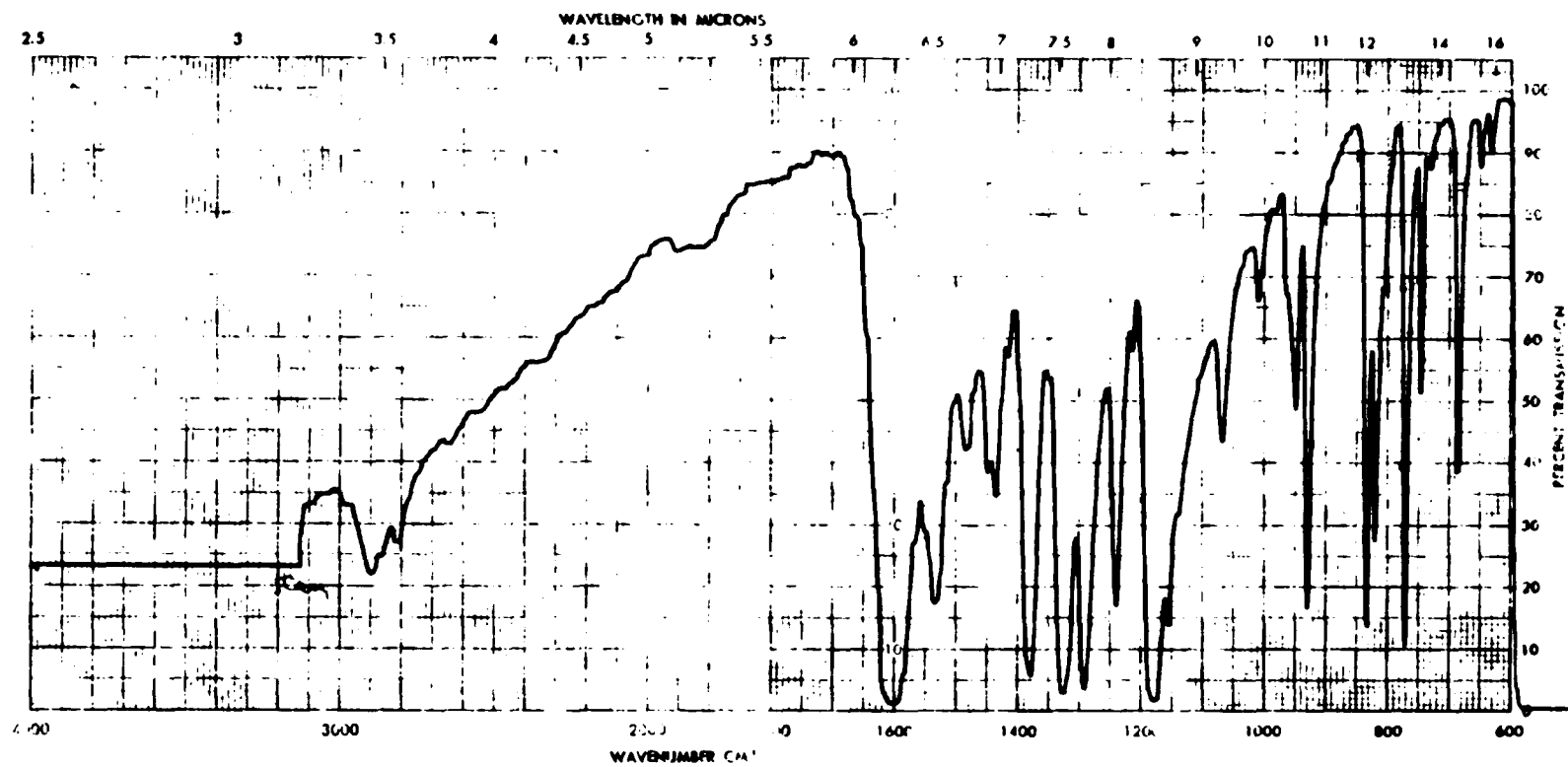


Figure 7c

C. Recovery of unaltered heparin following the catalysis.

The second criterion for justification of the term catalytic in describing heparin's acceleration of AuO hydrolysis lies in the proof that heparin itself is not consumed during the course of the reaction. To do this it will be demonstrated in this section that heparin can be quantitatively recovered following its catalysis of the reaction, and that the recovered heparin retains its ability to catalyze AuO hydrolysis at an unaltered specific activity.

The reaction is run at 25°C in a volume of 100 ml, containing 0.002 M HCl, 1.0×10^{-4} M AuO, and heparin at a final concentration of 200 ug/ml (added as 25 ml of a 0.8 mg/ml heparin solution). Based on previous studies, heparin is stable under these mildly acidic conditions (90). An identical reaction mixture without heparin is simultaneously set up on a smaller scale. The initial absorbances and hydrolysis rates for these two reactions are compared in Table IVa. Both the catalytic and metachromatic influences of the heparin are readily apparent- a 25% hypochromism and an 80-fold increase in hydrolysis rate being observed. Within 6 hours the heparin containing sample has completely faded (A_{430} = 0.018 and stable) and a precipitate has formed.

Solid NaCl is added to a final concentration of 0.2M to effect dissociation of any product or reactant complexed to heparin, thereby freeing the heparin and terminating the catalyzed reaction. Furthermore, NaCl is an absolute requirement for the ethanolic

precipitation step which is subsequently used to recover heparin (85). The reaction mixture cooled to 8°C, and filtered to remove the solid MK. NaOH (0.1M) is slowly added to the filtrate (initially at a measured pH of 3.08) to bring its pH to neutrality, and then cold 95% ethanol is added to a final concentration of 80%. Under these conditions any traces of MK should remain soluble, whereas essentially all of the subpopulations in a commercial heparin preparation should be precipitated (12). Though turbidity immediately develops, quantitative precipitation is ensured by maintaining the solution as such overnight at 8°C. The pellet is subsequently collected by centrifugation (10,000 xg) washed twice with cold 95% ethanol, twice with cold ether, and then thoroughly dried under N₂.

Rather than using a gravimetric procedure to determine the mass of heparin recovered following a round of catalysis, an analytical assay for heparin based on its uronic acid content was used to preclude any errors arising from contamination of the heparin pellet. The procedure chosen was the m-hydroxydiphenyl assay for uronic acid (88), a colorimetric procedure which does not require hydrolytic release of the uronic acid from the polymer backbone prior to analysis. This assay is quite similar to the classic carbazole procedure for quantitation of uronic acid, but proved to be more reproducible when the modified procedure described in the method section IIB2 was rigorously adhered to.

The heparin pellet, which was completely soluble in 5 ml of water, was quantitatively removed from the centrifuge tube, and brought to a final volume of 21 ml. This volume range was chosen to enable easy side by side comparison with the original 0.8 mg/ml heparin solution, 25 ml of which was used in setting up the experiment (Table 4b). When assayed by the *m*-hydroxydiphenyl procedure (Table 4b), and compared to a standard curve prepared with P_2O_5 dried heparin (see section IIIB2), the original reaction mixture set up for this experiment was determined to contain a total of 20.1 mg of heparin-uronic acid. The solubilized recovered heparin contains a total 17.8 mg of heparin-uronic acid. This amounts to a recovery of 89% of the heparin originally added to the reaction. Literature values for heparin recovery by alcohol precipitation range from 88-98% (89,93), and our own previous experiences with this technique have typically given recoveries of 90-95%. Thus the 89% recovery of the heparin following a round of catalysis provides strong (though not definitive) evidence that the heparin remains unchanged during the course of its accelerating influence on AuO hydrolysis.

Due to the microheterogeneity of heparin preparations (see section IB1), the possibility remains that only a specific heparin subpopulation is involved in the catalysis, and that this subpopulation represents the 11% of the original heparin that could not be recovered by alcohol precipitation. To rule out this possibility, it is imperative to compare the original and recovered

heparin's specific activity, here arbitrarily defined as the rate acceleration of the hydrolysis reaction ($-A_{430}/\text{min}$) per μg of heparin-uronic acid. This is done in Table IVc using reaction conditions which were subsequently adopted for development of new assay procedure for heparin based on its acceleration of AuO hydrolysis (see Appendix I for assay conditions). The fresh and recovered heparin solutions from above were diluted 1:5 and 1:5.6, respectively, so that both would be at approximately the same concentration, and well within the linear and most accurate range of the assay. As shown in the table, 100 μl of the fresh heparin dilution accelerated the rate of hydrolysis from a baseline of 0.0003 min^{-1} to 0.0025 min^{-1} ; 100 μl of the recovered heparin dilution accelerated the rate to $.0026 \text{ min}^{-1}$. Based on the m-hydroxydiphenyl assay, this corresponds to a rate acceleration of $1.5 \times 10^{-4} \text{ min}^{-1} / \mu\text{g}$ heparin-uronic acid for both the non-reacted heparin, and the recovered heparin. It can therefore be concluded that heparin is not consumed during the course of its acceleration of AuO hydrolysis.

TABLE IV

Comparison of the reaction conditions used to demonstrate that heparin is not consumed as a consequence of the rate acceleration. $1.0 \times 10^{-4}M$ AuO in 0.002M HCl containing:

(a) Comparison of reaction rates under these experimental conditions.

A - no addition;

B - containing 200 ug/ml heparin.

	<u>A</u>	<u>B</u>
Initial A_{430} (1mm pathlength)	.449	.305
A_{430}/min (1cm pathlength)	.0006 min^{-1}	.048 min^{-1}
A_{430} when reaction is terminated	.428 (1mm pathlength)	.018 (1cm pathlength)
% AuO hydrolyzed	4%	97%

(b) Calculation of % yield by comparison of recovered and fresh heparin.

A - recovered heparin;

B - original 0.8 mg/ml heparin solution.

	<u>A</u>	<u>B</u>
A_{520} for 20ul, assayed by the m-phenylphenol procedure	.422	.455
ug heparin-uronic acid (based on Fig. 4)	.005	.005
	16.1ug	16.9ug
Concentration (mg heparin-uronic acid/ml)	0.805mg/ml	0.845mg/ml

mass heparin-uronic acid originally added - 20.1mg
 mass heparin-uronic acid recovered - 17.8mg
 % heparin recovered = 89%

- (c) Comparison of the specific activity of fresh and recovered heparin. Rate measurements were performed as described in Appendix I. All determinations performed in triplicate. A and B from Table IVb were diluted 1:5 and 1:5.6, respectively, so both would be within the most accurate range for simultaneous determination of catalytic activity and uronic acid content.

	<u>A</u>	<u>B</u>
A ₅₂₀ (200 in m-phenyl phenol assay)	.865	.839
ug heparin-uronic acid per 100ul	17.0ug	16.5
Rate of AuO hydrolysis per ul	.0026	.0025
Specific activity (min ⁻¹ /ug)	1.5 x 10 ⁻⁴	1.5 x 10 ⁻⁴

D. Dependence of AuO hydrolysis on acidity of the medium.

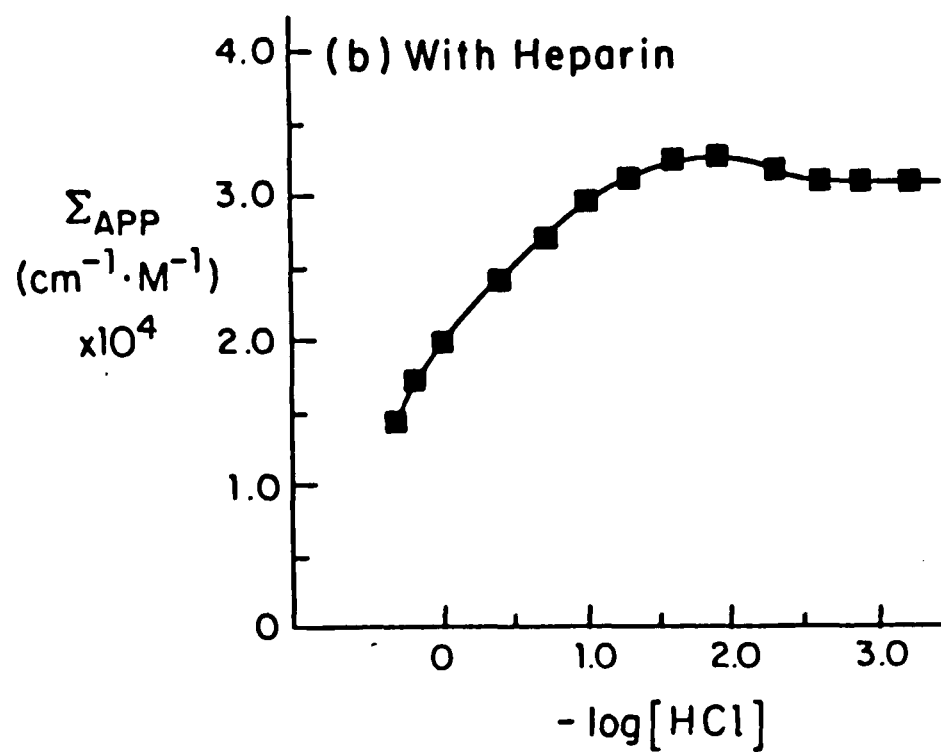
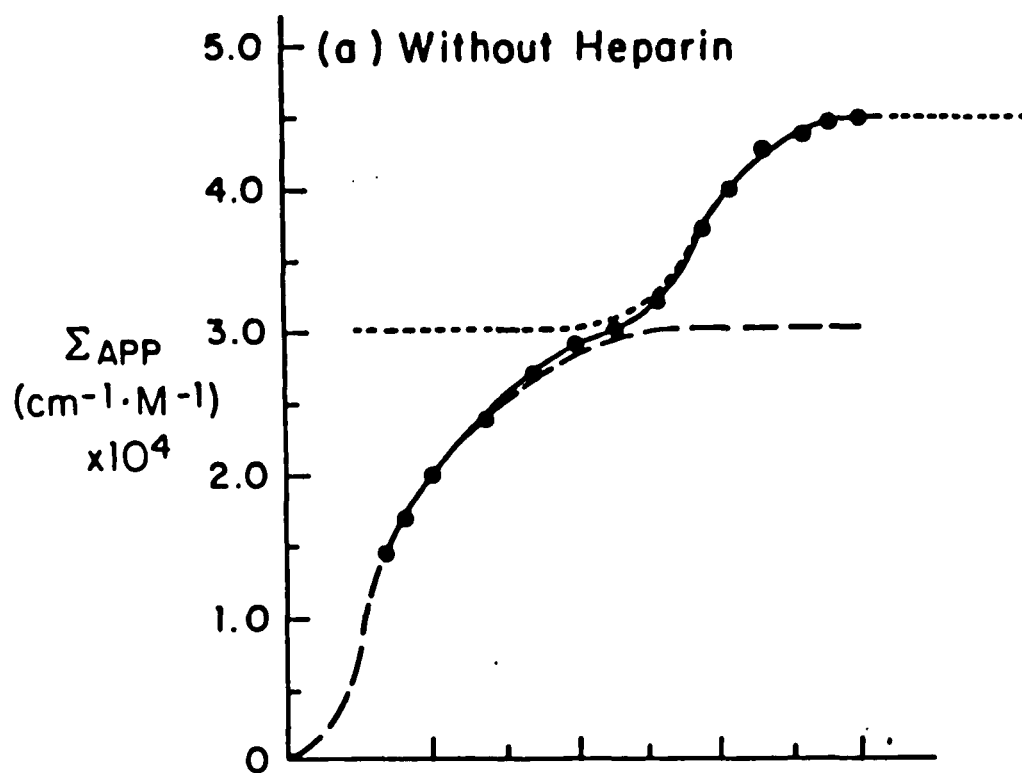
Investigations into the mechanism by which heparin influences the rate of AuO hydrolysis were begun with an examination of the pH dependence of the reaction, and heparin's effect on this rate profile. The acidity of the medium was varied using standardized HCl solutions, rather than a low pH constant ionic strength buffer. This was necessary for two reasons: i) no buffer is possible for the higher acidity region, $[H_3O^+] > 10^{-1}M$, which had to be investigated because it provided important data regarding the mechanism of the hydrolysis reaction; ii) in order to study the pH dependence of the heparin-catalyzed reaction under the same conditions as the non-catalyzed reaction, and yet maintain the influence of heparin at its optimal level, it was necessary to keep the ionic strength as low as possible.

When performing this experiment, it quickly became apparent that the initial A_{430} of the reaction varied significantly with both the HCl and heparin concentration. In order to convert rate data from units of $\Delta A_{430}/\text{minute}$ to units of moles AuO hydrolyzed/liter minute, it is necessary to know the reactant's extinction coefficient, or at least its apparent extinction coefficient, for each set of experimental conditions. At first attempts were made to accomplish this by extrapolating kinetic plots of the reaction to zero time, and then calculating an apparent extinction coefficient from the initial A_{430} value obtained. However at the higher acid concentrations, the initial rates were so large and non-linear that

the Σ_{430} thus graphically calculated showed too much of a variability to be useful. It was therefore necessary to use a stopped-flow spectrophotometer to determine the initial absorbance.

The apparent extinction coefficient of AuO at 430 nm (Σ_{App}) under experimental conditions similar to those used to demonstrate the pH dependence of the reaction, is graphically presented in Figures 8a and 8b. These values were determined from zero time A_{430} measurements made in the stopped flow chamber (as described in section IIB3). From the measured absorbance and known analytical concentration of AuO, the value of Σ_{App} is calculated and plotted as the ordinate. The abscissa is plotted as the $-\log$ of the analytical HCl concentration, rather than as a pH, because of the acidity range being considered (10^{-3} - 2 M HCl). It would not be strictly correct to use either the pH scale or an acidity function (e.g. the H_0 acidity scale) over this concentration range. It is true however, that $-\log[HCl]$ is approximately equal to both the pH and H_0 acidity scales for HCl concentrations within this range, which represents the limits of the respective scales (94). Higher acid concentrations were not studied because of the breakdown of this approximation at acid concentrations greater than 2 M (for HCl). In any event, the salient features of the reaction rate's dependence on acidity are observed within this range. For the sake of convenience, acidity values will still be referred to in terms of pH.

Figure 8: Σ_{App} as a function of pH for free and heparin-bound Au0.
 A_{430} was measured on solutions containing $2.1 \times 10^{-5} \text{M}$ Au0
with - (a) no addition; (b) heparin added to a final
concentration of 100 ug/ml.



A second series of samples was also prepared, expressly for the purpose of comparing the calculated analytical pH with the potentiometrically measured pH (using a glass combination electrode) (Table II, section IIB6). For HCl concentrations between 0.5 and 3×10^{-3} M, there was excellent agreement between the calculated and measured values (within 0.1 pH units). At higher acidities the glass electrode could not be used. At the lower acidities (pH > 2.8) there was still good agreement between the calculated and measured pH for the samples containing only AuO; but for the samples containing heparin (100 ug/ml), the measured pH was consistently higher than the calculated pH in this low acidity range. This is due to the fact that as the heparin and HCl concentrations become comparable protonation of the iduronate groups on heparin (pKa = 5.1 (95), can absorb enough protons to elevate the measured pH. For such samples, the pH plotted is that measured, rather than the calculated value.

Several important points are to be noted in this figure. First, it is quite apparent that the form of this curve is dramatically changed by heparin in the pH 1.5-3 acidity range. Whereas the extinction coefficient of AuO in the presence of heparin is either constant or slightly increasing with decreasing pH in this range, the extinction coefficient of free AuO is sharply decreasing with decreasing pH. At acidities greater than 6.3×10^{-2} M HCl (pH 1.2), the two curves superimpose. The reason for these differences, as well as the chemical origins of the observed spectral changes,

will be examined in greater detail in the next section. For now it suffices simply to know what value to use for the extinction coefficient when converting rate data from units of $\Delta A_{430}/\text{minute}$ to that of moles AuO hydrolyzed/liter minute.

Reaction rates thus calculated are plotted in Figure 9. The tremendous influence of acidity, readily apparent from the figure, is quite instructive regarding the mechanism of AuO hydrolysis. At pH values of 2.6 and above, the reaction rate for free AuO is immeasurably slow within the timescale of a kinetic recording (and thus to the best of our knowledge, is insensitive to pH). As the acidity of the medium is increased, the reaction undergoes a pronounced rate acceleration in a pH dependent fashion, increasing almost 300-fold between pH 2.5 and 1.0. Further increases in acidity result in the rate reaching a maximal value, which remains constant over the HCl concentration range from 0.40 M to 1.00 M. Increasing the HCl concentration further results in a rapid decline of the rate with increasing acidity. The pH dependence of the hydrolysis reaction in the presence of heparin (100 ug/ml), under otherwise identical conditions, can be described quite similarly, and in fact the curves superimpose at pH values below 1.2. However at higher pH values, not only is a significant rate acceleration due to heparin apparent, but in fact even the shape of the curve depicting the pH influence on the reaction rate is altered. In comparison to the curve for the non-catalyzed reaction in this pH range, the curve for the heparin catalyzed reaction is more linear

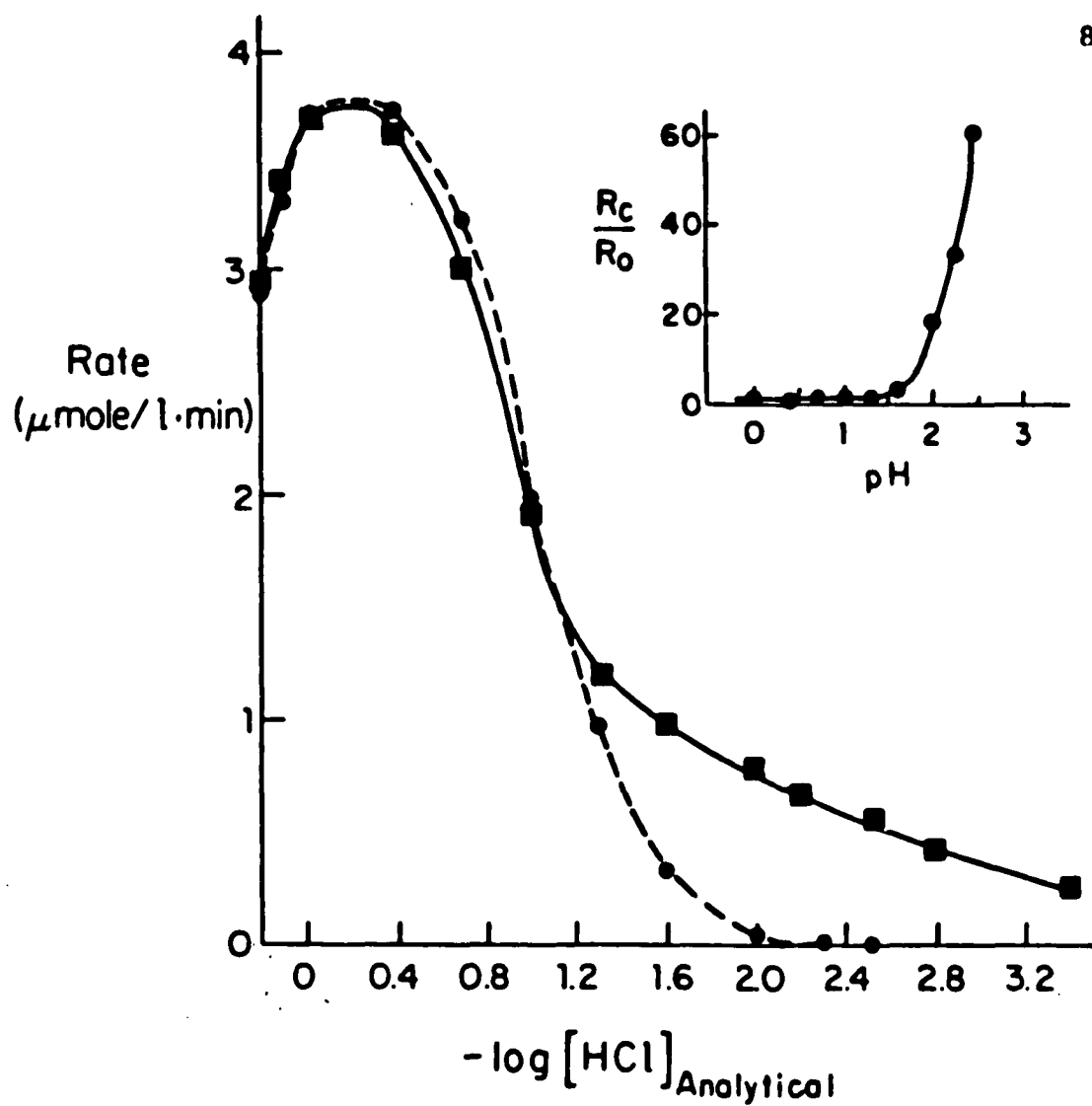


Figure 9: The rate of AuO hydrolysis as a function of pH;
influence of heparin. $(\text{AuO}) = 4.0 \times 10^{-5} \text{ M}$, ● - no heparin
added, ■ - heparin added to a final concentration of
100 $\mu\text{g/ml}$. Reaction conditions as described in the text.

and of shallower slope. Furthermore, the rate accelerating influence of heparin (R_c/R_o) is itself a function of pH, increasing dramatically as the pH is raised above 2.0, (see the insert to Figure 9). It is instructive to compare the way pH affects the reaction rate and Σ_{430} of AuO (Figures 8 and 9). Several parallels can be drawn between the way in which these two parameters vary with pH: i) The influence of heparin is only apparent in the same pH region of both curves (pH 1.2-3); (ii) for free AuO, both its extinction coefficient and reactivity begin to change at about the same pH (iii) in both figures, a crossover occurs between the curves obtained in the presence and absence of heparin, at approximately the same pH. The meaning of these parallels, and a fuller understanding of the mechanisms involved in both acid and heparin catalysis of AuO hydrolysis, requires a deeper understanding of the spectral properties of AuO, and the acid and heparin influence thereon.

E. Dependence of Auramine O's spectral characteristics on acidity;
influence of heparin.

In order to better understand the dramatic heparin-induced changes in the pH dependence of AuO's extinction coefficient (Fig. 8), the investigation of AuO's spectral properties was extended to a higher pH range, wherein the hydrolysis rate was slow enough to enable visible absorption spectra measurement on an ordinary spectrophotometer. In order to maintain consistency with the previous data, experiments were carried out in unbuffered, standardized HCl solutions; but due to the lower HCl concentrations considered, the pH values reported in the figure are those potentiometrically measured. In all other respects, sample preparation methods were the same as those of the previous section.

Figure 10 plots the pH dependence of Σ_{430} over the pH 2-6 range. In essence this figure is complimentary to Figure 8 and differs only in the method of spectral measurement and the pH range considered. It illustrates an important point that was not previously evident. In Figure 8, a sharp drop in Σ_{430} at about pH 2 was observed for free AuO, whereas in the presence of heparin this drop did not occur until about pH 1.5. This data in itself would lead to the erroneous interpretation that heparin lowers the sensitivity of this spectral parameter to pH, so that greater acidity is required to begin seeing the spectral changes. But examination of Figure 10 reveals that this is not the case, since in

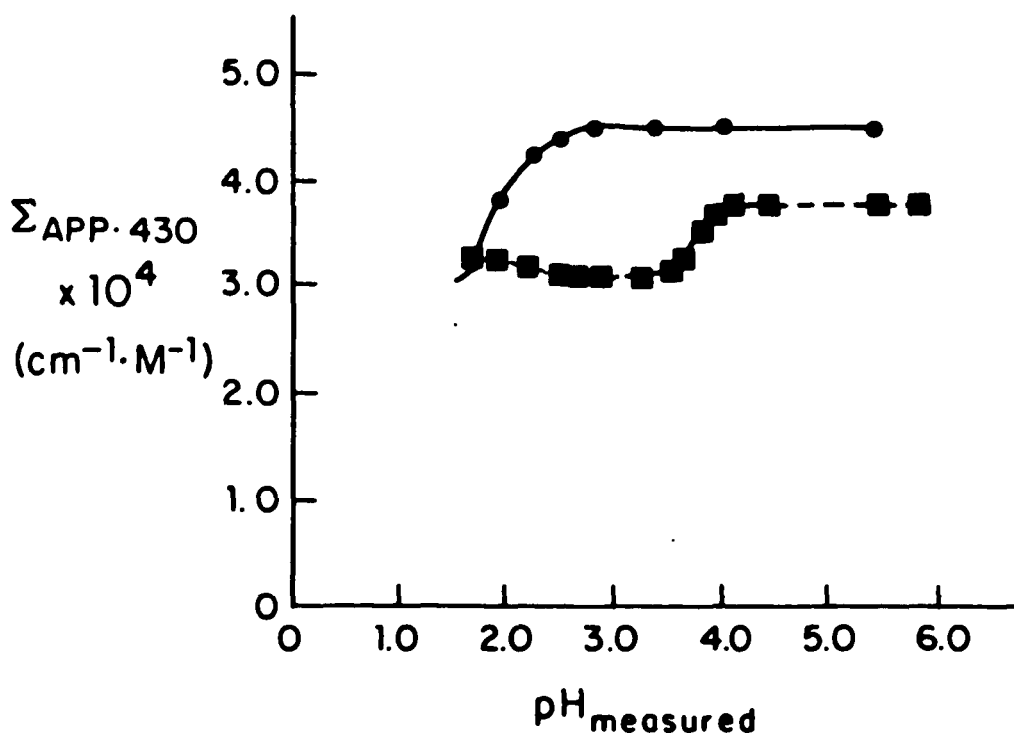


Figure 10: Σ_{app} as a function of pH for free and heparin-bound Au0; pH 1.5 - 6.0 range. A_{430} was measured on solutions containing 2.0×10^{-5} M Au0 with - ●, no addition; ■ - heparin added to a final concentration of 100 ug/ml.

the presence of heparin, a sharp drop in Σ_{430} is observed at about pH 4.0, a pH range wherein Σ_{430} for free AuO is stable and constant. Thus the drop in Σ_{430} of AuO in the presence of heparin at pH 1.5 (Figure 8) must be considered as the second step of a two-stage decline in the dye's extinction coefficient. Furthermore, careful reexamination of Figure 8a reveals that there is evidence of a second inflection point in the curve for free AuO (at about pH 1.0), indicating that here too the drop in Σ_{430} is a two stage process. Thus the only difference is that in the case of the free dye, the two steps are not sufficiently separated for their clear resolution.

The basis for these changes in Σ_{430} can be determined from the visible absorption spectra of AuO at several pH values along the curves of figure 10. These spectra were taken under conditions identical to those used to generate the data of Figure 10, except that the temperature was maintained at 8°C instead of 20°C, to minimize the extent of hydrolysis occurring during the time it takes to record an accurate spectrum. Figure 11 presents these spectra at pH 5.2, 2.9 and 1.6. Only at the lowest pH value did any appreciable hydrolysis occur during the time (about 3 min) it took to record the spectra (about 4 percent of the AuO hydrolyzed for the sample containing heparin, and 2 percent for the sample not containing heparin). At pH 5, free AuO displays two prominent peaks in its visible absorption spectra, at 430 nm and 368 nm. Heparin-bound AuO displays a qualitatively similar spectrum, but

Figure 11: Visible absorption spectra of 2×10^{-5} M Au0
as a function of pH. ● , pH 5.2; ■ pH 2.9;
▲ pH 1.6.
(a) without heparin.
(b) with heparin added to a final concentration
of 100 ug/ml.

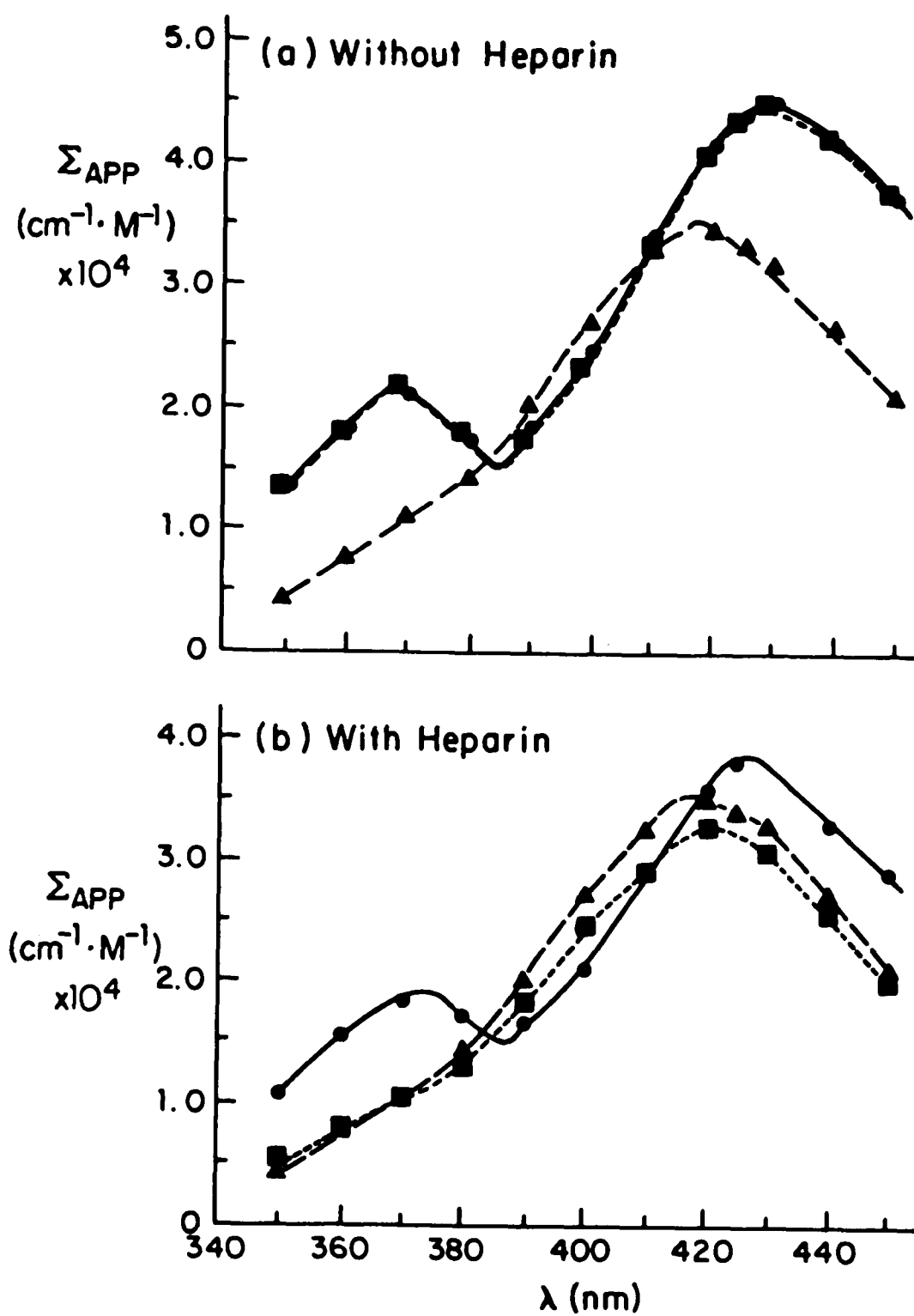


Figure 11

but a slight shift of the peaks to 427 nm and 371 nm. In addition, the bound dye shows a pronounced hypochromism throughout the spectrum. Lowering the pH to 3.0 has no effect on the free dye, but for heparin-bound AuO a dramatic change is observed. There is now only one peak in the visible spectrum (at 420 nm), and the overall oscillator strength of the absorption band has markedly decreased. At pH 1.6, the free and bound AuO now both have the same spectrum, characterized by a single peak at 419 nm and a decreased oscillator strength. The important point to note here is that whereas the spectrum of free AuO is constant in the pH 3.0-5.0 range and only changes as the pH is decreased to 1.6, that for heparin-bound AuO changes dramatically between pH 3.0 and 5.0, and is relatively constant between pH 1.6 and 3.0.

The plot of Σ_{430} vs pH for heparin-bound AuO (Figure 10) is in the form typical for a titration curve involving protonation of a single group, with the protonated species having an Σ_{430} of $3.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and the unprotonated species having an Σ_{430} of $3.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. If this is indeed the case, then the pK of the group involved can be determined from a plot of $\log (\Sigma_{\text{AuO}^{2+}} - \Sigma_{\text{APP}} / \Sigma_{\text{APP}} - \Sigma_{\text{AuO}^{1+}})$ vs pH (Figure 12), where $\Sigma_{\text{AuO}^{2+}}$ is the extinction coefficient of the diprotonated species, $\Sigma_{\text{AuO}^{1+}}$ is that for the monoprotonated species, and Σ_{APP} is the apparent extinction coefficient observed for a mixture of the two. The zero intercept of this plot, and hence the pK_a of the group involved, is 3.85.

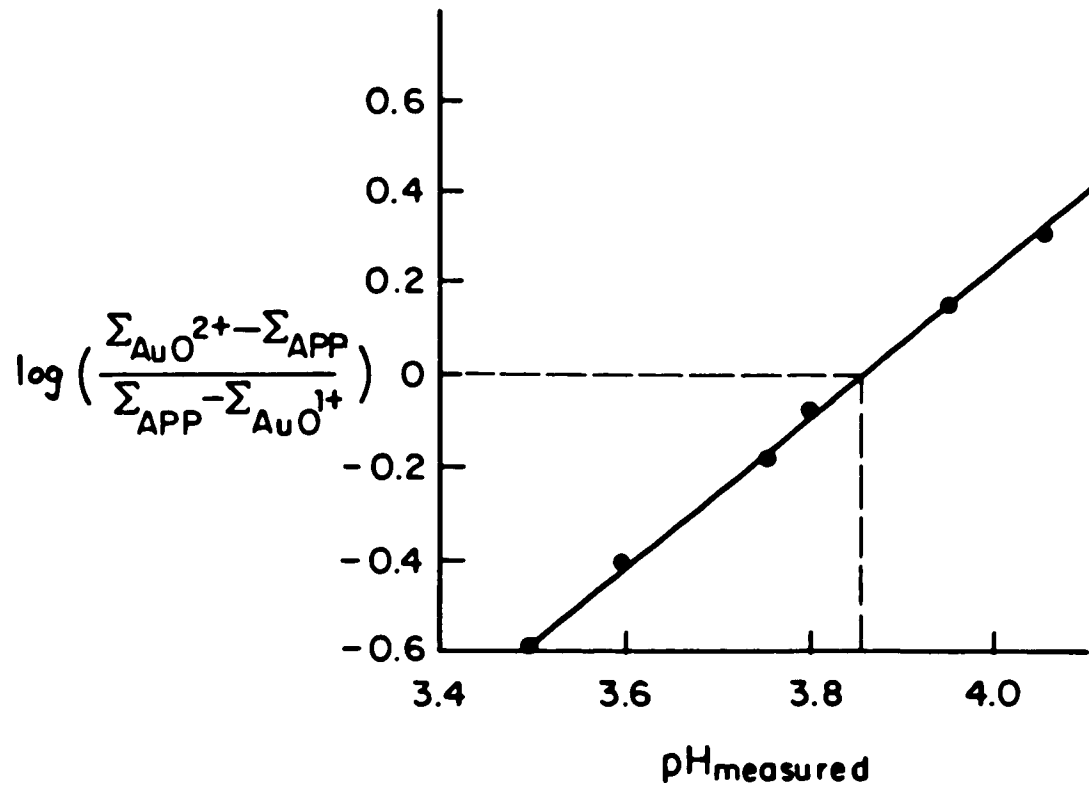


Figure 12: Log plot of the data in figure 10.

There is another aspect of figure 10 and 11 which needs to be elaborated. At pH values greater than 4.5, i.e. before the putative protonation has occurred, there is still a quite definite influence of heparin on AuO's extinction coefficient and $\lambda_{\max}$. Thus heparin can also alter the dye's spectral properties in a manner independent of its influence on the protonation equilibrium. Figure 13 illustrates the influence of added heparin on the extinction coefficient of AuO at pH 6 (solid line). The abscissa is plotted as the polymer site to dye ratio (P/D), rather than as some other unit expressing the progressive increase in heparin concentration, because it is the molar concentration of the polymer's monomeric sites that is critical here, rather than the concentration of the macromolecule itself. Polymer site concentration is calculated based on an average monomer molecular weight of 342, determined as described in methods. It is quite apparent from the figure that at pH 6 heparin decreases AuO's extinction coefficient in a manner very dependent on the relative concentrations of heparin sites and dye ions. The metachromasia is maximal at a P/D ratio of approximately 2, and is essentially lost when P/D is increased beyond 100. This behavior contrasts quite markedly with the P/D dependence of metachromasia at acid pH shown in the same figure as a dotted line. In this case the metachromasia doesn't reach its maximal value until a P/D ratio of approximately 30, and then remains relatively constant as P/D is further increased. This indicates that the molecular basis for heparin's metachromatic influence on AuO is quite different at the two pH values.

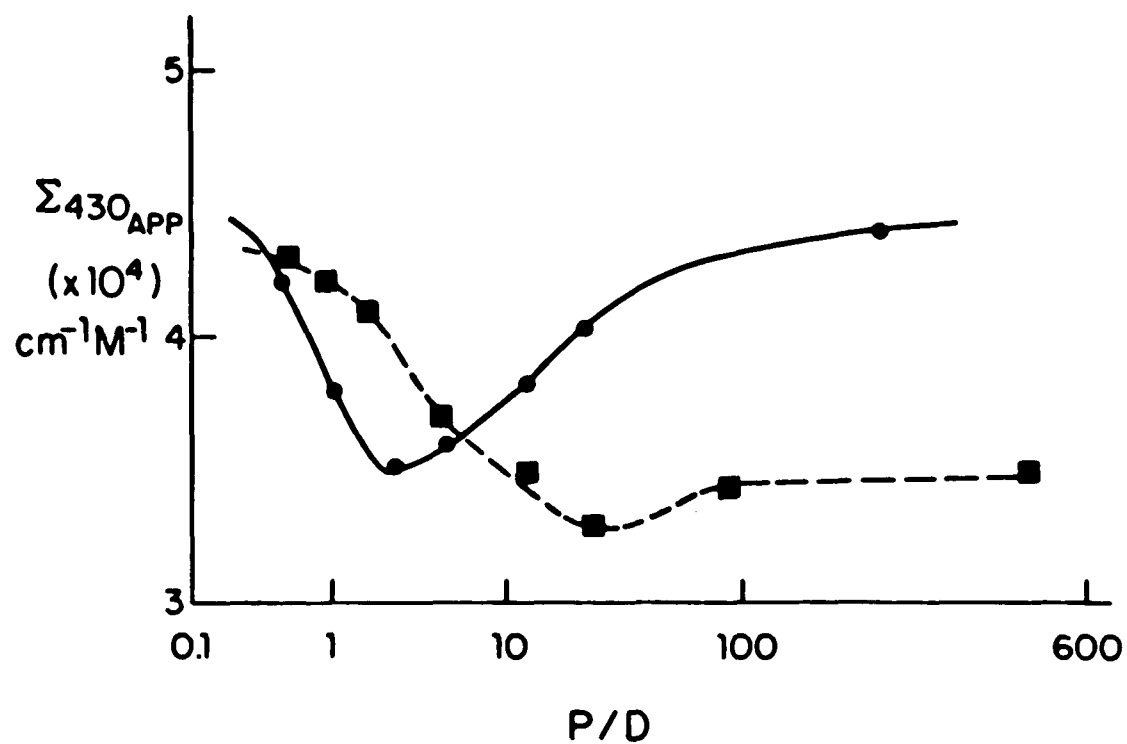


Figure 13: Dependence of Σ_{430_APP} on the P/D ratio at neutral and acidic pH. (Au0) constant at $3.3 \times 10^{-5} \text{ M}$; solid line - no exogenous HCl added, pH measured at 6.04; dashed line - containing 0.003M HCl, pH measured at 2.54.

F. The influence of AuO concentration on the rate of the catalyzed and non-catalyzed reaction.

The influence of concentration on rate is the standard diagnostic tool used to determine the order of a reaction with respect to a particular reactant. Furthermore, the alteration of reaction order by catalyst, and anomalous dependencies on substrate concentration in the presence of catalyst, can often yield insight into the nature of the catalytic complex. Therefore, in this section the reaction rate as a function of AuO concentration will be determined, at constant concentrations of heparin and HCl. The experiment was performed at pH 2.5 (measured potentiometrically) so that the rate of the non-catalyzed reaction would proceed at a measurable rate.

Rates were determined over an AuO concentration range from 6.1×10^{-6} M to 3.6×10^{-4} M, at final heparin concentrations of 0, 5 ug/ml and 16.5 ug/ml. The data obtained is directly plotted in Figure 14a. In the absence of heparin a straight line is obtained over the entire concentration range, implying that the reaction rate is proportional to AuO concentration, taken to the first power. The first order nature of the non-catalyzed reaction with respect to AuO is confirmed in the log-log plot presented in Figure 14b, which gives a straight line with unit slope and a correlation coefficient of 0.998.

In the presence of heparin, a quite different picture emerges with respect to the order of the reaction. At low AuO concentrations the rate of the catalyzed reaction increases linearly, just as it did for the non-catalyzed reaction, though the slope is steeper and proportional to the heparin concentration (Figure 14a). The log-log plot of the data at 16.5 ug/ml heparin (Figure 14b) shows linearity and unit slope up to 2×10^{-5} M AuO, indicating that the reaction is still first order with respect to [AuO] in this concentration range. However as the AuO concentration is further increased, the rate levels off and then actually decreases with increasing AuO concentration. This is reflected by the constantly decreasing slope of the log-log plot in this concentration range, which goes through zero and eventually reaches negative values. When the rate data are plotted on a semi-log scale this anomalous behavior is accentuated (figure 14c). With 5 ug/ml heparin, the maximal rate is reached at about 5×10^{-5} M AuO; with 16.5 ug/ml heparin, the maximal rate is reached at about 1.5×10^{-4} M AuO. Thus the AuO concentration giving a maximal rate is directly proportional to the concentration of heparin present. In both cases, increasing the AuO concentration further results in a rate decrease so sharp that the rate of the non-catalyzed reaction surpasses that of the catalyzed reaction. This precipitous drop in rate would not be expected to occur if it simply represented saturation of the catalyst. Rather it would seem to represent a real discontinuity in the reactivity of the bound AuO.

Figure 14: The influence of AuO concentration on reaction rate in the presence and absence of heparin: The reaction was run at a constant pH of 2.5 (± 0.04) as potentiometrically determined on a group of samples. Rates were measured as described in section IIB1, except at the higher AuO concentrations, where paired quartz spacers were used to shorten the pathlength of standard 1cm cells. ■ - no heparin added; o - heparin added to a final concentration of 5ug/ml ($1.6 \times 10^{-5}M$); ● - heparin added to a final concentration of 16.5ug/ml ($4.8 \times 10^{-5}M$).

- (a) Direct plot of rate vs concentration data.
- (b) Log-log plot for determination of reaction order.
- (c) Semi-log plot of the rate data.

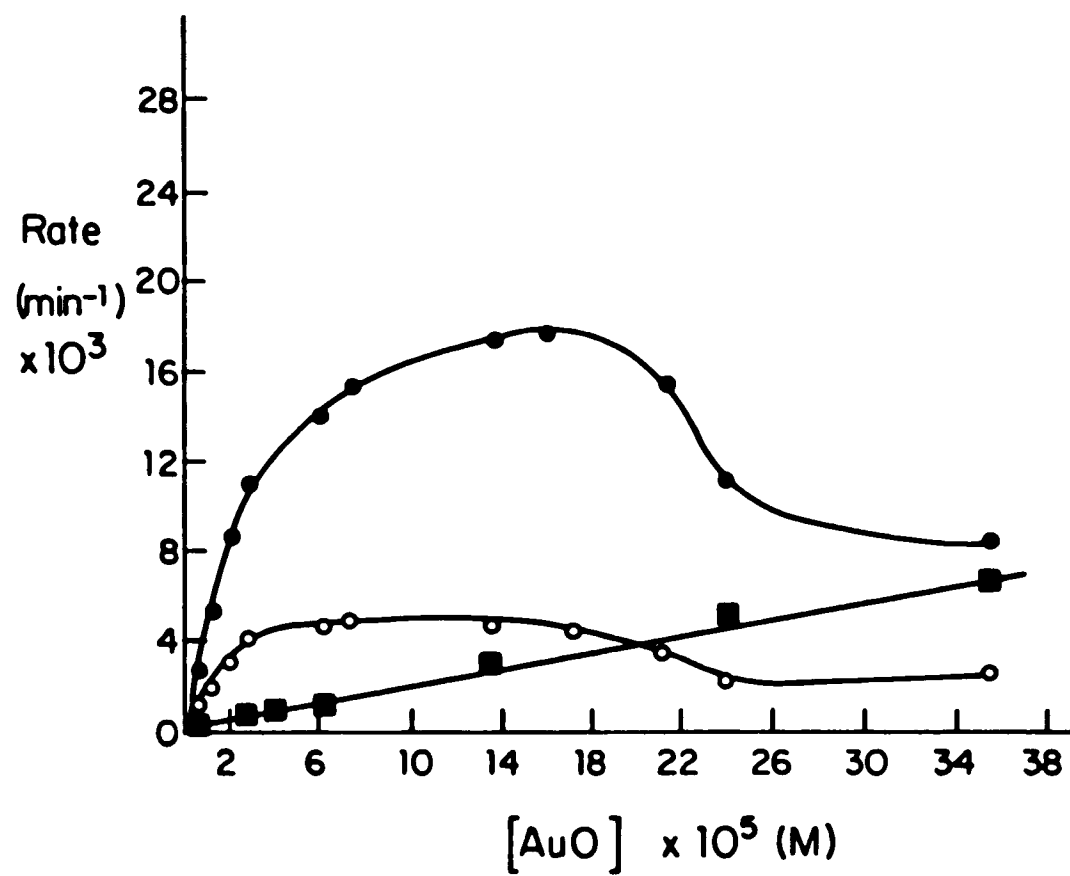


Figure 14a

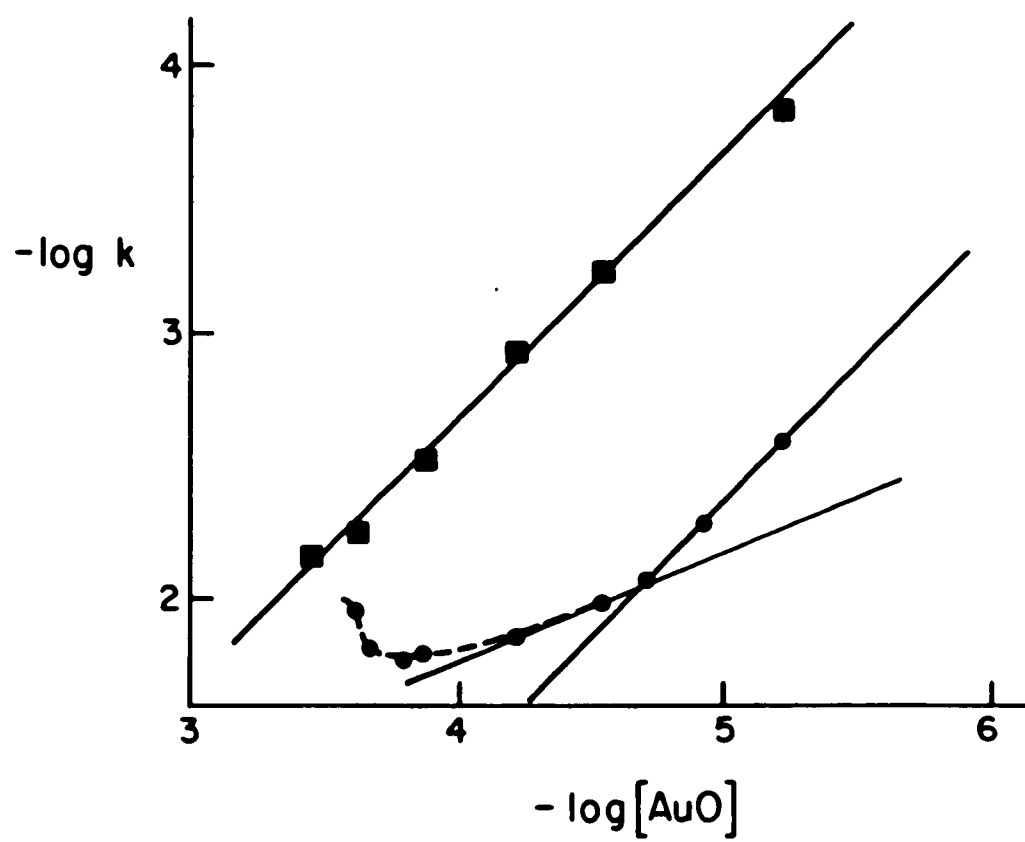


Figure 14b

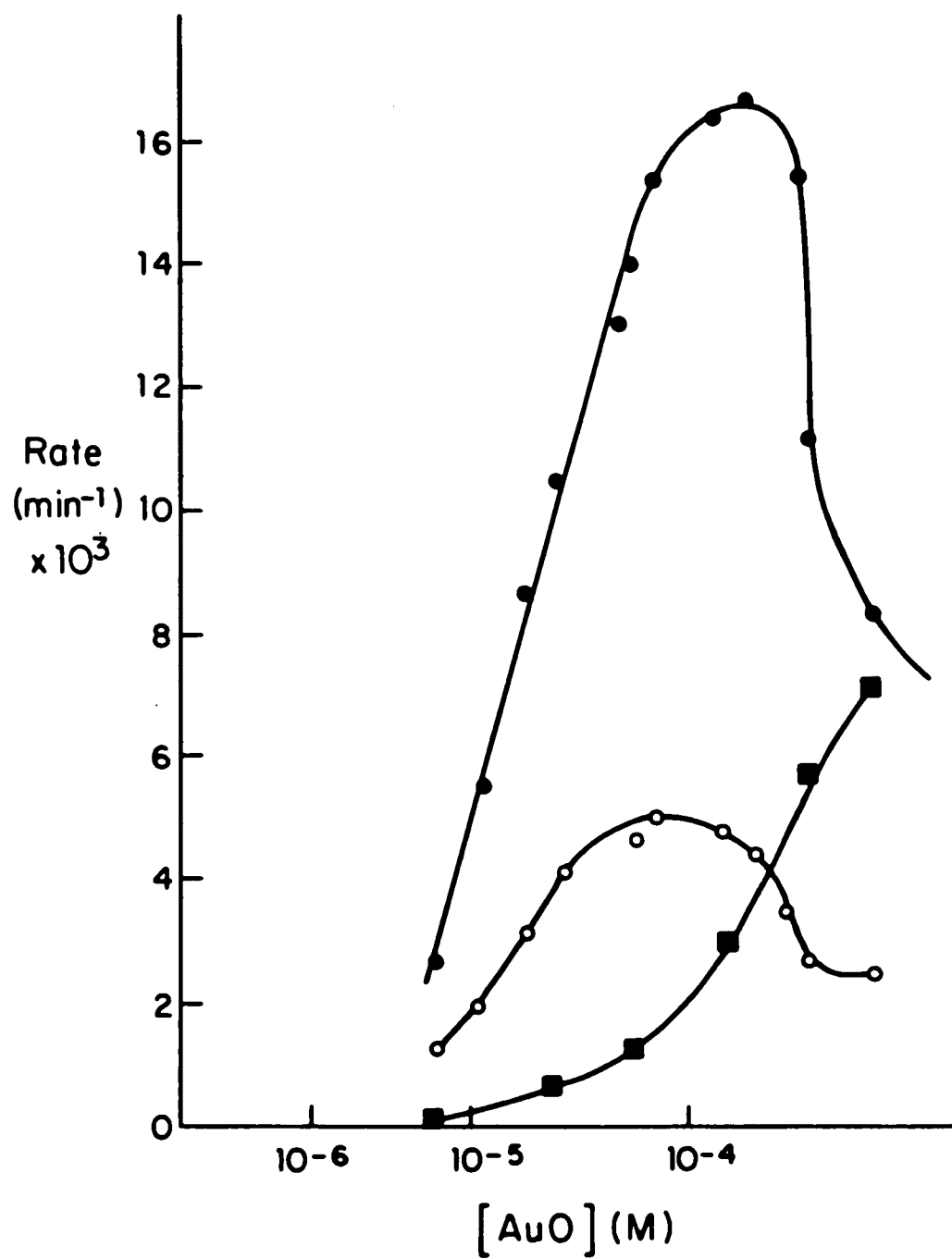


Figure 14c

To determine whether this discontinuity might be due to product inhibition, an experiment was performed to determine the extent to which MK would inhibit the catalyzed reaction (16.5 ug/ml heparin) in its zero order concentration range. Using a 5.0×10^{-3} M solution of Michler's ketone in ethanol, a 2.5×10^{-4} M AuO solution is made 1.2×10^{-5} M and 2.5×10^{-5} M, or 5 percent and 10 percent respectively, in Michler's ketone. A control with ethanol only added to the AuO is simultaneously prepared for comparison. The hydrolysis rates for these samples are measured in triplicate, and the results presented in Table V. It is apparent from the small effects observed in this table, that the precipitous drop in rate is not due to product inhibition.

Insight into the origins of AuO's inhibitory influence on the catalysis can be gained by replotting the data of Figure 14 in terms of the heparin site to dye concentration ratio (P/D). In Figure 15, wherein the rate of the catalyzed reaction is plotted vs the polymer site to dye ratio (P/D), an important point which was not evident in Figure 14 becomes readily apparent. It is the P/D ratio rather than the absolute AuO concentration, to which the catalysis is so sensitive. When thus plotted, the dependence of catalytic activity on AuO concentration for the two heparin concentrations examined are completely parallel, unlike the situation in Figure 14 where the two curves are displaced relative to each other along the concentration axis. The sharp loss of catalytic activity occurs at a P/D ratio of approximately 0.3 (Figure 15a), and is independent of both heparin and AuO

concentration. In Figure 15b the catalytic rate enhancement (R_c/R_o) rather than the actual rates, are plotted vs P/D. Presentation of the data in this manner, demonstrates that at a given heparin concentration the catalytic influence of heparin is constant and independent of the P/D ratio as long as the concentration of heparin sites is in sufficient excess. Thus although figures 15a and 15b present no new data, they are extremely instructive regarding the importance of the P/D ratio to the mechanism of heparin's catalytic influence.

TABLE V

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Effect of product inhibition by Michler's ketone on the catalyzed reaction: Rates were determined as described for figure 14, and were limited to the zero order (with respect to AuO) concentration range. The reaction was set up as described in the text, at a pH of 2.5, AuO concentration of $1.6 \times 10^{-4}M$, and heparin concentration of $4.8 \times 10^{-5}M$.

	<u>Rate</u> ($\text{min}^{-1} \times 10^{-3}$)
control - only ethanol added to 0.5%	16.3 $\pm$ 0.7
MK added to a final concentration of $1.2 \times 10^{-5}M$	15.9 $\pm$ 0.6
MK added to a final concentration of $2.5 \times 10^{-5}M$	15.7 $\pm$ 0.5

Figure 15: The influence of the P/D ratio on the catalyzed reaction. Rate data from figure 14 was used, and P/D determined for the points corresponding to the - ● , $4.8 \times 10^{-5}M$ heparin curve; ■ $1.6 \times 10^{-5}M$ heparin curve.

- (a) Reaction rate as a function of the P/D ratio.
- (b) Catalytic rate enhancement as a function of the P/D ratio.

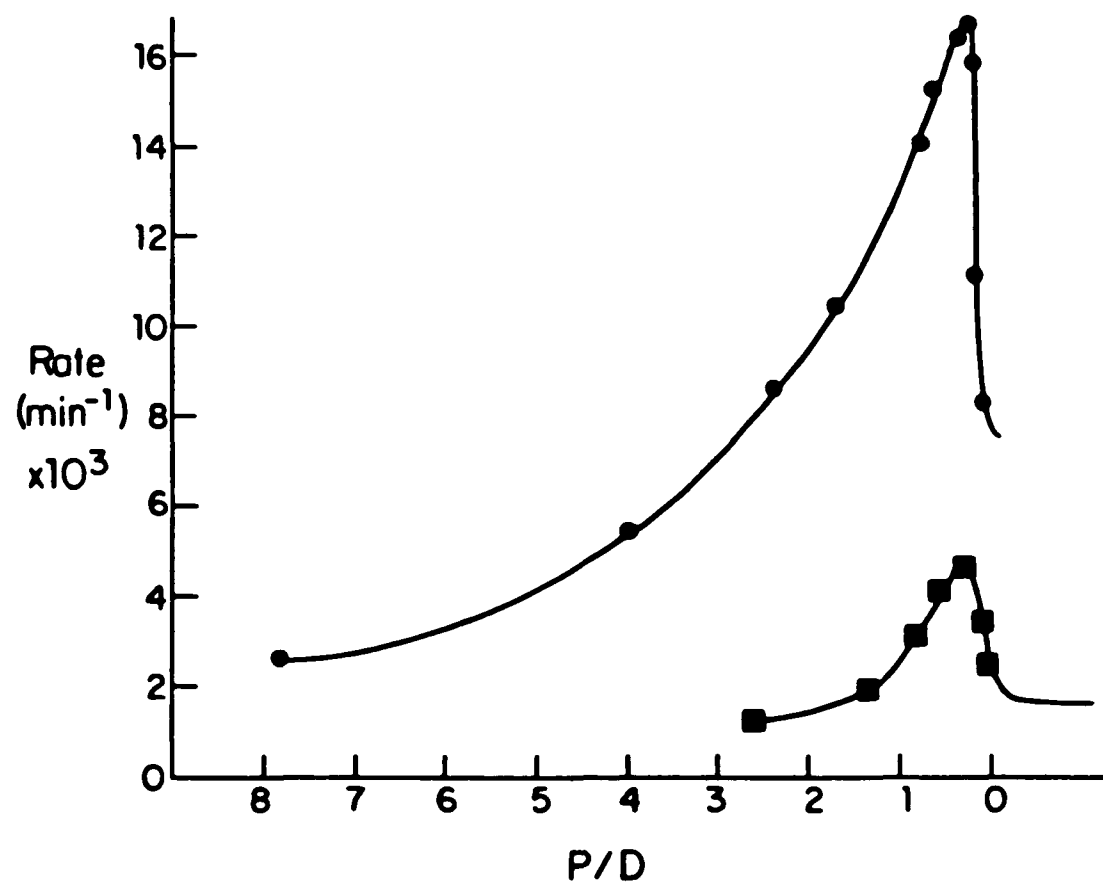


Figure 15a

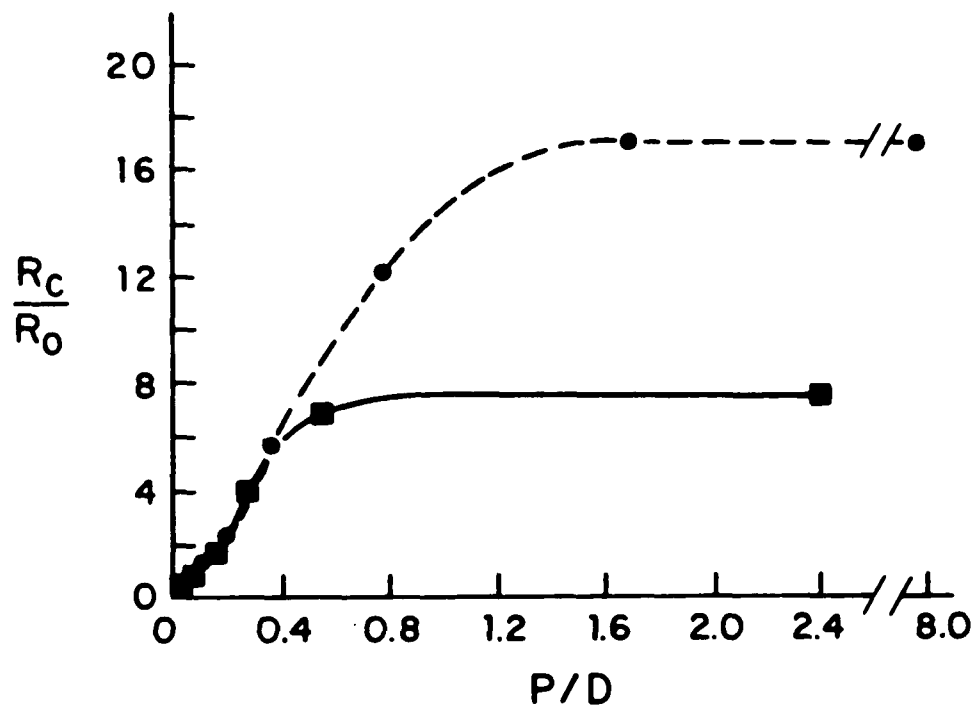


Figure 15b

G. Rate Dependence on Heparin Concentration.

In this section the dependence of AuO's hydrolysis rate on the concentration of added heparin will be examined. Although this dependence can serve as the basis for a sensitive and specific chemical assay of heparin (see Appendix I), this will not be the focus here. Rather this dependency will be examined over a heparin concentration range extending beyond the applicability limits of the assay, in order that an important point with respect to the mechanism of heparin's catalysis can be demonstrated.

This experiment was carried out on solutions having a measured pH of 2.6 ($.0025 \text{ M H}_3\text{O}^+$), and a final Auramine O concentration of $3.1 \times 10^{-5} \text{ M}$. Heparin dilutions were prepared from a 20 mg/ml ($5.8 \times 10^{-2} \text{ M}$) stock solution of the dried sodium salt. In general the .0060 N HCl stock solution was used for the preparation of these reaction mixtures. However for final heparin concentrations of $7.0 \times 10^{-4} \text{ M}$ and greater, the measured pH of the reaction mixtures was found to vary significantly from 2.60 (measured pH was 2.67 in this case and 2.97 for the $2.8 \times 10^{-3} \text{ M}$ heparin). This behavior is due to titration of the iduronate groups of the added sodium heparinate, which have a reported pKa of 5.1 (95). As the heparin concentration becomes comparable to the hydronium ion concentration, the removal of protons from solution by this group becomes measurable. The stoichiometry of the change in pH bears out this interpretation. For example the measured pH of 2.97 with the $2.8 \times 10^{-3} \text{ M}$ heparin corresponds to a ΔpH of 0.37,

indicating the uptake of 1.4×10^{-3} M H_3O^+ , which is exactly half of the heparin molarity, as would be expected based on the copolymeric nature of heparin. In order to maintain a pH of 2.6 in the reaction mixture, a more concentrated HCl stock solution had to be prepared, which would take this effect into account. The following equation:

$$\frac{[\text{HCl}] \text{ stock}}{2.1} - \frac{[\text{Heparin}] \text{ final}}{2} = .0025$$

was derived to enable calculation of the proper stock HCl concentration to be used with this and higher heparin concentrations, so as to achieve a final pH of 2.6 in the reaction mixture. (The factor 2.1 dividing the stock HCl concentration in to take into account its 1:2.1 dilution in the reaction mixture.) Using this equation it was determined that an HCl stock solution of 0.0082 M was needed for the 2.8×10^{-3} M heparin reaction mixture, and an HCl stock of 0.0347 was needed for the 2.8×10^{-2} M heparin reaction mixture. The HCl solutions were prepared and standardized, as described in the methods section. When the pH of the reaction mixtures thereby prepared were measured, their pH was indeed the expected 2.60 ± 0.1 .

Having overcome the technical difficulty of maintaining the measured pH constant at high heparin concentrations, this experiment could now be performed over a sufficiently wide concentration range to elicit information regarding the mechanism of the catalysis. The data is presented in Figure 16a on a semilogarithmic scale because

of the broad heparin concentration range being considered. The striking feature of this curve is the sharp decline in the rate of hydrolysis when the heparin concentration exceeds 10^{-3} M. It should be noted that this is just slightly below the bulk hydronium ion concentration measured for these samples. Replotting these data in terms of the P/D ratio (Figure 16b) reveals that the maximum catalytic influence of heparin under these conditions occurs at P/D ratio of 30. This is the same P/D ratio that was observed to give maximal metachromasia at acid pH (Figure 13, dotted line). But note the striking difference between Figure 16b and Figure 15b, which only differ in the way the P/D ratio was varied (in this case by varying the heparin concentration, whereas in the latter the AuO concentration was varied). The basis for the very differently shaped plots obtained will be discussed (section IV D4).

Figure 16: The dependence of reaction rate on heparin concentration. Reaction rates were determined as a function of the heparin concentration for samples containing 3.1×10^{-5} M AuO, and maintained at a constant measured pH of 2.6, as described in the text.

- (a) Catalytic rate enhancement (R_c/R_o) as a function of the heparin concentration.
- (b) Catalytic rate enhancement (R_c/R_o) as a function of the P/D ratio.

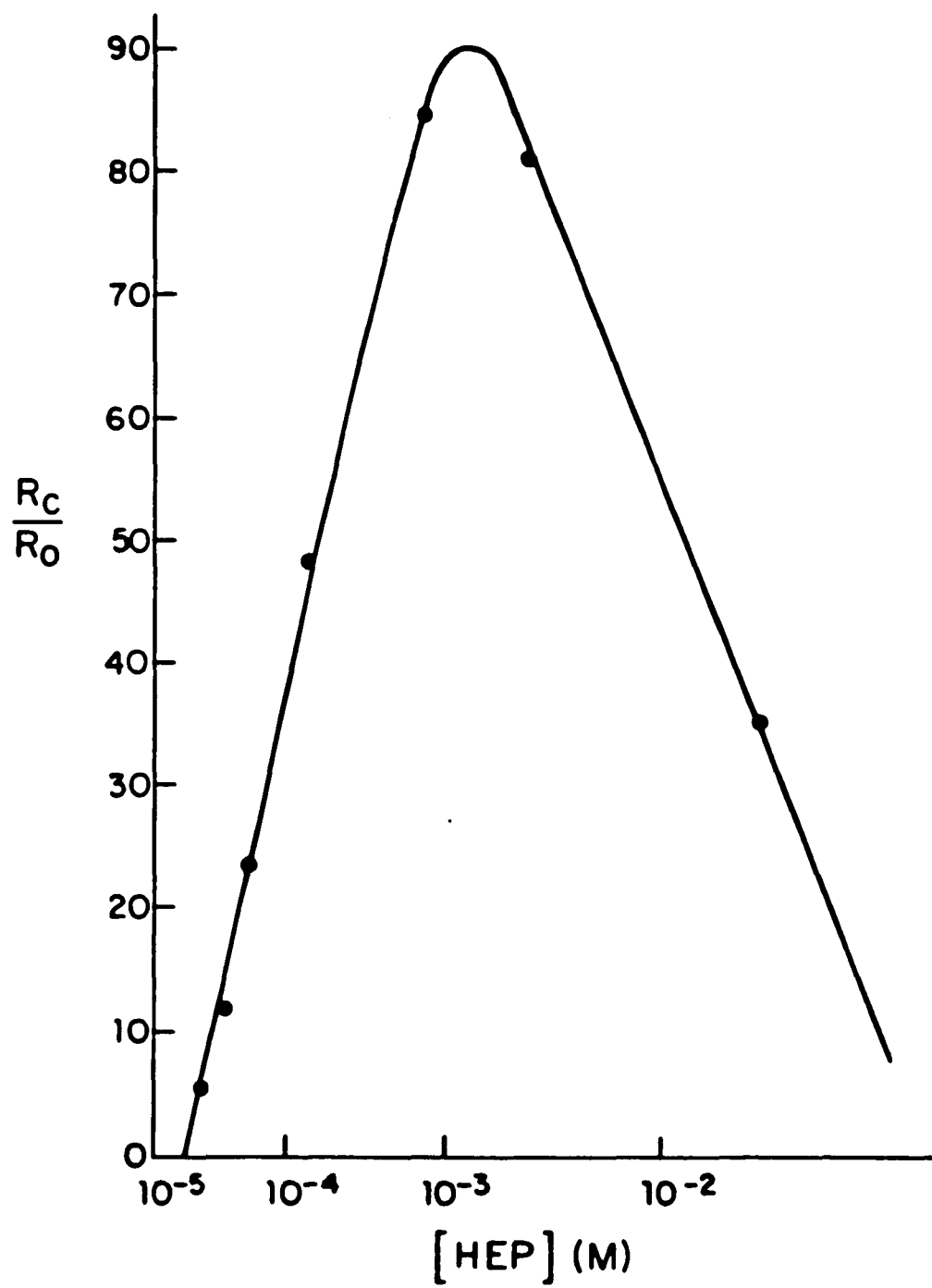


Figure 16a

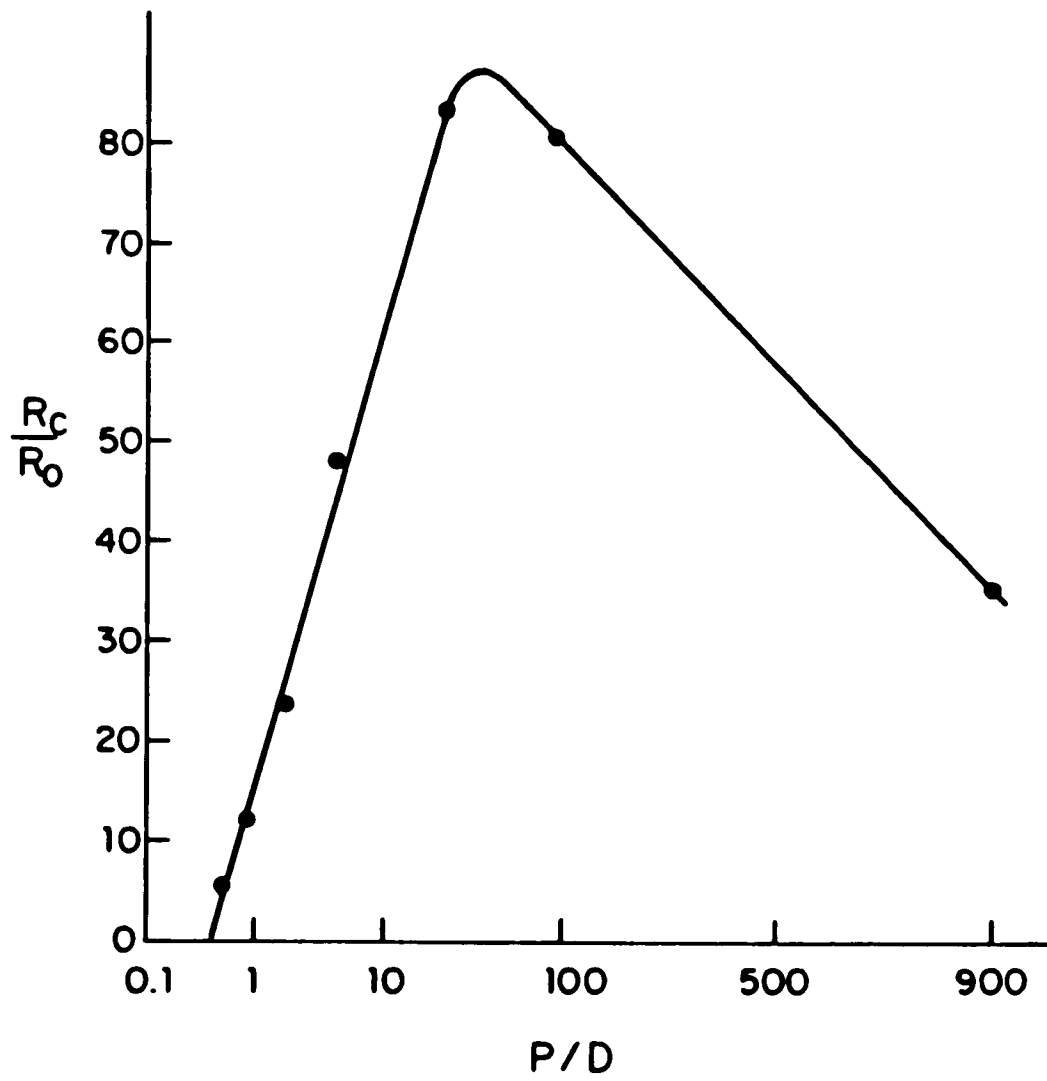


Figure 16b

H. The influence of extraneous ionic solutes on the catalysis.

The rate of AuO hydrolysis is sensitive to the addition of non-reacting salts, both in the presence and absence of added heparin. However the nature of these salt effects are quite different for the two cases, as will be here outlined. It is this difference which necessitated the use of low ionic strength conditions when studying catalysis of the reaction by heparin.

The experiment was set up with a final AuO concentration of 2.5×10^{-5} M, and a final HCl concentration of 0.01 M. Under these conditions the rate of the non-catalyzed reaction is large enough to be measured photometrically. Identical samples were prepared with and without 100 ug/ml heparin, containing NaCl over the concentration range 0-0.25 M. The resulting kinetic data is plotted in Figure 17.

In the case of the non-catalyzed reaction, the rate increases in a linear fashion as the sodium chloride concentration is increased. However, for the heparin-catalyzed reaction, a quite different dependence is observed. At the lowest NaCl concentrations tested the reaction rate decreased sharply with increasing NaCl. By 0.04 M added NaCl, the catalytic influence of heparin has been completely lost. Further increases in the NaCl concentration beyond this point influenced the reaction rate in parallel to the salt effect on the non-catalyzed reaction. It is thus quite clear from this data why the catalytic influence of heparin was best studied in unbuffered,

low ionic strength solutions. Extraneous ions in the system cause a leveling of the rates of the catalyzed and non-catalyzed reactions.

The influence of salts on the catalysis is rather non-specific, and dependent mainly on the valence of the positively charged counterion involved. Data demonstrating this fact can be found in Figure 5 of Appendix I, wherein the influence of ionic solutes on a heparin assay based on this reaction is presented. The divalent cations Ca^{++} and histamine are two orders of magnitude more effective than the monovalent Na^+ ion in inhibiting the catalysis. Furthermore, there is little discernable difference in the inhibitory capacity of Ca^{++} and histamine, despite the obviously large structural differences between these two ions. Thus the effect seems primarily electrostatic, with little contribution from more specific forces.

In the case of macromolecular ionic solutes, specificity plays more of a role. Figure 4 of Appendix I illustrates the inhibitory influence which proteins capable of interacting with heparin have on the catalysis. Of the two proteins examined, human serum albumin and human antithrombin III, the antithrombinIII is more effective at inhibiting the catalysis due to its interaction with a specific high affinity site on heparin (18). Conversely, when simple polycationic solutes are considered, such as protamine or polylysine, no difference can be observed with respect to their inhibitory influence on the catalysis. For both these polycations, their addition to the reaction results in formation of a flocculent precipitate with heparin.

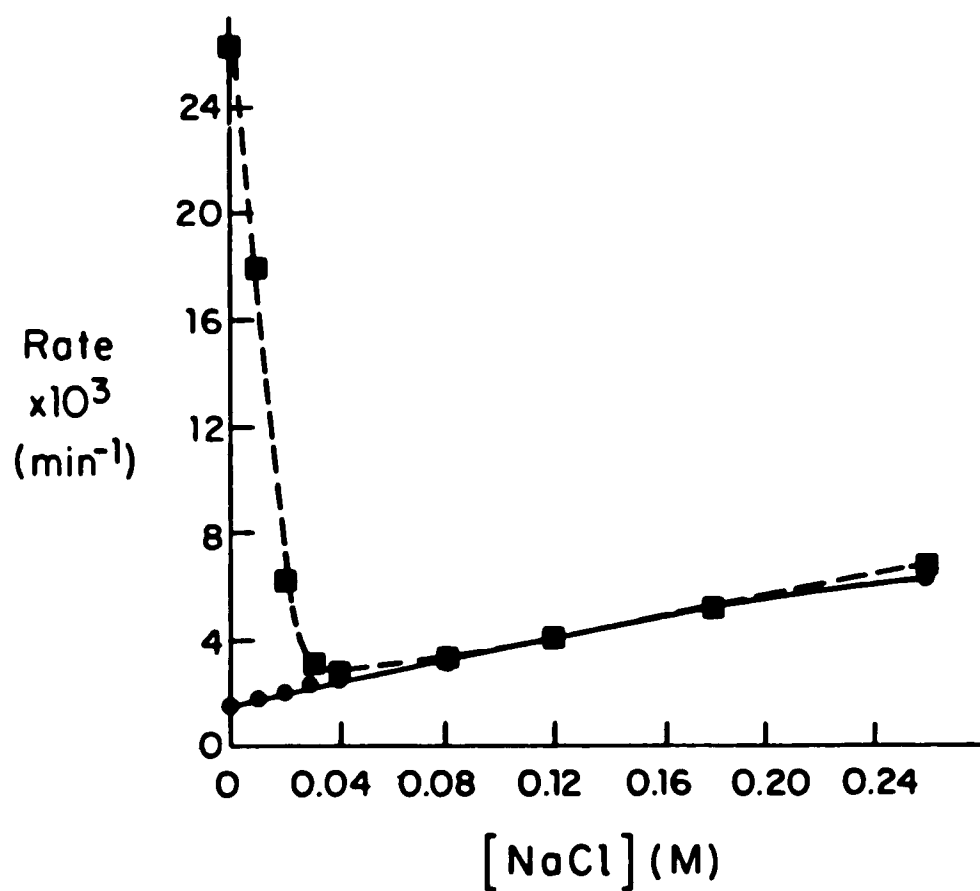


Figure 17: Influence of NaCl on the rate of AuO hydrolysis.

The reaction was run in 0.01M HCl, containing increasing concentrations of NaCl, and AuO at a final concentration of 2.5×10^{-5} M. ● - no heparin added; ■ - heparin added to a final concentration of 100 ug/ml.

I. Temperature dependence of the reaction; influence of heparin on the activation parameters.

In this section the temperature dependence of AuO hydrolysis is determined in the presence and absence of added heparin. From this data the activation energy of the reaction can be calculated, and the heparin influence thereon demonstrated. The experiment was performed at a constant pH of 2.6 so as to enable accurate rate measurements for the non-catalyzed reaction, while still optimizing the catalytic influence of heparin. Heparin concentration was maintained at 20 ug/ml ($5.9 \times 10^{-5} \text{ M}$), and AuO concentration at $3.5 \times 10^{-5} \text{ M}$, throughout the experiment. The data is presented as an Arrhenius plot in Figure 18.

Both the catalyzed and non-catalyzed reactions give Arrhenius plots which are quite linear (correlation coefficients greater than 0.98), indicating that the activation energies are relatively temperature independent over the temperature range considered. For the catalyzed reaction a slope of -2686 K° is obtained, yielding a calculated activation energy of 12.3 Kcal/mole. For the non-catalyzed reaction a slope of -3400 K° is obtained, yielding a calculated activation energy of 15.6 Kcal/mole.

From the intercept of the Arrhenius plots, A, the frequency factor of the reaction, can be determined. The intercepts are

obtained from the linear regression program on a Texas Instruments calculator. Then using the relationship (96):

$$A = \frac{eRT}{h} \exp \left(\frac{\Delta S^\ddagger}{R} \right)$$

$\Delta S^\ddagger$ at 25°C for the noncatalyzed reaction is calculated to be -25 eu, and for the catalyzed reaction, -30 eu.

$\Delta H^\ddagger$ of the reaction can also be calculated from the empirical Arrhenius activation energy, using the formula (96):

$$\Delta H^\ddagger = E_{\text{act}} - RT$$

For the noncatalyzed reaction a value of $\Delta H^\ddagger = 15.0$ Kcal/mole is thus calculated; for the catalyzed reaction, $\Delta H^\ddagger = 11.7$ Kcal/mole.

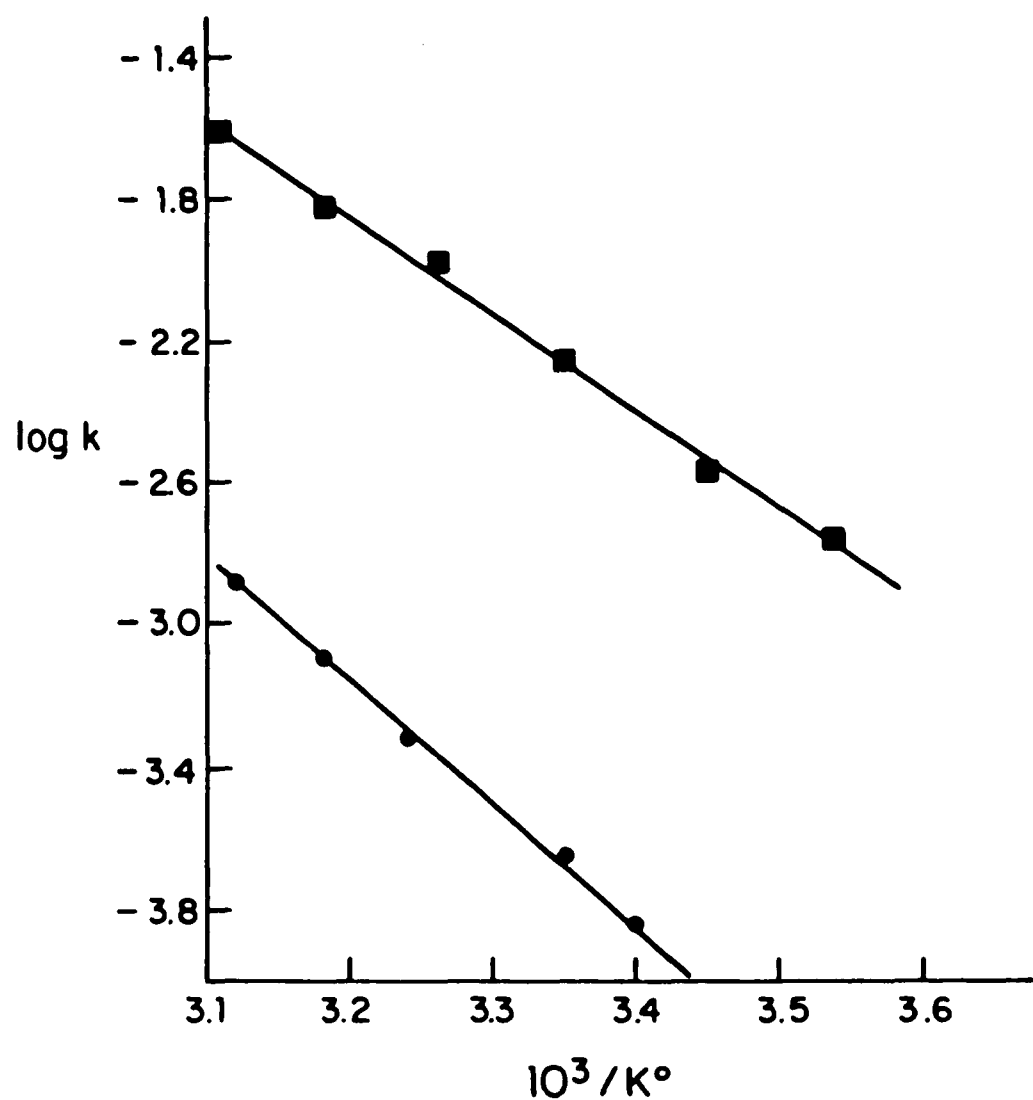


Figure 18: Arhenius plot of the temperature dependence of AuO hydrolysis. Reactions were run at pH 2.6, 3.5×10^{-5} M AuO, containing - ●, no added heparin; ■, heparin added to a final concentration of 200 μg/ml.

J. The influence of molecular weight on heparin's catalytic activity.

Two basic questions need to be answered with respect to the influence of molecular weight on heparin's catalytic activity. The first relates to the heterogeneity characterizing all commercial heparin preparations, which contain species varying in molecular weight from about 6000-30,000. Is there any relationship between size and catalytic activity. The second question relates to the fact that heparin is essentially a repeating copolymer. It is thus significant to ask what constitutes the minimum size oligomeric fragment still possessing catalytic activity. Both these questions are here answered using heparin preparations of defined sizes kindly supplied by other investigators, prepared as described in section IIA2.

The first question is answered using heparin fractions of defined molecular weight ranges, prepared by gel-filtration chromatography on Sephadex G-100 in Tris buffer (pH 7.5) containing 0.15 M NaCl. The eluate was arbitrarily divided into four pooled fractions, whose approximate molecular weight ranges, are listed in Table VI. The specific activities of these fractions were determined by simultaneous measurements of catalytic activity and glucuronic acid content, as described in IIB3. It is clear from the table that no correlation exists between these two parameters, in the molecular weight range here considered. The small differences

observed are most likely a reflection of chemical differences, which have been reported for heparin species in different molecular weight ranges. (97)

The second question, regarding the minimal size heparin oligosaccharide capable of exerting catalytic activity, was determined using fractions obtained from a partial chemical depolymerization of heparin, as described in section IIA2. The specific activity of these heparin fragments is given in Table VII. Though the smallest oligosaccharide tested can still accelerate AuO hydrolysis, in this size range a definite correlation is observed between chain length and catalytic activity. This relationship is graphically illustrated in Figure 19. Based on the data presented in this way, it appears that the minimum size heparin fragment capable of catalyzing the reaction would be the pentasaccharide, and that increasing chain length beyond the hexadecasaccharide causes little further increase in catalytic activity.

It should also be noted that the maximal activity observed for these heparin fragments is significantly lower than would be expected for the parent heparin preparation, based on its anticoagulant activity of 156 units/mg (see section K). This deviation is most likely due to the chemical procedures used to depolymerize the heparin. Despite the attempt to keep these conditions as mild as possible, some hydrolytic cleavage of sulfate residues undoubtedly occurs. Of the various types of covalently bound sulfate on heparin, the N-sulfate is most labile to acid

hydrolysis. It is thus a safe assumption that some of the N-sulfate on these oligosaccharides has been removed, and the resultant lowering of the charge density on heparin reduces its catalytic activity. However no definitive statement can be made regarding the absolute necessity of N-sulfate groups to the catalysis.

TABLE VI

Catalytic activity of the different molecular weight ranges in a commercial heparin preparation. The molecular weight fractions were prepared as described in section IIA2, and specific activity determined as described in IIB1.

<u>Approximate M.W. Range</u>	<u>Specific Activity</u> x 10 ⁴ (min ⁻¹ /ug)
less than 10,000	1.4
10,000 - 14,000	1.4
14,000 - 18,000	1.4
greater than 18,000	1.3

TABLE VII

Catalytic activity of heparin oligosaccharide fragments. Oligosaccharides prepared as described in section IIA2, and specific activity determined as described in section IIB1.

<u>Chain Length</u>	<u>Specific Activity</u> x 10 ⁵ (min ⁻¹ /ug)
hexasaccharide	1.5
octasaccharide	4.0
decasaccharide	5.5
dodecasaccharide	6.3
14-16 saccharide	7.3
low molecular weight pool	7.5

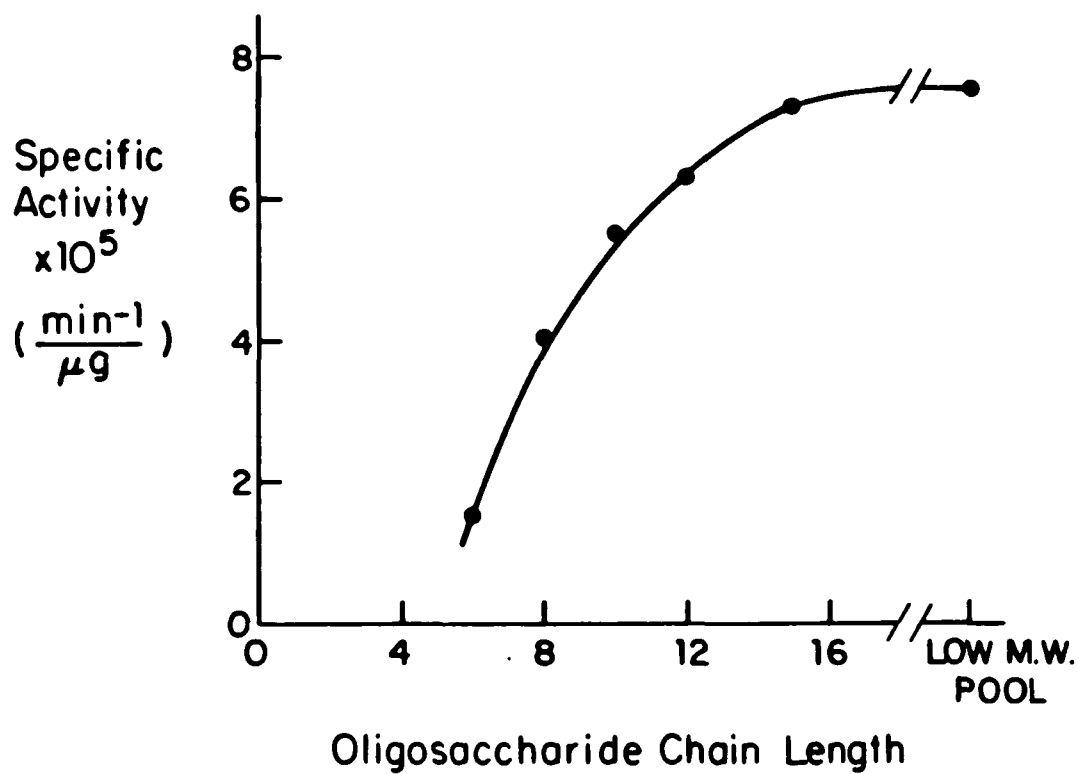


Figure 19: Specific activity of heparin oligosaccharide fragments as a function of chain length. The lyophilized oligosaccharides were prepared as described in section IIA2, and specific activity determined as described in section IIB1.

K. Relationship between the catalytic and anticoagulant activity of different heparin preparations and subfractions

During the course of this work, heparin preparations from various sources were examined for their catalytic activity. This is especially important for a complex biological material such as heparin, whose heterogeneity is evidenced by the widely varying anticoagulant activity of different preparations. In Table VIII the specific activity (with respect to AuO hydrolysis) and anticoagulant activity of all the heparins tested are listed. Specific activity was determined on P_2O_5 dried samples by simultaneous measurement of catalytic activity and glucuronic acid content (m-hydroxydiphenyl assay). From these data it would appear that although there is a general correlation between anticoagulant and catalytic activity, there is no steadfast relationship between these two parameters when comparing unfractionated commercial preparations. It can however be definitively stated that all of the commercial heparins tested do have catalytic activity with respect to AuO hydrolysis. The significance of this point will become apparent in the next section, when it is contrasted against the lack of catalytic activity demonstrated for preparations of all the other GAG.

A second experiment was performed to determine whether there was any systematic variation in the catalytic activity of heparin subfractions prepared from a single commercial preparation. A set

of such subfractions, essentially separated based on on their varying charge density (as described in section IIA2), and the original material from which they were prepared, was kindly supplied by Dr. Milton Smith of Hepar Industries. Their specific activities with respect to AuO hydrolysis were determined as above. These values, and the anticoagulant activities of the respective fractions, are given in Table IX. An obvious correlation between the two parameters is evident, as graphically illustrated in Figure 20. This result contrasts markedly with the lack of correlation when comparing different commercial preparations. Further substantiating this observation is the fact that the only point (■) in figure 20 which doesn't fall on a smooth curve is the point due to the parent heparin preparation.

Thus it would seem that different conclusions regarding the relationship between anticoagulant and catalytic activity would be drawn if either Table VIII or IX were considered alone. This is however not the case, and in fact the side by side comparison of the two cases itself makes an important statement with respect to how structural variations in heparin differently affect catalytic and anticoagulant activity.

TABLE VIII

Comparison of catalytic and anticoagulant activity of different heparin preparations. Heparin preparations are described in section IIA2, and specific activity determined as in section IIB1.

<u>Preparation</u>	Catalytic ($A_{430} \text{ min}^{-1}$) Activity <u>mg heparin-uronic acid</u>	Anticoagulant (USP Int. units/mg)
<u>A. From porcine intestinal mucosa</u>		
1. Sigma Grade I	1.5×10^{-4}	169
2. Sigma Grade II	1.1×10^{-4}	136
3. Calbiochem A grade	1.5×10^{-4}	162
4. Riker	1.7×10^{-4}	160
<u>B. From bovine lung</u>		
5. Sigma	1.6×10^{-4}	141
6. Hepar	1.6×10^{-4}	124
7. Upjohn	2.2×10^{-4}	183

TABLE IX

Relationship between the catalytic and anticoagulant activity of heparin subfractions with varying charge density. Subfractions prepared as described in section IIB2, and specific activity determined as described in IIB1.

<u>Sample No.</u>	<u>Anticoagulant (USP units)</u> <u>Activity</u> <u>mg</u>	<u>Specific (min-1)</u> <u>Activity</u> <u>ug uronic acid</u>
Unfractionated starting material, lot BL-160-81	124	1.6×10^{-4}
02-12-5	74	1.1×10^{-4}
02-12-G	114	1.5×10^{-4}
02-12-1	127	1.7×10^{-4}
02-12-A	152	2.3×10^{-4}

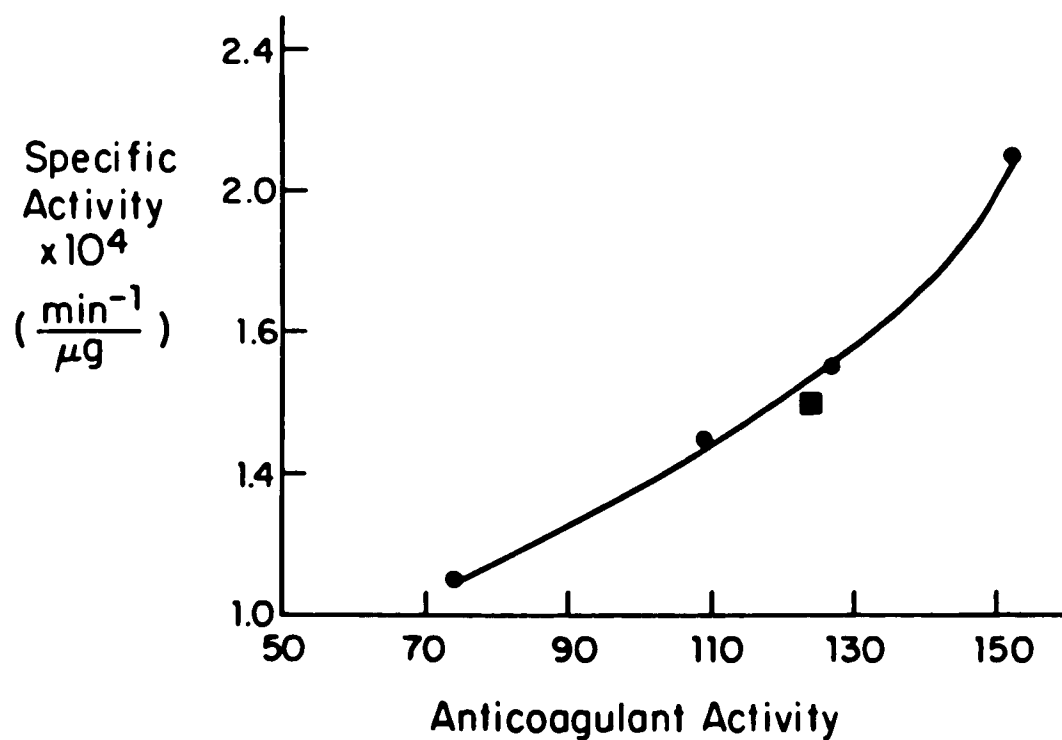


Figure 20: Specific activity as a function of anticoagulant activity. Heparin subfractions prepared as described in section IIA2, and specific activity determined as described in Section IIB.

- L. The specificity of the catalysis with respect to synthetic and natural polyelectrolytes.

So as to gain insight into the structural features of heparin responsible for its catalytic activity, the ability of a diverse group of polyelectrolytes to catalyze the same reaction was examined. Each PE was examined under reaction conditions which were found to be optimal for demonstrating the catalytic influence of heparin on AuO hydrolysis, and were subsequently adopted for the development of a heparin assay (see Appendix I). The PE were added to the reaction mixture at a final concentration of 10^{-4} M in monomer residues, and the initial rates of hydrolysis measured. The results of these experiment are presented in Table X, with the polyelectrolytes separated into groups of natural and synthetic origin. Among the natural PE, heparin is strikingly more effective in catalyzing AuO hydrolysis than all of the others examined. Among the synthetic polymers tested, dextran sulfate, polyvinyl sulfate and polyanethole sulfonate are all catalytic, and in fact more so than heparin.

The diverse structure of the synthetic PEs found to catalyze AuO hydrolysis precludes the possibility that a specific site is involved. One unifying feature of the polymers possessing catalytic activity is that they are all poly-sulfated. However the other GAG tested are also poly-sulfated, and yet are not catalytic. Thus the differentiation of heparin from other GAG with respect to catalysis

of this reaction must be based on a more subtle structural feature. That is, there must be some structural parallel between heparin and the synthetic polysulfates, which at the same time differentiates heparin from other GAG. This is reminiscent of the biological differentiation of heparin from other GAG.

TABLE X

Specificity of the catalysis with respect to PE structure. PE preparations are described in section IIA, and catalytic activity determined by the kinetic assay procedure in Appendix I.

<u>PE Added</u>	Rate of AuO hydrolysis ($10^3(\text{min}^{-1})$)
None	0.3
<u>Natural PE</u>	
heparin	17.5
chondroitin-6-sulfate	0.4
chondroitin-4-sulfate	0.4
hyaluronate	0.3
heparin sulfate	0.8
dermatan sulfate	0.7
keratin sulfate	0.7
RNA	0.5
DNA	0.8
protamine hydrochloride	0.3
<u>Synthetic PE</u>	
dextran sulfate	19.3
polyvinyl sulfate	20.6
polyanethole - sulfonate	22.9
polyvinyl - pyrrolidone	0.3
polylysine	0.3

IV. Discussion

A. Introductory remarks regarding the nature of the phenomenon examined.

1. Discovery of the heparin-accelerated fading of AuO

Auramine O is a basic diphenylemethane dye which dissolves in water to give a bright yellow solution. It has been used as a paper and textile dye (98), a reagent in fluorescent histochemical technic (99), and an index of viscosity via the dependence of its fluorescence in solution on viscosity parameters (100,101). This last property is related to utilization of AuO as a fluorescent probe of biologically important systems with which it complexes, such as the chromatophoric membranes of photosynthetic bacteria (102), yeast alcohol dehydrogenase (81) serum albumins (103,104), and polynucleotides (105). All of these applications are based on the low fluorescent quantum yield of AuO when free in solution, and the dramatic increases in quantum yield occurring upon change of the dye's microenvironment.

Based on this property of AuO, its cationic charge, and the strong tendency of MPS to bind cationic dyes, experimental work aimed at developing AuO as a fluorescent probe of mucopolysaccharides (MPS) was begun. Though extensive work had already been done showing changes in the emission characteristics of

other dyes bound to MPS (76,77), none of these dyes have the low quantum yield of AuO when in the unbound state. Thus application of AuO as a fluorescent probe of MPS would provide a more sensitive tool for studying the microenvironment of this interesting class of polyanions, because fluorescence of the bound dye could truly be studied against an almost black background.

As predicted, the addition of MPS to a non-fluorescent aqueous solution of AuO at neutral pH did in fact dramatically increase the dye's emission in a manner which was apparent even to the naked eye. However the nature of the fluorescent complex was anomalous in that it could only be demonstrated at AuO concentrations greater than 5×10^{-5} M (unpublished observations). The high extinction coefficient of AuO in the wavelength region involved ($\epsilon_{430} = 4.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) made quantitative fluorescence measurements difficult, due to inner filter effects. Furthermore, concentrations of AuO in excess of 5×10^{-4} M formed a colloidal precipitate upon the addition of MPS at neutral pH. Extensive purification of the dye by recrystallization or chromatography did not alleviate these problems. Since the formation of solid fluorescent complexes between MPS and alcian blue had already been described (106), further study of these colloidal fluorescent complexes did not seem warranted.

Attempts were made to find conditions applicable to the demonstration of fluorescent complexes in homogeneous single phase solutions. When examining the influence of MPS on AuO fluorescence in a pH 3.2 glycine buffer, an interesting visual observation was

made. The addition of heparin caused a rapid fading of the bright yellow AuO to a pale green, almost colorless solution. A detailed description of this observation is presented in section III A. The visible absorption spectrum of 2×10^{-5} M AuO in 0.01 M glycine buffer, pH 3.2, (figure 5a, solid line), is characterized by a $\lambda_{\max}$ at 430 nm with a shoulder at 370 nm. This spectra, and hence the yellow color of AuO is relatively stable, with less than a 2 percent change in the spectra occurring over five hours (Figure 5b). When heparin is added to an identical solution at a final concentration of 100 ug/ml, a metachromatic change in the AuO spectrum is immediately apparent at zero time (Figure 5a, dashed line). This metachromasia is characterized by the loss of the 370nm shoulder, an 11 nm blue shift in $\lambda_{\max}$ and accompanying hypochromism, similar to the optical changes described by other investigators for cationic dyes complexed to MPS (13,28,73). Though these metachromatic changes are smaller in magnitude than those typically reported, they are nonetheless indicative of an interaction between heparin and AuO.

This interaction is even more concretely evident from the dashed line in Figure 5b, which shows the spectrum of the complex five hours after its preparation. The absorption band responsible for AuO's yellow color is now completely absent, and only a single absorption band with $\lambda_{\max}$ at 370 nm remains. This is in sharp contrast to the relative stability of AuO free in solution under otherwise identical conditions (compare the solid lines of Figures 5a and 5b). From the kinetic rate determinations in Figure 6, it

can be seen that heparin, at a concentration of 100 ug/ml, decreases the half life of AuO from 12 days to 90 minutes, a dramatic 200-fold increase in the rate of the dye's fading.

Other MPS were incapable of exerting these influences on AuO. They neither formed a metachromatic complex with the dye, nor did they alter its rate of fading (Figure 6a). Thus it seemed that the chemistry of AuO was influenced in a unique way by heparin, paralleling the distinction of heparin from other MPS based on its biological and pharmacological actions (14-18,44,100,108). Moreover, use of a chemical reaction to characterize the microenvironment of MPS had not been previously attempted, though subsequent to our initial report of this system (110) additional reports regarding MPS influences on chemical reactions have appeared in the literature (111,80). Rather than continuing to pursue the fluorescence study, it was decided to characterize the fading reaction and the MPS influence thereon.

2. Identification of the reaction involved

The first necessity regarding characterization of this phenomenon was identification of the chemical reaction responsible for the rapid fading. Holmes and Darling had reported (but not demonstrated) that under acidic conditions and elevated temperatures, AuO could be hydrolyzed to form Michler's ketone (MK), as illustrated in Figure 3 (112). Based on the close correspondence

between the absorption spectrum reported by Adams for MK in glacial acetic acid (92), and the spectrum obtained for the reaction mixture when fading was essentially complete (Figure 5b), the hydrolysis of AuO to MK seemed the reaction most likely responsible for the rapid fading. Furthermore, Delben et al. had shown that some maleic acid copolymers could accelerate the fading of AuO in dilute acidic buffers, and implied that the product of the fading reaction was MK, though experimental proof for formation of this product was not presented (113). We therefore first set out to prove that hydrolysis was indeed the reaction responsible for the rapid fading, and that MK was the product obtained for both the accelerated and non-accelerated reactions.

The data presented in section IIIB describing the isolation and identification of the fading reaction product, provide strong evidence for the formation of MK. Table III and Figures 7a-7c compare the physical and spectral properties of the reaction products to each other and to a known sample of MK. For both the non-accelerated and heparin-accelerated fading, the crude precipitate formed during the reaction, could be recrystallized from methanol as pale green crystalline leaflets which melted at 171-172°C. There is a discrepancy in the literature regarding the melting point of MK. In the Handbook of Chemistry and Physics, 54th edition (82), the melting point is given as 179°C; in the Merck Index the melting point of MK is given as 172° (91). Since the recrystallized commercial sample of MK also melted at 171-172°C, it will be assumed that this is a positive identification. The UV-Visible spectral parameters of the products are also identical to

each other, to the known sample of MK and to the respective literature values (Table III). It is unlikely that such agreement could be obtained in two different solvents, glacial acetic acid and absolute ethanol, if the product of both reactions was not MK. The most definitive proof of this identification is the complete coincidence of the infra-red spectra presented in Figure 7.

3. Characterization of the acceleration as a catalytic process

Once the reaction involved in the fading had been identified, the next critical question regarded the nature of heparin's influence. Was heparin acting catalytically in this system, or did it participate in the reaction in a way which led to its consumption. Based on the demonstration by Delben et al (113) that other polyanions could accelerate AuO hydrolysis, the phenomenon seemed amenable to interpretation as an example of polyelectrolyte catalysis. However heparin could accelerate AuO hydrolysis by 200-fold, whereas the maximal rate acceleration reported by these authors was only 10-fold (113). Furthermore the inability of other GAG to catalyze AuO hydrolysis, despite their polyanionic nature, also complicates this straightforward interpretation. For the present, the rate enhancement will continue to be described in terms of PE catalysis, despite these sticking points, which will be dealt with later in the discussion.

Before going on, it is important to note at this point that the use of the term catalytic regarding PE influences on ionic reactions

needs to be qualified. According to Ostwald's classical definition of catalysis, which is based on thermodynamic arguments, a catalyst must effect its rate influence without altering the position of thermodynamic equilibrium, and thus must equivalently affect the forward and reverse reaction rates (65). This is in general not the case for PE "catalysts" since their influence on reaction rate is dependent on reactant charge, and for most ionic reactions the charge of the products is different from that of the reactants. Many examples of PE catalyzed reactions wherein the equilibrium constant is altered have been noted (57,64,114). With respect to AuO hydrolysis, the reaction can essentially be considered irreversible under the conditions studied, since reversibility would entail nucleophilic attack by ammonium ion. Nonetheless, use of the term PE catalysis has persisted in the literature, and we shall continue to use it based on the following criteria: that a catalyst will not alter the final product of a reaction, nor is it consumed during the course of the catalyzed reaction.

As has been just discussed, the product of the fading reaction is indeed the same in the presence or absence of heparin, thereby meeting the first criterion. Thus demonstration that the heparin is neither consumed nor altered during the course of the accelerated fading is necessary to meet the second criterion justifying use of the term catalytic in describing heparin's influence in this system. This is accomplished in section IIIC. 89% of the heparin added to the reaction could be recovered following a round of

catalysis, and the recovered heparin was capable of catalyzing a second round of hydrolysis. This compares favorably with the 88-98% recoveries reported in the literature for alcohol precipitation of heparin (85,93) and the 90-95% recovery typically obtained in our laboratory using this procedure.

However, due to the microheterogeneity of heparin preparations (see section IB1), the possibility remains that only a specific heparin subpopulation was involved in the catalysis, and that this subpopulation represented the 11% of the original heparin not recovered by alcohol precipitation. To rule out this possibility, the specific activity of the original and recovered heparin are compared. Specific activity is defined in terms of AuO hydrolysis, rather than heparin's biological activity, because this reaction would be most sensitive in picking up the loss of a heparin subpopulation specifically involved in the hydrolysis. The data presented in Table IV demonstrate not only that the recovered heparin is capable of catalyzing a second round of AuO hydrolysis, but moreover, that the recovered heparin has the same specific activity with respect to this reaction as the original heparin, and therefore could not have been altered in the course of this rate acceleration.

To briefly summarize, the reaction responsible for the originally observed fading has been identified; and heparin's influence on this reaction has been demonstrated to meet the criteria necessary for use of the term catalytic, at least in the

sense that it is ordinarily used when speaking of polyelectrolyte catalysts. It still remains to be demonstrated from more detailed analyses that the mechanism by which heparin influences this reaction can indeed be typified as a true example of PE catalysis.

B. The chemical and spectral properties of AuO, as they relate to heparin's acceleration of the hydrolysis reaction.

1. The structural basis for AuO's stability to hydrolysis at neutral pH

It is necessary to have an understanding of the chemistry of AuO in order to analyze the nature of heparin's influence on its reactivity. Little data specifically dealing with this dye could be found in the literature. The discussion that follows is therefore largely based on fundamental concepts and extrapolation of data from compounds structurally related to AuO in various ways. Careful examination of the structure of AuO, has important implications with respect to its reactivity. In Figure 3 the singly protonated form of AuO is illustrated. This is the predominant molecular species at neutral pH, based on the pKa of 10.7 reported by Goldacoe and Phillips for protonation of the free base (115). It is unlikely that this first protonation could occur at either of the other two basic positions on the molecule (i.e. the two aromatic dimethylamino groups); imino nitrogen atoms are typically much more basic than aromatic nitrogens. AuO is more basic than closely related compounds such as benzhydrylidineimine, $(C_6H_5)_2C=NH_2^+$, with a reported pKa of 7.0, and its p-methoxy derivative, pKa = 7.6 (116), not only because it has two electron-donating p-dimethylamino groups, but even more so due to the extensive resonance

stabilization of the singly protonated base. Figure 21 illustrates the type of canonical structure responsible for this stabilization. Resonance forms arise from conjugation of the lone pair electrons on the aromatic nitrogen atoms with the π -electron system of the ring. Thus the protonated imino nitrogen's positive charge is partially shared by the two aromatic nitrogens, resulting in a net stabilization.

The monoprotonated form of AuO can be considered as the protonated Schiff base derived from nucleophilic attack by ammonia on bis-p-dimethylaminobenzophenone (HK). Despite this analogy, its behavior differs significantly from that generally observed for protonated Schiff bases. The latter species are typically quite unstable in aqueous solution, rapidly hydrolyzing to form the corresponding carbonyl derivative (116-118). Protonated benzhydrylideneimine, a schiff base which can be considered as the unsubstituted analog of AuO, has a half-life of only 8.6 minutes in aqueous solution between pH 3-6 (116). Conversely, aqueous solutions of protonated AuO hydrolyze very slowly (112,113), and in our hands have been found to be stable for weeks when kept in the refrigerator at neutral pH. This atypical behavior for a Schiff base is due to a substituent effect of the p-dimethylamino group on the imino carbon's electrophilicity, as partially exerted through resonance forces. Ordinarily the imino carbon of a protonated Schiff base is extremely electrophilic, making it quite subject to nucleophilic attack by water. Placement of an electron donating

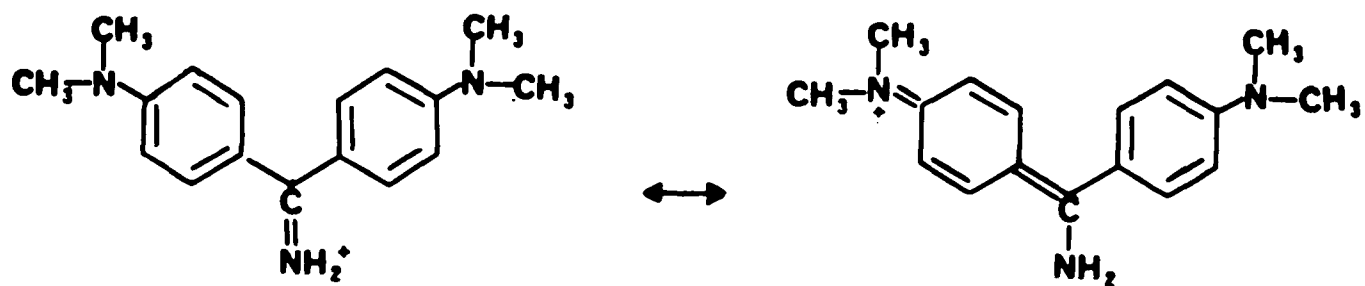


Figure 21: Canonical structures responsible for the resonance stabilization of Au0.

methoxy group para to the imino carbon in benzyhydrylideneamine decreases this protonated Schiff base's hydrolysis rate to one sixth its value in the parent compound (116). The p-dimethylamino derivative of N-benzylidene aniline, $[(CH_3)_2-N-C_6H_5-C=N-C_6H_5]$, is 300-fold more stable than its parent compound to hydrolysis (118). In the case of AuO, not only are there two p-dimethylamino groups capable of donating electron density into the imino carbon, but there is an additional large resonance stabilization of the reactant because the canonical structures in Figure 21 have similar energies. The net result of these factors is an extremely slow rate of attack at the imino carbon of AuO by a weak nucleophile like water.

2. The spectral properties and prototropic equilibria of free AuO

The straightforward interpretation of AuO's basicity and reactivity provided by resonance theory can also serve as a basis for understanding the spectral properties of the dye. Adams was the first to attempt a theoretical understanding of AuO's absorption spectrum (92). He ascribed the low energy electronic transitions responsible for the visible absorption bands to transitions between the "N states" of the molecule, which can be thought of as the wave functions describing structures such as those in Figure 21 (plus an additional structure with the positive charge on the imino carbon atom). Thus the relationship between the spectrum of AuO and its resonance forms is apparent.

Wicken and Woody analyzed the visible absorption bands of AuO based on molecular orbital calculations first performed for the simpler dimethylaminobenzaldiminium ion, which can be regarded as the monomeric analog of one half of the symmetrical AuO molecule (119). The lowest energy electronic absorption band of this ion corresponds to a charge transfer (CT) transition involving movement of electron density from the dimethylamino group (a good donor) to the protonated imino group (a good acceptor). In AuO, such a CT transition can occur in both the rings, and therefore two electronic absorption bands should be observed, corresponding to the interaction of the charge transfers in the individual rings in either a symmetric or antisymmetric fashion. The symmetric interaction, in which charge is transferred from the dimethylamino group in both rings to the central imino group, should be of higher energy and polarized along the molecule's C_2 axis of symmetry. The antisymmetric interaction, which corresponds to the transfer of electron density between the dimethylamino groups via the imino group, should therefore lead to a lower energy absorption band polarized along AuO's long molecular axis. These are in fact the polarizations previously reported by Adams. Furthermore when the molecular orbital calculations are carried out assuming an angle of 30° for the conrotary twist between the phenyl rings of AuO, good agreement with the experimentally determined oscillator strengths of these absorption bands are obtained. It is important to note here that even though coplanarity between the phenyl rings is prevented in diphenylmethanes by steric repulsions between ortho hydrogen

atoms (120), conjugation between the dimethylamino and the imino groups is not prevented. Indeed, if this conjugation were not possible, both the chemical and spectral properties of AuO would be difficult to explain.

Based on these principles, our experimental data detailing the spectral properties and chemical reactivity of AuO can be clearly interpreted. Over the pH 3-7 region, the visible spectral properties of AuO are constant, and in agreement with those reported by others (81,92,119). In 0.1 M phosphate buffer, pH 7.4, $\lambda_{\max}$ occurs at 430 nm, with a secondary peak at 371 nm; corresponding respectively to the antisymmetric and symmetric charge transfer transitions discussed above. An experimental value of $4.45 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ is obtained for Σ_{430} , in excellent agreement with the value given by Conrad et al (4.41×10^4) (81). We have also confirmed that this dye obeys Beer's Law up to a concentration of $1.0 \times 10^{-3} \text{ M}$, as previously reported (100), and is thus not likely subject to a concentration dependent aggregation.

The lack of an acidity influence on AuO's spectrum within the pH 3-7 range (Figure 10), indicates that no significant protonation of the aromatic nitrogens occurs within this range. Inasmuch as the lone pair electrons on these nitrogens are an integral part of the dye's chromophoric system, protonation would inevitably result in a spectral perturbation. Based on a pK_a of 5.15 for N,N dimethylaniline (82) this lack of protonation is somewhat surprising. However when one considers that the analagous nitrogen in p-nitroaniline has a pK of 1.0 (82), it is apparent that the

placement of a strongly electron withdrawing group para to the aromatic amine can reduce its basicity by several orders of magnitude.

This is indeed the case with respect to AuO, which does show a pronounced acidity influence on its spectrum at pH values below 3. As illustrated in Figure 8, the Σ_{430} of AuO does decrease with increasing acidity in this range. However the shape of this curve is not that which would be typical of a simple titration curve involving protonation of a single group, in that there appears to be an extra inflection point at about pH 1. Furthermore, the decrease in Σ_{430} with pH continues over an HCl concentration range corresponding to more than three orders of magnitude. This behavior implies that the change in Σ_{430} with pH is due to the stepwise protonation of both aromatic nitrogens, and that the pKa's for these two nitrogens are sufficiently close for their titration curves to overlap. Such an interpretation is supported by the pKa values for di- and triprotonation of the analogous positions in the singly charged crystal violet ion. These have been reported as 1.96 and 0.81 for the first and second protonation steps, respectively (121).

Because of this overlap, assignment of the corresponding pKa values for AuO cannot be accomplished directly from the spectrophotometric data alone. However based on the absorption spectra in figure 11, an approximation of these pKa's will shortly be made. In this figure, the visible absorption spectra of free and heparin-bound AuO are presented, at pH 5.0, 3.0 and 1.6. For free

AuO, the spectrum observed at pH 3.0 and 5.0 are identical and correspond to that previously described at neutral pH. Decreasing the pH to 1.6 causes a profound change in the spectrum. The resolved secondary peak at 370 nm is lost, and the 430 nm peak is shifted to 419 nm. This spectrum corresponds quite closely to that observed for heparin-bound AuO at pH 3.0. In this regard it should be noted that heparin-bound AuO has already undergone a pH induced spectral change by pH 3.0 ($pK_a = 3.85$ based on Figure 12) and is thereafter stable until about pH 1.5. Although the point will be discussed in greater detail later, it implies that the spectra recorded for free AuO at pH 1.6, and heparin-bound AuO at pH 3.0, correspond to that of the di-protonated form of the dye (AuO^{2+}).

This conclusion is supported by the fact that these spectral changes are in agreement with those which would have been predicted, based on Wicken and Woody's theoretical description of AuO's electronic transitions (119). As previously described, the two peaks in AuO's visible absorption spectrum are due to symmetric and antisymmetric interactions of the charge transfer transitions involving lone pair electrons on the aromatic nitrogen atoms. The protonation of one of the lone pairs abolishes the possibility of this interaction, and thus the reason for the two separate peaks in the visible spectra. Furthermore the single charge transfer which can still occur in AuO^{2+} might be expected to occur approximately midway between the two transitions observed in the monoprotonated dye. The 20 nm red-shift from the midway point actually observed

(419 nm vs 400 nm), probably reflects the fact that the single charge transfer transition remaining is of lower energy than expected because of the positive charge now present on one of the dimethylamino groups. Along this same line of reasoning, it would be predicted that the tri-protonated form of AuO (AuO^{3+}) should have no absorption in the visible region of the spectrum, because all possibilities for this charge transfer transition have now been abolished. This is in fact the behavior observed for methyl violet a dye with a chromophore similar to that present in AuO, wherein the color of the dye is completely destroyed by high acidity (122). Although the study of AuO's spectral properties as a function of pH was not carried out to high enough acidities to observe this total loss of visible absorption, it is clear from Figure 8 that this is the trend being followed.

The pKa values for di- and tri-protonation of free AuO can now be estimated if the extinction coefficient for AuO^{2+} is taken as approximately $3.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. This assumption is based on the following argument. The spectrum presented for AuO^{2+} bound to heparin (Figure 11b, pH 3.0) is almost identical to that observed for free AuO at pH 1.6 (Figure 11a) and in agreement with that theoretically predicted for the diprotonated form (as described above). Furthermore, the plot of Figure 8a can be considered as the sum of the two titration curves indicated by the dotted and dashed lines in the figure. The dashed line, corresponding to titration of the diprotonated species, is drawn so that it levels off at an

Σ_{430} value of $3.0 \times 10^4 \text{ m}^{-1} \text{ cm}^{-1}$, which is just slightly less than the extinction coefficient of AuO^{2+} bound to heparin. The dotted line, corresponding to titration of the triprotonated species, is drawn so that its extinction coefficient is approaching zero, for the theoretical reasons described above. Based on these two values for Σ_{430} , the pK_a of the di- and triprotonated species can be estimated as that corresponding to the pH of the midpoint between the respective values of Σ_{430} . For AuO^{2+} this gives a pK_a_2 of 1.9; for AuO^{3+} , a pK_a_3 of -0.3 is thus obtained. These values are in reasonable agreement with the corresponding values of 1.96 and 0.81 reported for crystal violet (121). The fact that the value of pK_a_3 for AuO is significantly lower than that of crystal violet, is probably reflective of the fact that in the sterically less hindered AuO molecule, the aromatic nitrogen is more conjugated to the central carbon, and thus its basicity is reduced.

3. The dependence of AuO's hydrolysis rate on acidity

Based on this understanding of AuO's prototropic equilibria, the form of the dependence of its hydrolysis rate on acidity can be discussed. This dependence is illustrated in Figure 9. To a first approximation, it seems that the increase in hydrolysis rate occurring at about pH 2 is linked to the increased protonation of the aromatic nitrogens occurring in this pH range. A deceptively simple explanation of this behavior can be offered in terms of the influence of protonation on the resonance stabilization of the imino

carbon. As the aromatic nitrogens become protonated, their lone pair electrons can no longer donate electron density into the imino carbon, hence this carbon becomes more electrophilic, and thus more reactive with a weak nucleophile like water. As an extension of this argument, AuO^{3+} should be even more reactive, since there will be even less electron density on the imino carbon. However examination of Figure 9 indicates that this cannot be the case, since at the lowest pH values where the concentration of AuO^{3+} is increasing, the rate of hydrolysis is decreasing with decreasing pH. Furthermore, careful comparison of Figures 8 and 9 reveals that they are not parallel in the way that one would expect if the influence of acidity on the reaction were simply exerted via the acidity dependence of a rapid pre-equilibrium protonation. In fact, the only point that is immediately apparent from the Figures is that the variation of rate with pH is complex in a manner which would be difficult to explain if the rate determining step remained nucleophilic attack at the imino carbon throughout the pH range examined.

In order to better examine the relationship between the prototropic equilibria and hydrolysis rate, equations are derived to calculate the relative concentrations of di- and triprotonated AuO as a function of pH. These are:

$$\frac{[\text{AuO}^{2+}]}{[\text{AuO}]_{\text{total}}} = \frac{K_{a_3} [\text{H}_3\text{O}^+]}{K_{a_3} [\text{H}_3\text{O}^+] + K_{a_2} K_{a_3} + [\text{H}_3\text{O}^+]^2}$$

$$\frac{[\text{AuO}^{3+}]}{[\text{AuO}]_{\text{tot}}} = \frac{[\text{H}_3\text{O}^+]^2}{[\text{H}_3\text{O}^+]^2 + K_{a_3}[\text{H}_3\text{O}^+] + K_{a_2}K_{a_3}}$$

Using these equations, and values of 1.9 and -0.3 for $\text{p}K_{a_2}$ and $\text{p}K_{a_3}$ respectively, the percentages of di- and tri-protonated AuO as a function of pH are plotted in figure 23. With the help of these graphs, the influence of the protonation equilibrium on hydrolysis rate can be concretely discussed.

Between pH 3.0 and 1.0, the concentration of AuO^{2+} is increasing (Figure 23), in a similar (though not parallel) fashion to the rate increase. Further decreases in pH over the range 1.0-0.5 causes no net change in the concentration of AuO^{2+} , but the hydrolysis rate continues to increase. Since the concentration of AuO^{3+} is increasing in this acidity range, it might be argued that the increasing concentration of this species is the source of the rate increase. However, it is clear from Figure 23 that this argument is untenable, because at pH values below 0.5, where the concentration of AuO^{3+} is increasing most rapidly, the rate at first remains constant, and then sharply decreases. It is thus apparent from comparison of Figures 23 and 9 that the hydrolysis rate for AuO^{3+} is lower than that for AuO^{2+} .

This behavior implies that for AuO^{3+} the rate determining step is no longer nucleophilic attack at the imino carbon, since the rate

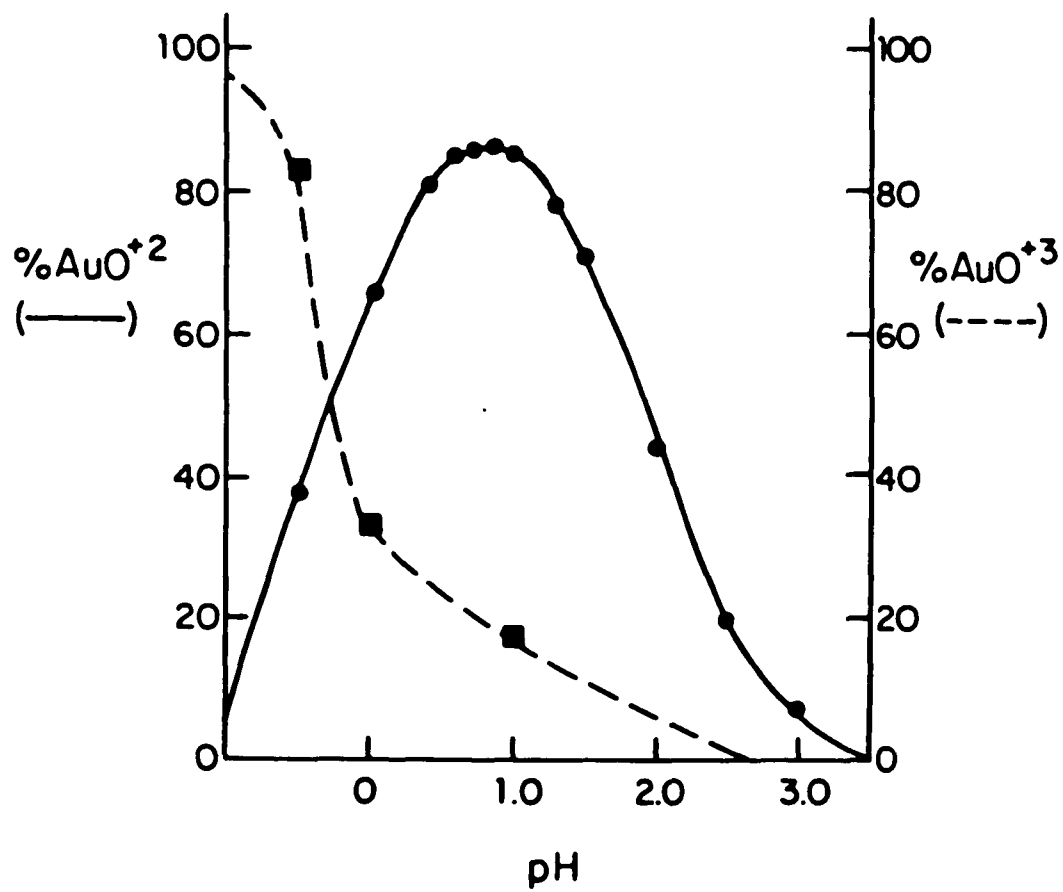


Figure 22: Relative distribution of protonated AuO species as a function of pH.

of this attack would definitely be greater for the tri- compared to the di-protonated species. Rather the rate determining step must now be at some subsequent point in the reaction. The most likely candidate for the new rate determining step would be decomposition of a tetrahedral carbinol-amine intermediate (123). The breakdown of this intermediate to form product involves the homolytic breakage of the C-N bond, and thus the transition state for this step must necessarily entail the accumulation of a partial positive charge on the imino carbon. In the case of both mono- and diprotonated AuO , electron donation from the aromatic nitrogens can stabilize this transition state, and thereby lower the activation energy for its breakdown. However, in the case of AuO^{3+} , the possibility for this resonance stabilization of the transition state is lost, thereby elevating the activation energy for breakdown of the intermediate. Thus the decrease in rate observed at the highest acidities in figure 9 is due to AuO^{3+} becoming the predominant form of the dye in this pH range, and the fact that for this species breakdown of the intermediate has become rate determining, rather than the initial nucleophilic attack.

However, if this is the only point at which the rate determining step has changed, then why is the rate of hydrolysis increasing between pH 1.0 and 0.5, since according to figure 22, the concentration of AuO^{2+} is constant over this pH range. If the rate determining step were truly nucleophilic attack in this pH range, then the hydrolysis rate should be constant where the

concentration of AuO^{2+} is constant, because it is only via this species that acidity can influence the rate of nucleophilic attack. The fact that the hydrolysis rate is not constant, indicates that in this acidity range also the rate determining step is no longer nucleophilic attack. Thus acid is increasing the hydrolysis rate not via its influence on the rapid pre-equilibrium protonation, but rather via an increase in the rate of decomposition of the carbinolamine intermediate. A similar conclusion was reached by Reeves in his analysis of the acid catalyzed hydrolysis of p-trimethylammoniumbenzylidene p-hydroxyaniline, a Schiff base reaction involving a similar carbinol-amine intermediate (124). The pH dependence of this compound's hydrolysis could likewise not be explained by a mechanism involving only pre-equilibrium conversion of the substrate to its conjugate acid; rather a mechanism wherein rate-limiting breakdown of the intermediate occurs by uncatalyzed and acid-catalyzed pathways had to be proposed.

With respect to AuO hydrolysis, the basis for acid catalysis of the intermediate's decomposition most likely lies in the influence of acidity on protonation of the leaving amino group. In the initial intermediate formed by nucleophilic attack, the amino group is not charged, since it is directly derived from the $\text{C}=\text{NH}_2^+$ group by movement of the electrons in the double bond onto the nitrogen atom. Breakage of the carbon nitrogen bond in this state is extremely difficult, as it would entail separation of a negatively charged amide group from a partial positively charged

carbon atom. Therefore, it is most likely that protonation of the amine occurs before the intermediate breaks down, since in this case the transition state would only involve separation of a neutral ammonia molecule from the partial positively charged carbon, which is an energetically much more favorable process. Although amino groups generally have pK_a 's in the pH 9-10 range, protonation of the amino group in the intermediate might still be acid catalyzed in this low pH range because the intermediate is already positively charged (due to protonation of the aromatic nitrogen), and thus its pK_a would be expected to be markedly reduced. Thus even for AuO^{2+} , the rate determining step is not simply nucleophilic attack by water as modulated by a rapid, acid-dependent, pre-equilibrium protonation.

Thus to summarize, the pH dependence of AuO hydrolysis is complex, and indicative of several changes in rate determining step as a function of pH. At neutral pH, the rate determining step is either nucleophilic attack on monoprotonated AuO , or the rapid pre-equilibrium protonation to form AuO^{2+} . As the pH is decreased down to the pH 2-3 range, wherein a significant concentration of AuO^{2+} begins to form, the rate determining step becomes the rapid pre-equilibrium protonation, and the rate increases in parallel with the increasing concentration of this species. However, acid catalysis of the reaction is not limited to this influence on protonation of the aromatic nitrogens; acid also catalyzes the breakdown of the carbinol-amine tetrahedral intermediate. Therefore

the continued rate increase with acidity until a pH value of approximately 0.4 is reached indicates that decomposition of the intermediate has become rate determining in this range. At this point, the rate is observed to remain constant until the pH is reduced to about 0. The reason for this constancy is that acid has opposing influences on the rate of hydrolysis within this pH range. On the one hand, acid continues to catalyze the decomposition of the intermediate; but on the other hand, acid is beginning to cause significant triprotonation in this range; and since decomposition of the intermediate is much slower for the tri- compared to the di-protonated species, increasing the acidity will tend to decrease the reaction rate. It is thus the balance of these two influences of acidity that results in the rate remaining constant with pH over the range 0-0.4. As the acidity is increased further, the concentration of AuO^{3+} continues to increase, and therefore the reaction rate decreases, as this species begins to become the predominant form present.

C. The Mechanism of Heparin's Influence on AuO Hydrolysis.

In the previous section, attention was focused on how and why acidity determines the rate of AuO hydrolysis. However, as briefly mentioned, the reaction is a complex multistep transformation, involving several intermediates of varying stabilities. Consequently, there are many other points along the reaction pathway where environmental factors can influence the rate of hydrolysis. Ion pair formation, solvation phenomena, steric-conformational factors, and stabilization of species with partial carbonium ion character, could all exert modulatory influences on the rate of reaction. It is within this broad context that the catalysis of AuO hydrolysis by heparin must be examined.

1. Heparin as a polyelectrolyte:

In a solution containing a PE and oppositely charged simple electrolytes, the predominant manifestation of the interaction between these two species is the phenomenon of counterion condensation. This phenomenon has its origins in the same electrostatic forces which are responsible for the properties of simple electrolyte solutions. Manning has derived a series of equations describing the properties of PE solutions, which are in many ways analogous to the Debye-Huckel limiting laws for simple electrolyte solutions (56,58) (as described in section Ic). These equations can be used to describe the PE behavior of heparin as it pertains to the ability of this MPS to catalyze AuO hydrolysis.

The most critical structural parameter of a PE determining its counterion condensation properties is the linear charge density parameter ξ . For heparin, as is the case with other MPS, no single value for this parameter can be given. Because of the microheterogeneity of molecular species present in any given preparation as isolated from tissue (17), only an average charge density can be determined. Values of ξ ranging from 1.66 to 2.45 have been reported for different heparin preparations (54,78,125). It has been found that there is a correlation between the anticoagulant activity of a heparin preparation and its linear charge density parameter (78). Based on this correlation, it can be estimated that for the commercial heparin preparation used throughout most of this study, (anticoagulant activity of 169.7 units/mg), the value of the ξ parameter should be approximately 2.5.

However, the above value of ξ is only that which would be expected at neutral pH. With respect to the catalytic influence of heparin on AuO hydrolysis, it is necessary to know the value of ξ at acid pH. This could be expected to differ significantly from its value at neutral pH because part of the charge at neutral pH is due to ionized carboxyl groups, which become protonated in the acidic pH range. Heparin's carboxyl groups are predominantly due to the iduronic acid moieties on the polymer backbone, which have been reported to have a pKa of 5.1 (95). Thus at the pH value of 2.6, where most of the work aimed at deducing the mechanism of catalysis was carried out, these carboxyl groups can be considered to be

completely protonated. Based on the predominant repeating unit of the heparin copolymer (Figure 1), the carboxyl group is responsible for 25% of the charge along the polymer backbone. Since ξ is proportional to $1/b$ (56, 58), where b is the average linear charge spacing of the polyion, a 25% decrease in charge density could be expected to approximately decrease ξ by 25%, thereby giving a calculated value of $\xi = 1.88$ in the pH range where catalysis is observed. This calculation assumes that no major conformational changes occur as a result of the protonation, which has been demonstrated to be the case for heparin (126). Even if a conformational change were to occur as a function of pH, carboxyl group protonation would be expected to cause a contraction of the heparin polyion, if anything, and such a contraction would only tend to increase the value of ξ . In any event, a small deviation of ξ at acid pH from the value thus calculated will not affect the qualitative argument which follows.

Based on Manning's description of counterion condensation, this process is more of an all-or-none phenomenon than a traditional ligand binding process, obeying a classical mass action formulation (58). Thus the theory predicts that counterion condensation will only occur when ξ is greater than $|Z_i Z_p|^{-1}$, where Z_i is the valence of the counterion being considered, and Z_p the valence of the charged groups on the polymer backbone. For the case of sodium heparinate, in a dilute HCl solution containing AuO as the only additional salt, the critical ξ value is one. Thus for heparin, an ξ value of 1.88 indicates that counterion condensation will occur.

This condensation does not occur in an unlimited fashion, but rather only proceeds until ξ has been reduced to its minimal value, given by the equation:

$$\xi_{\text{net}} = |Z_i Z_p|^{-1}$$

which is equal to unity for this system. The theory makes no provision for treating the different counterions present (Na^+ , H_3O^+ , and AuO^+) with respect to their varying tendencies to participate in this reduction of ξ to ξ_{net} ; since all are of the same charge, they are identical from a straight electrostatic point of view. In actuality, other interactive forces between the polyion and counterion may lead to the unequal participation of the various counterions in the reduction of the polyion's net charge density, sometimes even resulting in the partial breakdown of the equations describing the condensation process (58). An example where such breakdown might occur, would be in a case where loci existed on the polyion which could participate in specific site binding of the counterion. In this situation, site-bound counterions reduce the polyion's charge density until all specific sites are saturated, and only then, if the residual charge density is still greater than the critical value, will condensation of territorially bound counterions occur. At present, no distinction will be made as to whether AuO behaves as a territorially or site-bound counterion, since the consequences with respect to the chemistry of AuO are basically the same, and the point is more appropriately discussed when competition between the various counterions is dealt with. For now, it is sufficient to state that the phenomenon of condensation itself has never been negated (58).

2. The influence of heparin on AuO's prototropic equilibria:

The most striking consequence of counterion condensation in the heparin-AuO system is the dramatic change in the pK_{a2} of AuO when it is present in solution with heparin. Whereas for free AuO $pK_{a2} = 1.9$, in the presence of heparin a value of 3.85 is obtained for pK_{a2} (Figure 12), which amounts to a two order of magnitude change in the equilibrium constant for protonation of the first aromatic nitrogen. The ability of PE to change the pK_a of bound ligands has been known since the beginning of this century, when Sorenson first described the "colloid error" occurring when indicator dyes are used in protein solutions (128). Hartley and Roe later demonstrated the same phenomenon in solutions of surfactant micelles (129). Furthermore, since these investigators were working with surfactants of known ionic charge, they were able to demonstrate the electrostatic origin of the effect, and thereby formulate an empirical "sign rule" for predicting whether or not a pK_a change would be observed in a particular dye-surfactant system, and whether the pK_a could be expected to increase or decrease. This rule states that a pK_a change will only occur when the dye and surfactant are of opposite charge, and that for negatively charged surfactants the apparent pK_a will always increase, while for positively charged surfactants the apparent pK_a will always decrease.

Baumgartner et al., demonstrated that PE - indicator systems conform to this same sign rule (130). The magnitude of the pK_a change was found to be as high as 2.5 pH units in some cases. In

addition the effect was demonstrated to be more than a simple complexation effect by the observation that comparable pK changes are not induced by monomeric analogs of the PE backbone, and that in fact the magnitude of the effect was dependent on PE chain length. A more recent investigation provides the strongest evidence that AuO's pK_a_2 could be expected to increase when bound to heparin. It was demonstrated that the pKa of the aromatic nitrogen in 3,8-diamino-diphenyl-phenanthridine was increased from a value of 2.4 when free in solution, to a value of approximately 5 when bound to DNA (131). This is a clear cut analogy to the observed 2 pH unit increase in the pKa of the aromatic nitrogen of heparin-bound AuO. Furthermore, these investigators reached the conclusion that the ionic strength dependence of the pKa was in quantitative agreement with that predicted by the theory of counterion condensation, and that in fact no absolute value can be given for the pKa of a PE-bound ligand, but only an ionic strength dependent value.

The electrostatic basis for the sign rule further brings into focus the parallels between this phenomenon and that of counterion condensation. It is clear that for both PE and colloids, electrostatic potential plays an important role in driving the indicator into the polyion's domain, just as any other counterion would be statistically localized within this domain. It is, however, somewhat less clear as to what the actual physicochemical basis for the pKa change is. In Hartley and Roe's original description of the phenomenon, they envisioned the indicators as

being adsorbed onto the colloid, at the surface of which the pH differs from that in the bulk solution (29). A negatively charged colloid would concentrate hydronium ion at its surface, whereas a positively charged colloid would concentrate hydroxide ion at its surface. From this point of view, the change in the pKa of the indicator is only an apparent change due to the dye being localized at a phase boundary where a local pH differing significantly from the bulk pH can exist. This viewpoint is supported by the fact that in cases where exceptions to the sign rule do exist, they are always exceptions to that part of the rule predicting when a pKa change will occur, but never to the part of the rule predicting the direction of the pK change (132). For example, the pKa of crystal violet (cationic) does change in the presence of hexadecylpyridinium bromide, contrary to what would be predicted by the sign rule. But the pKa decreases instead of increasing as it does for the same dye bound to sodium dodecyl sulfate. This anomaly is interpreted as indicating that hydrophobic forces can be sufficiently strong to drive an interaction between a colloid and indicator of the same charge type; but the sign of the pKa change is absolutely dependent on the charge type of the colloid. Positively charged colloids can only concentrate anionic hydroxide ions at their surface, hence the surface pH of the micelle will always be higher than the pH of the bulk solution, and consequently indicators localized in this region, will behave as if they have an apparently lower pK. The converse argument holds equally well for negatively charged colloids, which

could be expected to always cause an increase in pK_a apparent. Based on this approach it has even been suggested that the surface pH of micelles could be determined using solubilized indicator dyes (133). However, among other problems, this application brings into sharp focus the question of how one distinguishes changes in apparent pK_a due only to local pH effects, from real alterations in an indicator's intrinsic pK_a , as a true chemical consequence of the dye-polyion complexation.

The same problem arises regarding the interpretation of pK_a changes in ligands bound to PE, but even more so. Because no clear-cut phase boundaries exist, the concept of a localized pH is even more tenuous than is the case with respect to micelles. Despite this shortcoming, Baumgartner et al., concluded from their study of the pK_a of PE-bound dyes that the major causative factor of the pK_a changes observed were alterations in apparent, rather than intrinsic pK_a , and thus were predominantly due to differences between the pH in the bulk solution and that in the region close to the PE chains (130). However, it must be noted that despite this conclusion, these authors were cautious and did point out that some portion of the observed change in pK_a was probably due to a real change in intrinsic pK_a , over and above the change in apparent pK_a which is a consequence only of the "polyelectrolyte effect" (130).

A number of forces other than the local pH effects discussed above may contribute to changes in the pK_a of polyion-bound ligands. The well known influences of PE on the spectral properties

of dyes in a manner not related to their prototropic equilibria attests to the ability of PE to alter the electronic distribution of bound species (134). Inasmuch as pKa values are very dependent on electronic distribution, this implies that polyanions can definitely influence the intrinsic pKa of a dye. With respect to heparin-bound AuO, Figures 11 and 13 demonstrate that even in the pH range where heparin is not significantly altering the distribution of mono- and di-protonated AuO, it causes a perturbation in the spectrum of bound mono-protonated AuO, characterized by a blue shift in $\lambda_{\max}$ and decrease in oscillator strength. Since the same electrons are involved in the protonation equilibrium as in the charge transfer transition responsible for this absorption band, these spectral changes clearly imply that heparin can induce a change in AuO's intrinsic pK.

Another part of the observed pKa change may be due to stabilization of diprotonated AuO in heparin's negative electrostatic potential, thereby shifting the equilibrium in favor of the dispositive form, relative to the equilibrium in water. A related mechanism by which PE have been demonstrated to change the pKa of bound ligands is by the process of linked equilibria, whereby preferential binding of one member of an acid-base conjugate shifts the prototropic equilibrium in favor of that species. This was found to be the case for bromphenol blue binding to neurophysin; a 22-fold greater affinity of the protein for the deprotonated, relative to the protonated form of the dye, resulted in a decrease

in pK_a from 3.95 to 2.6 (135). Linked equilibria may contribute somewhat to the heparin induced increase in AuO's pK_a , but the effect is not likely to be as appreciable as in the case just mentioned. Both the mono- and di-protonated forms of AuO are positively charged, and so from the viewpoint of counterion condensation both would be expected to bind to the extent necessary to reduce ξ of heparin to its limiting value. Furthermore, it is unlikely that a protonated aromatic nitrogen could be involved in specific site binding to heparin, since the pK_a change is induced by polyanions of widely divergent structure (see section IIK), as will be more fully discussed in connection with the specificity of the catalysis.

Thus it is clear that heparin changes the pK_{a2} of bound AuO, and that a number of different forces may contribute to the observed changes in pK_a . At this point, the relative contribution of these forces was only briefly addressed. The question will be more fully dealt with after the basis for the catalysis has been laid out, since some conclusions in this regard can be drawn from data on how salts, AuO concentration and heparin concentration influence the catalysis.

It should be pointed out at this time that no comparable elevation in pK_{a3} of heparin-bound AuO occurs. Based on a pK_{a3} of -0.3 for free AuO, and the demonstration of a 2pH unit increase in pK_{a2} for heparin-bound AuO, it might be expected that the pK_{a3} for heparin-bound AuO would be elevated to about 1.7.

Examination of Figure 10 reveals that this is obviously not the case, since in the pH 2.5-1.5 range, the Σ_{430} of AuO in the presence of heparin is not decreasing sharply, but is in fact either constant or slightly increasing. It is unlikely that these data could be obtained if the AuO was still heparin-bound in this pH range, since the same factors causing an increase in pK_{a2} would cause an increase in pK_{a3} , and a decrease in Σ_{430} would have been observed. Therefore, the only possible explanation of the data is that increasing acidity over this pH range results in loss of the heparin-AuO binding interaction, a conclusion which is supported by the fact that catalytic activity is also lost over this pH range (Figure 9).

There are two possible explanations for the loss of binding. The first is that hydronium ion in this concentration range becomes better able to compete with AuO cations in reducing heparin's charge density, and thus heparin-bound AuO is displaced; counterion condensation does not proceed indefinitely, but only until a limiting charge density is reached (58). The second possibility is related to the fact that in this pH range the sulfate groups on heparin are becoming protonated (136,137). Since the sulfate groups represent the only remaining negative charges on the polymer, their titration results in a progressive decrease in charge density; at the point where ξ is reduced to unity, the driving force for counterion condensation is lost. This conclusion is strengthened by careful comparison of Figures 8 and 9. In Figure 8, the curve

representing the rate as a function of pH in the presence of heparin undergoes a rather abrupt slope change at pH 1.2, the same point (on the pH axis) where Σ_{430} begins to decrease for AuO in the presence of heparin. Both these sets of data are consistent with the viewpoint that at about pH 1.2 the sulfate groups on heparin have become sufficiently protonated for ξ to be reduced below unity, with the result that all formal counterion condensation is lost. The abruptness of the change is consistent with the all-or-none nature of counterion condensation, and provides further evidence that it is not necessary to propose a conventional mass action formulation to describe the heparin-AuO interaction.

3. Alterations in the mechanism of AuO hydrolysis in the presence of heparin.

Thus far, the discussion of heparin's catalytic influence on AuO hydrolysis has focused on heparin's perturbation of AuO's prototropic equilibria. This was obviously the place to start, because of the crucial role played by protonation of the aromatic nitrogens in the mechanism of the unbound dye's hydrolysis. However, in the case of heparin-bound AuO, protonation does not seem to predispose the dye to hydrolysis to the same extent that is the case for the unbound dye. This is clearly seen by considering the hydrolysis rate at pH values corresponding to 80% protonation of bound and free species. For heparin bound AuO, this corresponds to a pH of approximately 3.2 ($pK_a = 3.85$), whereas for free AuO, 80% is

diprotonated at about pH 1.2 ($pK_{a2} = 1.9$). The hydrolysis rates at these pH values for the bound and free species are, respectively $0.31 \text{ } \mu\text{moles} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$ and $1.26 \text{ } \mu\text{moles} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$. Thus under conditions where bound and free AuO are diprotonated to the same extent, the free dye is undergoing hydrolysis at a 4-fold faster rate than the heparin-bound dye. This is only possible if heparin can alter the rate of AuO hydrolysis in a manner independent of its influence on the prototropic equilibrium. Furthermore, it implies that this secondary influence on reaction rate stabilizes the dye to hydrolysis, and thus is opposite to the destabilizing influence heparin has on AuO, by virtue of its ability to increase pK_{a2} . Based on this conclusion, it would seem that in the absence of this secondary stabilization heparin would be an even more potent catalyst of AuO hydrolysis.

This viewpoint is supported by the data in Figure 8. Between pH 3-2, where little if any additional protonation of AuO is occurring, the rate continues to increase with decreasing pH. This region of the curve is analogous to the pH 1.2 - 0.4 region of the curve for the non-catalyzed reaction, where likewise the rate is increasing despite the fact that no additional protonation is occurring. As previously discussed with respect to the non-catalyzed reaction, this is taken to imply that the tetrahedral carbinol-amine intermediate breaks down by an acid catalyzed pathway (124). However comparison of the slopes for these corresponding regions of the two curves (between pH 3-2 for the catalyzed reaction, and 1.2 - 0.4 for the non-catalyzed reaction) reveals that the catalytic

influence of acid is much more pronounced for the non-catalyzed reaction, since the slope of the rate vs. pH curve is much steeper than that of the corresponding curve for the heparin-catalyzed hydrolysis. Thus both in terms of the absolute rate of hydrolysis and the sensitivity of the intermediate's breakdown to acid, heparin's influence on diprotonated AuO can be characterized as a stabilization, rather than the destabilization that might have been assumed by comparison of hydrolysis rates at a single pH value.

There are several ways in which it might be possible for heparin to stabilize the diprotonated form of AuO toward hydrolysis. The most obvious is by stabilization of the dipositive species in heparin's negative electrostatic potential, thereby raising the activation energy for the reaction. A second possibility is that heparin facilitates electron donation by resonance from the lone pair electrons on the remaining aromatic amino group, thereby making the imino carbon less electrophilic, and thus less subject to nucleophilic attack. A third possibility is that the solvation sphere of heparin-bound AuO differs from that of free AuO in a manner which makes the bound dye less susceptible to nucleophilic attack by the water in this solvation sphere. Solvation has been demonstrated to play an extremely important role in the PE catalyzed hydrolysis of ionic esters (66). It may even be the case that due to the hydrophobicity of portions of the AuO ion, it is bound in such a way that the accessibility of the imino carbon to water is sterically hindered. Although Manning's description of

counterion condensation specifically refers to counterions being territorially bound, and this means that they retain their inner hydration spheres (58), this last possibility is not contradictory to counterion condensation being the driving force underlying the catalysis, because the theory does not preclude site bound counterions from participating in the lowering of the PE's charge density; it just does not require any site binding to be assumed.

Regardless of any such changes in the hydrolysis mechanism for AuO^{2+} bound to heparin, the fact remains that the predominant influence of heparin on the reaction is still exerted via its perturbation of AuO's prototropic equilibria. Despite the increased stability of heparin-bound AuO^{2+} , it is still significantly more reactive than the monoprotonated species. Thus in the pH range where catalysis is most evident (pH 2-3), the rate enhancement is due to the heparin promoted formation of AuO^{2+} , since this species would not even be present in the absence of heparin. That is, even though heparin-bound AuO^{2+} is more stable than free AuO^{2+} , the bound diprotonated species is still much more reactive than the monoprotonated AuO which would predominate in this pH range, were it free in solution.

From this point of view, it is evident that heparin's influence on AuO hydrolysis represents an example of how polyanions can accelerate acid catalyzed reactions. As such, it is directly comparable to the numerous other examples in the literature wherein polyanionic acceleration of acid catalyzed reactions has been

demonstrated (62,138-143). Polystyrene sulfonic acid was found to be 115 times more effective than hydrochloric acid in catalyzing the acid hydrolysis of dipeptides (138). Though this was a heterogeneous system, and thus not directly comparable to the case of a soluble polyanion, Morawetz cited this work as evidence that a polyanion could generate locally elevated hydronium ion concentrations, and predicted that examples of soluble polyanions accelerating acid catalyzed reactions would soon be found (139). This prediction has been amply borne out (140-143). The concept of a local pH in PE solutions has been invoked to explain the enhancement by polyanions of acid catalyzed ester hydrolysis (142), the acid catalyzed benzidine rearrangement (140), and the autocatalyzed hydrolysis of acrylate polymer colloids (143). In addition, indirect evidence for this concept has been presented based on studies of polyvinylsulfonate's catalytic activity with respect to the reduction of cis and trans $\text{Co}(\text{NH}_3)_4(\text{N}_3)^{2+}$ (62). The greater catalytic activity displayed for the reaction of the trans isomer in this system is explained based on the fact that only for the trans isomer is the reduction acid catalyzed.

An analogous situation exists with respect to micelles in aqueous solution containing no added counterions. As previously described, local pH effects are believed to underly the influence of micelles on indicator equilibria (131-133,144). It is thus not unexpected that anionic micelles have been found to accelerate several acid catalyzed reactions (146). In fact, it has even proven

possible to measure an increase in the bulk pH of dilute HCl solutions upon the addition of sodium dodecylsulfate at concentrations above its critical micelle concentration (146). Since no titration of the surfactant's sulfate groups is occurring in the considered pH range, these measured pH elevations represent a direct demonstration of hydronium ion behaving as a typical counterion, in condensing onto the polyanionic surface.

The influence of polyanions on acid catalyzed reactions is clearly distinct from the secondary kinetic salt effect displayed by simple electrolytes (147). PE induced changes in pKa are likewise clearly differentiated mechanistically from the influence of simple electrolytes on acidity constants (130). In both instances the basis for the differentiation between poly- and simple electrolytes is the same. Firstly, the magnitude of the effects observed with PE is much greater than those observed with simple electrolytes. Secondly, unlike the situation with simple electrolytes, the direction of the influence can be predicted a priori for PE. Thirdly, the addition of simple electrolytes to a PE system displaying these effects rapidly abolishes the PE influence, rather than the additivity that would be expected if both effects originated from the same underlying forces. It is thus clear that for polyelectrolytes the observed influences originate in the phenomena of counterion condensation, a process which has no counterpart in simple electrolyte solutions.

This relationship between counterion condensation and the catalytic properties of PE has led some investigators to attempt the derivation of quantitative equations describing the catalysis (57,60,64). These derivations are based on the principle that the differences just mentioned between the kinetic influences of poly- and simple electrolytes lies not in their origination in totally different forces, but only in the qualitatively different manifestation of these forces in the two systems. Thus in both cases the kinetic effects can be dealt with in terms of the influence of added electrolyte on the activity coefficient term in the Bronsted-Bjerrum equation. For simple electrolytes this is the well known primary salt effect, and evaluation of the activity coefficient term based on the Debye-Huckel theory yields an equation whose validity has been amply demonstrated (65). For PE, this term must be evaluated using Manning's limiting laws, in conjunction with assumptions regarding the relative participation of condensed and uncondensed species in the ionic reaction. Thus depending on reaction type (i.e. the relative magnitude and charge type of the reactant ions) different equations are obtained, in some cases yielding quite good quantitative agreement with the observed kinetic data (57,60,64). However the assumptions involved in these derivations are by no means universally accepted, and thus they cannot be indiscriminately applied to all types of reactions (55).

No attempts have been made to apply a Bronsted-Bjerrum-Manning type treatment to the polyanionic enhancement of acid catalyzed

reactions. The reasons for this are several-fold. In the first place, the unique solvation properties of the proton, and its relationship to the structure of water, differentiate this counterion from all others considered (50). Secondly, the role of hydronium ion in acid catalyzed reactions is generally not akin to that of a simple reactant ion, which the Bronsted-Bjerrum theory was originally derived to deal with. Rather, hydronium ion often plays a more complex role in reactions, sometimes participating in several steps of the mechanism. This is the case with respect to the hydrolysis of AuO , where hydronium ion participates both in a rapid pre-equilibrium protonation, and in the decomposition of a reactive intermediate. Thus quantitative treatment of PE catalysis in these systems cannot be accomplished in the same way as for simple ionic reactions; both the Manning treatment of activity coefficients, and the Bronsted-Bjerrum treatment of ionic reactions, cannot be straightforwardly applied.

4. Further characterization of heparin's influence on AuO hydrolysis as a PE effect.

Since heparin's influence on AuO hydrolysis falls into the category of a polyanion enhanced, acid catalyzed reaction, it cannot be dealt with by quantitative application of a Bronsted-Bjerrum-Manning type treatment. Nonetheless, from a qualitative point of view, this system displays many of the typical features observed in other PE catalyzed reactions, and all of the

salient features observed when other polyanions (or anionic micelles) influence acid dependent reactions. These parallels will now be drawn so as to solidify the position that heparin's influence on AuO hydrolysis is grounded solely in its properties as a polyelectrolyte, and that there is no need to invoke the existence of a specific catalytic site. This point will be seen to be especially important when the basis for heparin's differentiation from other GAG with respect to AuO hydrolysis is subsequently discussed.

The influence of AuO concentration on the rate of hydrolysis is presented in section IIIF. Though the non-catalyzed reaction shows a simple first order dependence on AuO concentration, the catalyzed reaction displays a complex variation of order with respect to AuO (Figure 14). At low concentrations the catalyzed reaction is first order with respect to AuO, as demonstrated by the linearity and unit slope of the first three points on the log-log plot of the rate data in Figure 14b. However as the AuO concentration is increased, the linearity of this plot is lost and the slope is observed to continually decrease, passing through zero and finally assuming negative values. This changing order is a reflection of the fact that high AuO concentrations have a pronounced inhibitory influence on the catalyzed reaction, a point clearly illustrated in Figure 14c. The magnitude of the inhibition precludes any explanation in terms of product inhibition (Table V), and is certainly quite different from the saturation kinetics displayed by enzymatic or other site-specific catalysts (94). This pattern is, however, typical of PE catalyzed reactions involving ionic species with the

same sign, and has been generally explained in terms of the accumulation effect (55.62.63) (see Section IC2).

The explanation of this behavior is a bit different in the heparin-AuO system. Here, the drop in rate at high AuO concentrations is a consequence of site-bound AuO reducing heparin's charge density below its critical value. Once this is the case, counterion condensation, and consequently the catalysis, is lost. This behavior solidifies the viewpoint that the local pH effects observed in PE solutions are a reflection of the participation of hydronium ion in counterion condensation. The rate drop-off cannot simply be a consequence of counterion competition; it is too sharp, and the AuO concentration at which it occurs is lower than the hydronium ion concentration. Rather the heparin-AuO interaction behaves as though it obeys a mass action formulation, as evidenced by the way AuO influences the catalyzed reaction at two different heparin concentrations (Figure 14c). It can be seen that the AuO concentration at which inhibition is observed is dependent on the heparin concentration; the higher the heparin concentration, the higher the AuO concentration before inhibition is observed. This relationship is clearly observed in Figure 15a. When reaction rate is plotted as a function of the P/D ratio, the curves for both heparin concentrations are now seen to exactly coincide. Catalytic activity smoothly increases with increasing AuO concentration until a P/D value of approximately 0.3 is reached, at which point the reaction rate abruptly declines with further increases in AuO concentration (decreasing P/D ratio). The significance of this

point is that when 3 dye molecules are present per polymer residue, the ξ value of heparin has been reduced below one.

The fact that the order of the reaction remains one at high values of the P/D ratio (Figure 14b) indicates that aggregative forces between AuO ions within the PE domain do not at all contribute to the catalysis. If anything, such forces would be inhibitory, based on the loss of catalytic activity as P/D is decreased below 0.3. This is clearly seen in figure 15b, wherein the rate enhancement (R_c/R_o) is plotted vs the P/D ratio. For both heparin concentrations studied, the rate enhancement increases with P/D only until a P/D value of approximately one is reached, and is thereafter constant with P/D. Thus the catalytic influence of heparin is independent of the P/D ratio as long as enough heparin is present to ensure that all of the available AuO is bound in a non-aggregated state. Based on this fact and the unimolecular order of the reaction at high values of P/D, the catalyzed reaction can be envisioned as involving singular AuO molecules bound in a region of high local acidity within heparin's electrostatic domain.

This picture of the catalyzed reaction is also supported by the influence of heparin concentration on reaction rate. As illustrated in Figure 16a the catalytic rate enhancement increases with heparin concentration up to the point where the reaction mixture is approximately $10^{-3}M$ in heparin monomer residues. Further increases in heparin concentration result in a dramatic loss of catalytic activity. Once again this is the behavior expected for PE catalysts (55,57,60-64) and quite contrary to what would be expected

for other types of polymeric catalysts (94,117). The basis for this effect can be thought of in terms of dilution of the local reactant ion concentration within the PE domain. Increasing the PE concentration beyond the point where all of the reactants are localized within the PE domain results in a spreading out dilution of the available counterions, and thus a decrease in the elevated local concentrations responsible for the catalysis. It is thus not coincidental that this dilution begins to take place at $10^{-3}M$ heparin when the analytical hydronium ion concentration is $2.5 \times 10^{-3}M$. A similar pattern is observed with respect to the influence of sodium dodecylsulfate concentration on the acid catalyzed hydrolysis of nitrobenzaldehyde diethyl acetal; the loss of micellar catalysis at high concentrations of surfactant is likewise attributed to a dilution of the elevated hydronium ion concentration at the surface of the micelle (146). In fact by calculating the concentration of micellar bound hydronium ion, and thereby correcting the observed reaction rates for the decrease occurring at high surfactant concentration, these investigators were able to demonstrate that the corrected rate constants do in fact level off at high micelle concentrations, rather than the decrease observed when uncorrected rates are considered. This correction is based on an empirical calculation of the micellar hydronium ion concentration from potentiometrically measured bulk pH values. This increase in measured pH over the analytical value is quite distinct from that which the equation in section IIIG was derived to deal

with. This equation is meant to deal only with the increase in pH due to titration of heparin's iduronic acid groups, a process which entails site binding of the proton, rather than its territorial localization as a counterion. Although in the heparin-AuO system no systematic study of increases in measured pH as a consequence of territorially bound hydronium ion was performed, this trend is evident from the data in Table II.

An additional point can be made regarding the influence of heparin concentration on the catalysis when these data are plotted in terms of the P/D ratio in Figure 16b. Maximal catalytic activity is observed at a P/D ratio of 30. This does not contradict the conclusion drawn from Figure 15b, that catalytic activity is constant above a P/D value of one. Figure 15b was generated under conditions wherein the heparin concentration was held constant, whereas for Figure 16b, the AuO concentration was held constant. The difference between these two conditions is quite real. When the heparin concentration is varied to get P/D values greater than one, R_c/R_o can increase because the availability of more heparin domain results in a greater fraction of the available AuO becoming bound. But as the AuO concentration is decreased to get higher values of P/D at a constant heparin concentration, the fraction of AuO bound remains constant, because even at the lowest AuO concentrations displaying the maximal rate, there is enough of the dye available to reduce heparin's charge density to the value dictated by Manning's limiting laws (56,58). This is not a trivial point, because it

demonstrates that at high P/D ratios the binding of AuO to heparin behaves to a large extent in the manner expected for a counterion condensation process, rather than a mass action formulation. If a true K_D expression was valid for the heparin-AuO system, then decreasing the AuO concentration would result in a decrease in the concentration of the catalytic complex, and thus R_c/R_o would be expected to decrease at high values of P/D, rather than the constancy observed.

Thus the significance of the fact that increasing the heparin concentration causes a decline in the rate enhancement after a P/D value of 30 is reached, lies not in the influence that high heparin concentrations have on the binding of AuO, but rather in the influence high heparin concentrations have on the local pH in the PE domain. Since the reaction is first order with respect to AuO at high P/D, dilution of the AuO within the heparin domain does not affect the catalysis. All that is necessary for the catalysis to take place is for the AuO to be localized in a microenvironment of elevated acidity, so that its diprotonated form will predominate. This view point is supported by the influence of the P/D ratio on metachromasia at acid pH, as illustrated in Figure 13. It must be remembered that the change in Σ_{430} upon adding heparin at acid pH is due to heparin's perturbation of AuO's prototropic equilibria. Thus the demonstration in this Figure that Σ_{430} reaches its minimum value at P/D = 30 is to be expected, since the acid-pH metachromasia and the catalysis arise from the same phenomena. This behavior contrasts quite markedly with the

influence of the P/D ratio on metachromasia at neutral pH, shown in the same figure. In this pH range, the metachromatic influences of heparin on AuO are not the result of an apparent change in pKa, but rather more akin to those observed in other dye-chromotrope systems (13,28,73,134), and thus reach a maximum at a P/D value of approximately one, where stacking type interactions are most favored.

If the catalytic activity of heparin is indeed predominantly a consequence of counterion condensation, it would be expected that any extraneous ionic solute would have a marked inhibitory influence on the catalysis. Addition of any other counterion to the system would have the effect of reducing the concentration of reactant ions (AuO and H_3O^+) in the heparin domain. This is in fact the behavior observed, as illustrated in Figure 17 of results, and figure 5 of the Appendix I. In Figure 17, the influence of added NaCl on the catalyzed and non-catalyzed reaction is compared. In the case of the non-catalyzed hydrolysis, NaCl has a mild stimulatory influence, on the same order of magnitude as is observed for other acid catalyzed hydrolyses (148,149). However, in the case of the heparin-catalyzed reaction, NaCl dramatically lowers the reaction rate, such that by 0.04M NaCl the rate in the presence of heparin has been decreased to that in its absence. Further increases in the NaCl concentration then accelerate the rate in a fashion parallel to the influence of equivalent concentrations on the non-catalyzed reaction. Thus at NaCl concentrations greater

than 0.04M the reaction is no longer influenced by heparin. Figure 5 of Appendix I demonstrates similar results, but makes the additional point that divalent cations are almost two orders of magnitude more effective. Furthermore, the two divalent cations tested, Ca^{+2} and histamine, are practically indistinguishable with respect to their inhibitory influence on the catalysis. Considering the grossly different structure of these two divalent cations, it is therefore unlikely that anything other than electrostatic forces is involved in this inhibition.

An additional point relating this inhibition to counterion condensation can be made by comparing the NaCl concentrations reducing the rate of the catalyzed reaction to that of the non-catalyzed reaction in these two figures. The only difference between the Figures is the HCl concentration at which they were generated. In Figure 17, where 0.01M HCl was used, maximal inhibition was observed at 0.04M NaCl, which corresponds to a $\text{Na}^+/\text{H}_3\text{O}^+$ concentration ratio of 4. In Figure 5 of Appendix I, the HCl concentration was 0.0028M, and maximal inhibition of the catalysis was observed at .01M NaCl, which likewise corresponds to a $\text{Na}^+/\text{H}_3\text{O}^+$ concentration ratio of approximately 4. This behavior supports the notion that the inhibitory influence of salts is simply due to counterion competition.

Another important piece of data relating heparin's catalytic activity in this system to PE effects is the dependence of catalytic activity on molecular size (section III K). When catalytic activity

was determined as a function of chain length for a series of heparin-derived oligosaccharides, it was found to increase regularly between the hexa- and tetradeca-saccharide, and then level off (Figure 19). This was also consistent with the observation that catalytic activity is independent of molecular weight, for the molecular weight range from 6,000-30,000 D (Table VI). The leveling off of catalytic activity above a minimal chain length is typical of the behavior observed in other systems arising as a consequence of PE effects. For example, the molecular weight of polystyrene sulfonate was found not to influence the catalytic activity of the polymer with respect to amino ester hydrolysis, in the molecular weight range examined (67). With respect to the PE influence on the pKa of bound ligands, once again it is only dependent on chain length when the polymer is below a minimal size (about 10 monomeric units) (130). In general it can be stated that all PE properties resulting from counterion condensation will display this threshold effect at low chain length, and be independent of chain length when sufficiently beyond the threshold (150,151).

From the temperature dependence of AuO hydrolysis, the activation parameters for the catalyzed and non-catalyzed reaction were calculated in section I of results. Using these values, some tentative statements can be made regarding the information this reaction gives us with respect to heparin's microenvironment. The concept that some type of medium effect is operative in PE catalysis is not limited to those proponents of the viewpoint that the

catalysis is explicable in terms of activated complex theory (i.e. as an extended primary salt effect) (57). Even those stressing the "accumulation effect" agree that a local medium effect is superimposed on the concentration effect (55,61-63). If this were in fact not the case, the activation parameters of the reaction would be unaltered by PE.

The Arrhenius activation energy (E_{act}) is empirically calculated from a plot of the reaction's temperature dependence as $\log K$ vs $1/T$. E_{act} values for the catalyzed and non-catalyzed reactions are, respectively 12.3 Kcal/mole and 15.6 Kcal/mole. The lowering of E_{act} for the catalyzed reaction is the result anticipated based on the relationship expected between E_{act} and rate. However, in light of the previous statement that AuO^{2+} behaves as though it is more stable to hydrolysis in the presence of heparin, this point needs clarification. It must be remembered that the predominant factor underlying heparin's catalytic activity is its perturbation of AuO 's prototropic equilibria. Thus at the constant pH of 2.6 used to determine the reaction's temperature dependence, AuO bound to heparin can be considered as completely in its diprotonated form. Conversely, free AuO at this pH is a mixture of 16% and 84% respectively, of the di- and mono-protonated species. Therefore the measured E_{act} is a weighted average of this value for these two species, which have very different reactivities toward hydrolysis. The relative contribution from these two species can be determined from estimates of their

respective hydrolysis rate constants. That of monoprotonated AuO can be estimated from the measured hydrolysis rate of AuO at pH 3.2 and 20° (section IIIA, results), where based on Figure 23, the dye is 97% in its monoprotonated form. The observed half-life under these conditions is 12 days, yielding a pseudo first order rate constant of $5.6 \times 10^{-5} \text{ min}^{-1}$. (This is the maximal value of the rate constant for the monoprotonated species, because the contribution to the observed rate from AuO^{2+} is neglected). At pH 2.6, the pseudo-first order rate constant for the non-catalyzed reaction is $1.25 \times 10^{-4} \text{ min}^{-1}$. Thus under the experimental conditions used to determine E_{act} , the reaction of the monoprotonated species can contribute as much as 45% to the calculated activation parameters. In light of this fact it is clear why E_{act} is decreased by heparin. Though AuO^{2+} is less reactive when bound to heparin than when free, the E_{act} for heparin-bound AuO^{2+} is still lower than that of free AuO^{1+} , which constitutes 84% of the mixture of free dye species under the conditions of this experiment.

This complication also makes it impossible to draw useful conclusions from heparin's influence on the other activation parameters of the reaction. Even in the simplest cases this would be difficult, since PE can exert medium effects on reactions occurring in their electrostatic domain in multiple ways (66-69). Local dielectric constants, changes in ion hydration, and electrostatic stabilization of the transition state may all

contribute to the PE induced changes in the reactions activation parameters. Nonetheless comparisons can be drawn to other PE catalyzed systems. In their study of the PE catalyzed alkaline hydrolysis of triphenylmethane dyes structurally related to AuO (e.g. crystal violet, brilliant green, malachite green), Okubo and Ise (68) found that the catalysis of this reaction by polycations with hydrophobic character was accompanied by a decrease in $\Delta H^\ddagger$ from 8.8 Kcal/mole for the non-catalyzed reaction to 7.2 Kcal/mole for the reaction in the presence of poly (4-vinyl-N-cetylpridinium bromide). The small decrease in activation enthalpy is reflective of the fact that electrostatic repulsive forces must be overcome. For the same system $\Delta S^\ddagger$ was found to be -30 eu and -32 eu, for the non-catalyzed and catalyzed reactions respectively, indicating that the polymer has little effect on the entropy of activation. This compares favorably with the calculated $\Delta S^\ddagger$ of -25 eu for free AuO hydrolysis, and the heparin-mediated decrease in this value to -30 eu. The observation of a negative $\Delta S^\ddagger$ is unusual for a reaction between ionic species of opposite charge, though it has been reported by others in similar systems, such as the alkaline hydrolysis of ethyl violet (152). However the negative $\Delta S^\ddagger$ observed for AuO hydrolysis is what would be expected for a reaction involving hydronium ion and a dye cation (147).

D. The specificity of the catalysis with respect to PE structure.

The two most striking results reported have not yet been discussed. These are the relationship between anticoagulant and catalytic activity (section IIK) and the differentiation of heparin from other GAG with respect to the catalysis (section IIIL). The latter has been utilized as the basis for a chemical assay of heparin, in which regard it has the advantage of specificity over all other chemical assays for heparin (see Appendix I). Here it will be demonstrated that both of these observations are reflective of the fact that heparin's unique influence on the reaction is a consequence of counterion condensation, as strictly defined by Manning's limiting laws of PE solutions. Furthermore, the question of whether the same factors that make heparin unique in this system are relevant to the basis for its unique biochemical roles will be addressed.

1. The basis for the differentiation of heparin from other GAG with respect to catalytic activity.

First the reason why other GAG do not catalyze AuO hydrolysis. When one examines the GAG structures illustrated in Figure I, it is apparent that all are polyanions, and thus should concentrate cations into their electrostatic domain. However, this qualitative description is quite different from the precise mathematical description of counterion condensation set forth in Manning's

theories. Qualitatively it might be expected that the ability of a PE to condense counterions would be a continuous property, with the charge density of a PE only dictating the magnitude of the force underlying the condensation, but not the presence or absence of the phenomenon. This viewpoint is fostered by the image of an electrolyte solution presented in Debye-Huckel theory, where small ions, of much lower electrostatic potential than even the weakest of PE, are envisioned to support the development of an oppositely charged ionic cloud (65). But the difference between what is meant by an ionic atmosphere as it relates to poly- and simple-electrolytes is brought sharply into focus by considering the very different dependence of these two phenomena on ionic strength. Counterion condensation, as defined in Manning's limiting laws, is an ionic strength independent process, occurring in an all or none fashion, and theoretically persisting even at infinite dilution (56,58). Conversely, in Debye-Huckel theory, the development of an ionic atmosphere is a graded, rather than an all or none process, theoretically vanishing at infinite dilution, and whose ionic strength dependence is evident in the mathematical description of the Debye-length, the thickness of the ionic atmosphere (65). The analogous description of V_p , the volume of the region surrounding a PE within which counterions are said to be bound, is independent of ionic strength, and dependent only on the structural parameters of the PE (58).

This is not meant to imply that nothing analogous to the ionic atmosphere of Debye-Huckel theory exists in PE solutions. Indeed there are continuing attempts to describe PE solutions by fully dealing with non-linear Poisson-Boltzman equations, and thereby approaching questions about PE solutions which Manning theory is not addressed to (153, 154). However, the fact remains that only Manning satisfactorily describes a theoretical basis for the empirical PE charge fraction rule ($f = |\xi Z|^{-1}$), and thus explains the experimental fact that counterion condensation is a strictly all or none phenomena. It occurs only for PE whose ξ parameter (charge density) is above a limiting value ($\xi = 1$ for a system containing only univalent counterions), and always reduces the net charge density of a PE to the same final value ($\xi_{\text{net}} = 1/\text{counterion valence}$), regardless of the original structural charge density of the polymer. It is this observation with which Manning's limiting laws specifically deal.

Thus in deciding whether counterion condensation, as thus strictly defined, will occur for a particular PE, it is only necessary to know the applicable ξ parameter, as determined by the polymer's structural charge density. The application of Manning's limiting laws to biological PE is a relatively new approach, and thus ξ parameters for all of the GAG tested for catalytic activity could not be found in the literature. However there have been several recent studies treating GAG from the viewpoint of Manning theory (54,78,125,136,155-157). These studies not only demonstrate

the validity of the theory as applied to anionic polysaccharides, but also provide an internal consistency check of the ξ parameter value determined from structural considerations (157). This is especially important, considering the microheterogeneity characterizing all biological GAG preparations, resulting in the charge density calculated being an average rather than absolute value, and thus requiring an empirical measure of the charge density in a particular preparation (78).

In the argument that follows, we will refer to ξ values from the literature when available. However, due to the microheterogeneity problem, it is necessary to estimate the actual ξ values for the preparations tested. This will be accomplished based on the analytically determined sulfate contents of the samples, presented in Table I. The sulfate content, expressed as moles sulfate per mole hexoseamine, gives a measure of the number of ionic charges per dimer of the copolymer (adding one to the sulfate content to account for the charge due to the carboxy group, (in all cases except keratin sulfate). The length of a disaccharide unit is known from the average axial periodicities of the GAG, determined by X-ray diffraction analysis. The values used are those determined for a 2_1 , helical symmetry, which seems to be the most prevalent conformation for the biological GAG (23). Based on these data, b , the average linear charge spacing of the polyanion, is determined (b = average axial periodicity/number of charges per

dimer). Manning's linear charge density parameter, ξ can then be simply calculated as:

$$\xi = \frac{0.7092}{b}$$

at 20°C, with b expressed in nm, $q = 1.603 \times 10^{19} \text{C}$, $\epsilon = 80.37$, and $k = 1.38 \times 10^{-23} \text{J molecule}^{-1} \text{K}^{-1}$. The ξ values thus calculated are only valid to the extent that the axial periodicities determined from X-ray diffraction are reflective of the true solution conformations of GAG. Previous work comparing experimentally determined ξ values with those calculated as above have shown reasonable agreement (155-157). Furthermore the chiroptical properties of GAG-acridine orange complexes (28,73) support the viewpoint that the chromotropic backbone of these polyanions in solution is in the form of a helical array. These data are especially supportive in light of the structural similarities between AuO and acridine orange. In any event, the uncertainty arising in the calculated ξ values for this reason is of a magnitude which would not affect the validity of the argument which follows.

The calculated linear charge density (ξ) for the seven standard GAG tested are listed in Table XI. These values are in good agreement with those reported by others. For example, Cleland reported calculated ξ values of 1.4 and 0.75 for chondroitin-4-sulfate and hyaluronate, respectively (155). Tivant et al., (157) experimentally determined ξ for chondroitin-4-sulfate

to be 1.15 (from counterion tracer diffusion measurements) and noted that the lower experimental, compared to the calculated value, was probably a reflection of the fact that some site binding of counterions lowers the structural value of ξ , i.e. before the process of counterion condensation, as strictly defined by Manning, occurs. A wide range of ξ values have been reported for heparin. Ander et al., (156) calculated an ξ of 3.0 for heparin, based on an equivalent weight of 183.5 (determined by titration of the sample, as heparinic acid, vs standardized NaOH), and an axial charge spacing of 0.24nm. Wedlock et al. (125) calculated for a heparin preparation of anticoagulant activity 165 USP units/mg, an ξ of 2.45, by using acridine orange titrations to determine the anionic site equivalent weight. This latter group also reported that there was a correlation between anticoagulant activity and charge density (78). Since the anticoagulant activity of the NIH standard heparin listed in Table I and XI is 184 USP units/mg, our calculated ξ value of 3.22 is still reasonable, despite the very different ways in which ξ was calculated for the two cases.

It is not immediately apparent from examination of the ξ values in Table XI why heparin is the only one of these GAG capable of accelerating AuO hydrolysis. With the exception of hyaluronic acid and keratin sulfate, all of the GAG have charge densities greater than the critical value necessary for counterion condensation to occur ($\xi_{\text{critical}} = [Z/Z_p]^{-1} = 1$). Thus if counterion condensation is indeed the force underlying the catalysis, why is

TABLE XI

Calculated linear charge density parameters for NIH standard GAG preparations.

<u>Preparation</u>	<u>Average axial periodicity (23) (nm/disaccharide)</u>	<u>Average # charges Disaccharide</u>	<u>(nm) b</u>	<u>ξ</u>
Heparin	0.83	3.77	.22	3.22
Hyaluronate	0.98	1.0	.98	.72
Chondroitin-4- sulfate	0.98	1.98	.49	1.45
Chondroitin-6 sulfate	0.93	1.90	.49	1.45
Heparin sulfate	0.93	1.97	.47	1.51
Dermatan sulfate	0.96	2.13	.45	1.58
Keratin sulfate	0.93	1.26	.74	.96

heparin unique? The answer to this question becomes apparent when it is realized that the ξ values listed in this table pertain only to the GAG at neutral pH; since the charge density is partly due to ionized carboxylate groups, ξ could be expected to decrease significantly under acidic conditions. It is, in fact, at pH 2.6 that the differentiation of heparin from other GAG with respect to the catalysis is demonstrated (Table X). Based on the reported pKa for carboxy groups in GAG (158,159), which is in all cases is in the pH 3.5-5 range, their contribution to charge density at pH 2.6 should be negligible. The relevant ξ values at pH 2.6 can thus be simply calculated by considering what effect protonation of the carboxylates will have on b , based on the structural formulas for GAG illustrated in figure 1. In the case of heparin, the carboxy group only contributes 1 of 4 negative charges per disaccharide; thus ξ at pH 2.6 would be 25% lower than its value at neutral pH. This would give an ξ value of 2.42, and thus well in excess of the critical value necessary for the counterion condensation to occur. However, in the case of the other GAG, the carboxy group contributes 50% of the negative charge. Since even at neutral pH the maximal ξ parameter for any of the GAG (excluding heparin) is 1.58, at acid pH, all have charge densities well below the critical value supporting counterion condensation. It is thus clear that heparin is unique in retaining the ability to support counterion condensation at acid pH, and that this may be the factor underlying heparin's differentiation from other GAG with respect to the capacity to catalyze AuO hydrolysis.

The above explanation is supported by the structural characteristics of the other PE shown to catalyze AuO hydrolysis in Table X. Only three other polyanions were found to significantly accelerate the reaction-dextran sulfate, polyvinylsulfate and polyanethole sulfonate. The grossly different structural characteristics of these polymers compared to each other and heparin, rules out the possibility that some type of specific site on the polymer is involved in the catalysis. Rather the only structural parallel between the four PE is that in all cases, these polyanions have ξ parameters at acid pH sufficiently large to promote counterion condensation. Dextran sulfate, with approximately two sulfate groups per glucosyl residue, corresponds to a distance between ionic groups (b) of 0.25nm, and an ξ parameter of 2.85 (156); for polyvinylsulfate b is likewise 0.25nm (60), yielding an ξ parameter of 2.85. Though no value of b for polyanetholesulfonate could be found, it also has an ξ parameter of approximately 2.8, by analogy with polystyrenesulfonate, which has $b=0.25\text{nm}$ (60). It is interesting to note that all three of these synthetic polyanions have some degree of anticoagulant activity (91,160). Though their therapeutic application is limited by side effects, the point remains that a qualitative correspondence between catalytic and anticoagulant activity is illustrated in Table X. More with regard to this relationship will be said shortly.

One more point remains to be clarified regarding the role of the ξ parameter in determining whether a PE will catalyze AuO hydrolysis. High charge density seems to be a necessary, but not sufficient condition for catalytic activity. This is illustrated by the inability of DNA and RNA to accelerate the reaction. Both of these bipolymers have an ξ parameter of approximately 4 at neutral pH (161), and should still be sufficiently ionized at pH 2.6 to accelerate AuO hydrolysis. The fact that they are not catalytic illustrates nicely that it is binding of the diffuse territorial type corresponding to counterion condensation that promotes catalysis. In the case of both these polynucleotides, their binding to AuO is characterized by a more specific type of association, as evidenced by the altered fluorescence properties of the complex (105). Fluorescent AuO-polynucleotide complexes are visible to the naked eye even at pH 2.6 and high P/D ratios, whereas GAG-AuO complexes show no fluorescence under these conditions (unpublished observations). When specific interactive forces exist between a PE and a counterion, the extent of binding dictated by these forces first takes place. If such site binding of the substrate dye takes place to an extent where the structural ξ parameter of the polyanion is reduced below the critical value for counterion condensation, then catalysis will not occur, because the further condensation of hydronium ion as counterion will be precluded. Evidently in the case of DNA and RNA, site binding of AuO is sufficient to reduce ξ below its critical value, so that no counterion condensation, as strictly defined, takes place.

2. The relationship between catalytic and anticoagulant activities.

It is appropriate at this point to discuss more fully the relationship between catalytic and anticoagulant activity. As previously mentioned, there is a correlation between the ξ parameter and anticoagulant activity of a heparin preparation, and we have noted a qualitative correspondence between those polyanions possessing anticoagulant and catalytic activity (Table X). In addition, Hurst et al., have reported that the anticoagulant activity of heparin is dependent on the parameter " Z^2 " where Z is the number of chemically measured anionic sites per uronic acid moiety (162). Furthermore, these same investigators reported that the same parameter, Z^2 , correlated with the ability of a heparin preparation to promote the photochemical oxidation of acridine orange (80). It is thus quite evident that among polyanions, there is a relationship between charge density, anticoagulant activity, and the reactivity of bound cationic dyes. However, the exact physicochemical basis of this relationship has remained elusive. To an extent, the data presented in this thesis may clarify the reason why no clear-cut relationship can be found.

In Figure 20, we have plotted the relationship between specific catalytic activity and anticoagulant activity for a series of heparin subfractions prepared by trimethylhexadecylammonium chloride fractionation from a parent commercial preparation. For the curve indicated by the circles, a smooth correlation is observed between the anticoagulant activity of a subfraction and its effectiveness in

catalyzing AuO hydrolysis. This is in fact the result to be anticipated based on the literature and our description of the forces underlying the catalysis. Both Diakun et al., (70) and Hurst et. al. (162) have demonstrated the relationship between anticoagulant activity and anionic charge density, with the former group relating their data to the same quantitative physiochemical parameter which has been here invoked as the basis for heparin's catalytic activity. Thus for the subfractions tested, it can be expected that the lower their anticoagulant activity, the lower their ξ value. The lower the ξ value of a heparin preparation, the smaller the quantity of counterions which must condense in order to reduce $\xi_{\text{structural}}$ to its limiting value ($\xi_{\text{net}} = |Z|Z_p|^{-1}$). Thus the larger $\xi_{\text{structural}}$ is, the greater the fraction of AuO which will be present in the condensed state, and thus the greater the overall rate of hydrolysis observed.

However the situation is not quite as simple as might appear from the preceding argument. This is seen by careful examination of Table VIII, where it can be seen that for unfractionated commercial heparin preparations, it is not strictly true that anticoagulant and catalytic activity are smoothly correlated. For example, compare the Sigma grade II porcine mucosal heparin (specific activity = $1.1 \times 10^{-4} \text{ min}^{-1}/\mu\text{g heparin uronic acid}$, anticoagulant activity 136 USP units/mg) with the Hepar Industries bovine lung heparin (specific activity = $1.6 \times 10^{-4} \text{ min}^{-1}/\mu\text{g heparin uronic acid}$, anticoagulant activity = 124 USP units/mg).

The lack of a smooth correlation is even observed when comparing heparins prepared from the same tissue, e.g. the Riker and Sigma grade I porcine intestinal mucosa heparins. These inconsistencies are not contradictory to the premise set forth here, but rather point out an important aspect of when and why this correlation holds true.

It is only when comparing heparin fractionated by a procedure sensitive to charge density that a relationship between catalytic and anticoagulant activity is observed; and here it occurs more because of the fractionation procedure itself, rather than the existence of an absolute parallel between the structural parameters important to these two properties of heparin. That is, the correlation occurs because catalytic and anticoagulant activity are both related to charge density, and the fractionation procedure itself is based on charge density. Insofar as other structural parameters of heparin are uniquely important to anticoagulant activity, e.g. the presence of specific, high affinity, antithrombin III binding sequences (16,18), the correlation will be violated. This is illustrated by the departure of the point denoted by a square from the rest of the curve in Figure 20; this point corresponds to the parent heparin from which the subfractions were prepared by hexadecylammonium bromide fractionation, a technique largely based on charge density. Thus in comparing heparins prepared either from different tissues, or by different procedures, one cannot expect a straightforward parallel between catalytic and

anticoagulant activity. However, when comparing heparin subfractions prepared by a procedure based on charge density, a correlation will be observed.

In fact, when carefully examining the reports of Diakun et al., (78) Hurst et al. (162), and Menter et. al. (80), supporting the relationship between charge density and anticoagulant activity, it seems that the same point holds true in their data. For example, Hurst et al. (162) prepared the heparin fractions used in their study from a single hog mucosal heparin, fractionated by a procedure involving the partitioning of heparin-hexadecylpyridinium complexes between n-butanol and aqueous NaCl phases (163), a technique demonstrated to be based only on anionic charge density, and insensitive to chain length in excess of a critical value of about 10,000 D (164). The authors themselves note (162) that had a fractionation procedure based on affinity for antithrombin been used to prepare heparins of different anticoagulant activity (e.g.), no correlation to charge density would have been observed. This viewpoint is supported by the recently reported sequence for the putative specific antithrombin binding site in heparin (18), which apparently is characterized by the presence of a non-sulfated L-iduronic acid residue, and is thus of lower charge density than the "typical" heparin disaccharide (14).

The above argument is not meant to imply that the often reported relationship between anticoagulant activity and charge density is simply fortuitous. Rather it is meant to express the viewpoint

that the ability of heparin to interact with antithrombin is not necessarily the only mechanism of its in vivo anticoagulant action (165-167), though it may be most important to some methods of in vitro anticoagulant assay (168,169). The large degree of attention focused on antithrombin is largely a consequence of the importance of this reaction to many of the methods for in vitro anticoagulant assay. The differences between in vitro and in vivo anticoagulation are brought sharply into focus by the dependence of the pharmacokinetics of heparin's action on its mode of administration, leading Mahadoo and Jaques (170) to postulate that it is the release of heparin from an endogenous cellular pool that is most important to the level of in vivo anticoagulation achieved.

Many suggestions have been set forth regarding the basis for the relationship between anticoagulant activity and charge density. Early theories focused on the influence of heparin as a PE on the colloidal stability of blood, and in particular that of fibrinogen (167). The role in coagulation of reactions occurring at liquid - solid interfaces is a well known fact, and the negative surface potential of the solid has been demonstrated to be a critical parameter governing the interaction of the surface with the soluble and cellular elements involved in thrombus formation (165). This relationship has proven important to the development of intravascular prosthetic devices. For example, it has been

demonstrated that the coating of a plastic catheter with a heparin-benzalkonium complex reduced the problem of thrombus formation on the catheter (165,171,172). However, the precise nature of the physicochemical forces involved in these phenomena is still a subject of debate. Though the point cannot be dealt with in detail here, heparin's uniquely high ξ parameter and the resultant high concentration of counterions existing in the condensed state, may prove important to the clarification of these phenomena.

E. Conclusions and Significance

It has been the aim of this thesis to demonstrate how the catalytic properties of PE represent a viable new approach toward elucidating the chemical microenvironment of GAG. Through the insights derived from such a system, it was hoped that some aspects of the unique biochemical attributes of specific GAG might be illuminated. The extent to which these goals have been attained will now be outlined.

The first of these two goals has largely been met. A system based on the ability of polyanions to catalyze AuO hydrolysis has been exploited as a new type of extrinsic probe. Insofar as the rate of this reaction is reflective of chemical conditions within the GAG microenvironment, heparin's differentiation from other GAG with respect to the ability to accelerate AuO hydrolysis, demonstrates a unique feature of heparin's electrostatic domain.

The basis for heparin's catalytic activity has been straightforwardly offered in terms of Manning's polyelectrolyte limiting laws, and the strict definition of counterion condensation presented therein (58). Because heparin is a polyanion with an ξ parameter greater than the critical value necessary for counterion condensation, in low ionic strength solutions it induces condensation of hydronium ion into its electrostatic domain. AuO, which is likewise bound to heparin, is therefore subjected to a locally elevated hydronium ion concentration. Since the hydrolysis of AuO has been demonstrated to be critically dependent on acidity,

this localization results in an enhanced rate of hydrolysis. The other GAG do not likewise catalyze the reaction, because at acid pH none have ξ parameters in excess of the critical value necessary for counterion condensation to occur. Thus only heparin can generate the locally elevated acidity necessary for catalysis to take place.

This differentiation of heparin from other GAG with respect to AuO hydrolysis parallels the unique biochemical and pharmacological actions of heparin. Though many of these actions have been related to the fact that heparin is a polyanion (14-16, 44, 165-167), they are not shared by the other GAG despite their analogous polyanionic nature. Thus the demonstration here that catalytic activity requires not only a polyanion, but one with a ξ parameter in excess of the critical value necessary for counterion condensation, bears relevance to the origin of heparin's biological uniqueness. The question to be asked is whether under physiological conditions, the same differentiation might exist.

The question, which is tied to the second goal of the thesis, can only be partially answered here. It is clear that to the limits of our current measurement capabilities, the physiological milieu must be considered to be at neutral pH, and moderately high in ionic strength. This sharply contrasts with the conditions of our model system, thereby precluding a straightforward comparison. However, the possibility that heparin's biological uniqueness may be a consequence of the same factors which make it singular with respect to catalyzing AuO hydrolysis, remains a viable alternative lesson.

To draw this parallel, the structural features which led to heparin alone maintaining an ξ parameter above the critical value at acid pH must be recalled. It was the fact that carboxy groups contribute only 25% of the anionic charge per heparin dimer, compared to 50% per dimer for the other GAG, that gave rise to this differentiation. Thus only in the case of heparin will neutralization of the carboxylate charge not reduce ξ below its critical value. Now, although protonation of carboxylate groups would not be expected at neutral pH, other types of site bound counterions might be able to specifically neutralize that portion of a GAG's structural charge density due to carboxylates. An example of how this type of specific charge neutralization might take place can be drawn from some recent data which have been interpreted to indicate that monovalent counterions can site-bind specifically to carboxylate groups (136). Such site bound counterions are treated by Manning in terms of their contribution to the polyion's structural charge density, rather than as condensed counterions (58). When this type of site binding reduces ξ below its critical value, no counterion condensation will occur. If this is the case in the physiological milieu, then heparin will be the only GAG maintaining a ξ parameter sufficiently high to promote counterion condensation. The applicability of this idea to the differentiation of heparin from other GAG in physiological systems merits further investigation.

The Specific Assay of Heparin by Its Chemical Properties

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A sensitive and specific chemical assay for heparin is herein presented. It is based on heparin's ability to catalyze the acidic hydrolysis of the cationic dye Auramine O. The assay can detect as little as 1 μ g of heparin, and is specific in that it distinguishes heparin from all other glycosaminoglycans tested. The applicability of the assay and interference due to the presence of proteins, organic cations, and inorganic salts is discussed.

The assay of heparin by chemical means can be accomplished in several ways. Among the most widely used techniques are assays based on metachromatic interactions with dyes (1), turbidimetric interactions with polycations (2), and analysis for monosaccharide constituents in the intact polysaccharide chain (3,4). However, all these methods have the drawback that they are not capable of distinguishing heparin from the other naturally occurring glycosaminoglycans. For this reason, differentiation of heparin has typically been based on heparin's unique physiological activity as an anticoagulant. The most commonly employed systems for this purpose involve either direct measurement of heparin's inhibitory effect on *in vitro* blood coagulation (5), or quantitation of heparin enhancement of clotting enzyme inactivation by antithrombin III (6). However, these methods have deficiencies stemming from their biological nature. Most notable in this regard are problems stemming from the microheterogeneity of heparin preparations. Different heparin subpopulations have varying effects with respect to the different coagulation proteins (7). Thus, the heparin activity determined by these

methods is very dependent on the potency of a particular preparation in the specific assay system used (8). The result of this situation is relatively poor reproducibility between laboratories, and a persistent inability to develop a global standard for heparin (9).

The substitution of these biological methods with a chemical assay specific for heparin will enable the circumvention of many of these problems. Toward this end, we have sought to take advantage of the ability of heparin to catalyze the hydrolysis of Auramine O, a capability it does not share with all the other naturally occurring glycosaminoglycans we have tested (10). In this communication, we present suitable conditions for the specific, sensitive, low cost assay of heparin.

MATERIALS AND METHODS

Auramine O (bis[*p*-dimethylaminophenyl]methylidene ammonium chloride) was purchased from Aldrich Chemical, Lot 86 103-0, and purified by recrystallization from 0.02 M NaCl as described by Conrad *et al.* (11). Stock solutions of the dye were prepared in Millipore Type I water, and adjusted to a final concentration of 7.3×10^{-5} M, using the ϵ_{430} value of 4.4×10^4 M⁻¹ cm⁻¹ given by Conrad *et al.*, and verified for our preparation. This stock solution was sta-

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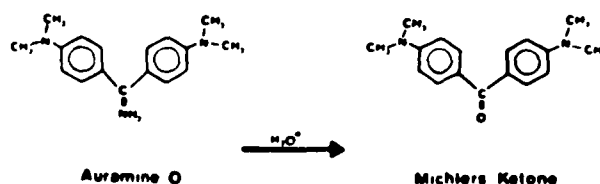


FIG. 1. Hydrolysis of Auramine O.

ble for several weeks when kept in the dark at 4°C.

The following polyelectrolytes were obtained from the Sigma Chemical Company: heparin, sodium salt, Grade I, 169.7 USP units/mg, Lot 96c-0093; chondroitin sulfate Type A, sodium salt, Lot 77c-0187; chondroitin sulfate Type B, sodium salt, Lot 66c-3975; chondroitin sulfate Type C, sodium salt, Lot 56c-3881; hyaluronic acid, Grade I, Lot 115c-009; histamine dihydrochloride was likewise a product of Sigma (Lot 87c-0373). Human serum albumin was a fatty acid-free fraction obtained from Miles Laboratories (Lot 13). Human antithrombin III was the kind gift of Dr. M. Wickerhauser of the American Red Cross Blood Services Laboratories (MDPH Lot 9). The acid stock solution used throughout was 0.0060 N HCl, standardized by titration, with potassium biphthalate as primary standard. The heparin used as a standard was the Sigma product previously mentioned, but was dried for 30 days over phosphorous pentoxide prior to use. NIH reference glycosaminoglycans were obtained from Dr. M. B. Mathews. All other reagents used were of the highest quality commercially available.

Reaction rates were monitored by following the decrease in absorbance at 430 nm, as the bright yellow Auramine is converted into the pale green Michler's ketone. Measurements were performed on a Gilford Model 2400-S spectrophotometer, unless the rates were exceedingly slow, in which case a Cary Model 17 was used. All runs were thermostated at 20°C, and were performed in triplicate.

Procedures for both a kinetic and fixed-

RESULTS AND DISCUSSION

The conversion of Auramine O into Michler's ketone, illustrated in Fig. 1, was originally reported to be an acid-catalyzed hydrolysis by Holmes and Darling (12). More recently, Crescenzi *et al.* demonstrated this reaction to be subject to acceleration by maleic acid copolymers in acidic buffer solutions (13). This is not unusual behavior, and the extensive literature describing polyelectrolyte catalysis predicts that a reaction involving a cationic dye and hydronium ion should be catalyzed by polyanions (14). In this communication, we have attempted to show how this property of polyelectrolytes can be used as a tool for studying mucopolysaccharides, a group of biological polyanions whose importance has become increasingly apparent (15).

Kinetic plots of the hydrolysis of Auramine O under varying conditions are shown in Fig. 2. As expected, heparin has a marked accelerating influence on the rate. However, a rather unexpected result demonstrated in

SPECIFIC ASSAY OF HEPARIN BY ITS CHEMICAL PROPERTIES

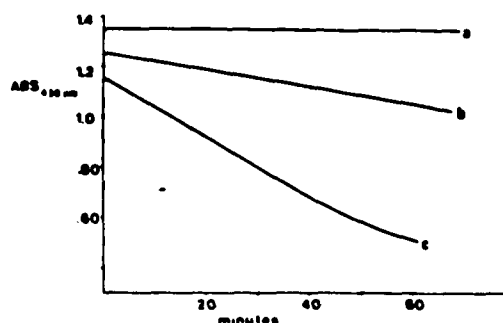


FIG. 2. Kinetic plot of the course of the hydrolysis reaction under the conditions described under Materials and Methods: (a) In the absence of any polyelectrolyte, or in the presence of 100 μ g of chondroitin sulfate A, chondroitin sulfate C, dermatan sulfate, or hyaluronic acid. (b) In the presence of 20 μ g heparin. (c) In the presence of 100 μ g heparin.

this figure, is the apparent lack of catalytic influence for the commercial glycosaminoglycan preparations tested, despite their polyanionic nature. The chondroitin sulfates and hyaluronic acid have no influence on the rate of the reaction even at concentrations manyfold higher than heparin. It should also be noted that heparin alone induces a decrease in the apparent extinction coefficient of Auramine O. The formation of metachromatic complexes between dyes and polyanions is well known (16), and provides a secondary confirmation of the unique interaction between heparin and the dye. The specificity of this rate enhancement is quantitatively examined with NIH standard GAG² preparations in Table I. Even heparan sulfate, which is structurally quite similar to heparin (this preparation has an anticoagulant activity of 2.3 U/mg) is more than an order of magnitude less effective in catalyzing the reaction. Heparin preparations with varying anticoagulant activity differ in their ability to catalyze this reaction, but all examined thus far demonstrate this property, in sharp contrast to other glycosaminoglycans. This parallels the differentiation of heparin from other GAGs based on anticoagulant and

other biological properties (17). Furthermore, the synthetic polyelectrolytes dextran sulfate, polyvinyl sulfate and polyanethole sulfonate, which are weakly anticoagulant, catalyze Auramine O hydrolysis, and in general a qualitative correspondence exists between anticoagulant and catalytic activity (10).

Based on this unique ability of heparin, it is possible to specifically assay for this glycosaminoglycan without relying on its biological activities. The standard curves obtained for kinetic and fixed-time methods of analysis are shown in Figs. 3 and 4, respectively. The rate of hydrolysis reaction is a linear function of heparin added in the range 1–50 μ g. At higher heparin concentrations, the catalytic influence levels off, and when more than 500 μ g of heparin are added, the rate begins to decrease with concentration, as detailed elsewhere (18).

The use of this assay is not hindered by the presence of other glycosaminoglycans in mixture with heparin. As shown in Fig. 4, an identical standard curve is obtained whether or not 100 μ g of chondroitin sulfate is present. In Fig. 4, the effect of macromolecular solutes which interact with heparin is also illustrated, with human serum

TABLE I
INFLUENCE OF STANDARD NIH GAG PREPARATIONS
ON THE RATE OF AURAMINE O HYDROLYSIS^a

GAG added ^a	Rate of Auramine O hydrolysis ($\Delta A_{410}/\text{min} \times 10^3$)
None	0.3
Heparin	17.5
Chondroitin 6-sulfate	0.4
Chondroitin 4-sulfate	0.4
Hyaluronate	0.3
Heparan sulfate	0.8
Dermatan sulfate	0.7
Keratin sulfate	0.7

^a The final GAG concentration in each sample is 10^{-4} M, based on an average monomer molecular weight calculated from analytical data.

² Abbreviation used: GAG, glycosaminoglycan.

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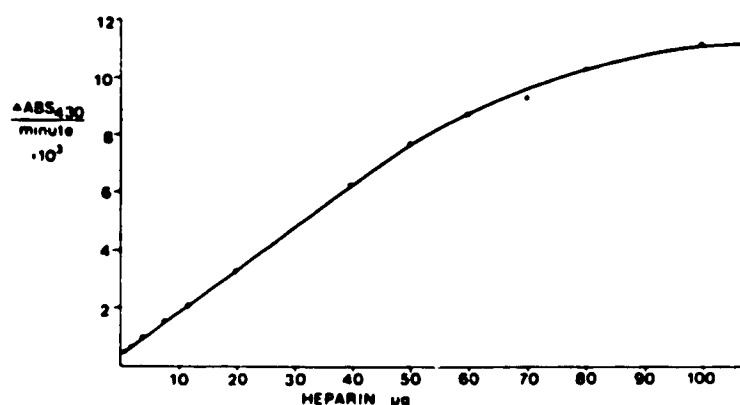


FIG. 3. Standard curve for the kinetic assay procedure, described under Materials and Methods.

albumin and human antithrombin III given as examples. The slope of the standard curve in the presence of these macromolecules is relatively unchanged; however, the minimum heparin concentration capable of exerting catalytic influence is increased. This points out a major consideration when deciding on the applicability of this method to a particular situation. Only heparin free in solution will catalyze the reaction. Thus, any species which has a greater binding affinity for heparin than Auramine O will decrease

the apparent quantity of heparin as determined by this assay. Problems of this sort are common to many of the methods used for quantitating heparin. When the influence of the interfering substance can be adopted into the standardization procedure, assay is still possible. In fact, heparin can even be assayed under conditions wherein it is involved in the formation of a colloidal precipitate, as would be the case in low pH heparin-albumin solutions at concentrations higher than those we have employed here. If desired, standard procedures, for separating GAGs from biological mixtures (3) can be attempted before assay. For example, the GAG can first be precipitated from solution as an alkylpyridium complex, brought back into solution with excess inorganic salts, and then separated from the excess salt by precipitation from 80% ethanol. From another point of view, interference of this sort is in itself useful, since it provides a kinetic technique for detecting macromolecular interactions with heparin, and can thereby complement equilibrium displacement techniques (19).

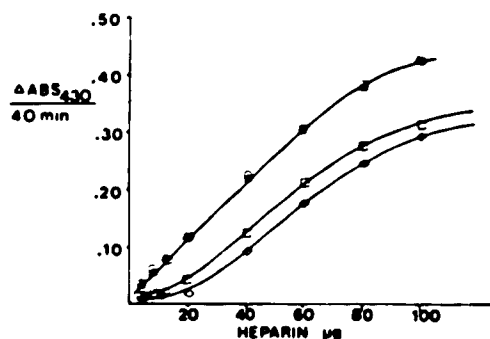


FIG. 4. Standard curve for the fixed-time assay procedure in the presence and absence of interfering macromolecules. O, Heparin alone; Δ, with 100 μg chondroitin sulfate A; □, with 50 μg human serum albumin; ○, with 100 μg human antithrombin III.

Figure 5 illustrates the interfering influence of metal cations on the catalysis. At a concentration of 5 mM, NaCl reduces the catalytic influence of 50 μg of heparin by

SPECIFIC ASSAY OF HEPARIN BY ITS CHEMICAL PROPERTIES

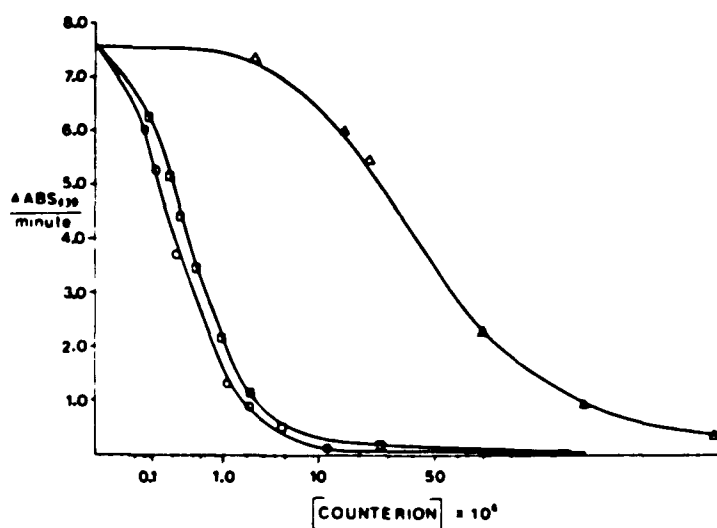


FIG. 5. Effect of ionic solutes on the catalytic influence of 50 μ g heparin, in the kinetic assay procedure. Δ , NaCl; $\square$, CaCl_2 ; O, histamine.

50%; divalent cations in the micromolar concentration range similarly reduce heparin's activity. This result would be anticipated if the catalytic influence of heparin is due to its tendency as a polyelectrolyte to induce counterion condensation within its electrostatic atmosphere. As such, both dye cations and hydronium ions are localized, causing the reaction to proceed at a quicker rate than that which would be expected from the analytical concentrations of the reactants in the bulk solution. Because other cations likewise become concentrated in the electrostatic domain of the polyanion, they can exclude reactant cations; condensation only proceeds until a limiting charge density is reached (20). The greater effectiveness of divalent cations is also in accord with current polyelectrolyte theory (20).

In summary, we have presented a preliminary report of a phenomenon which should be of practical interest for the assay of heparin and for the detection of its interaction with other solutes. The methodology is simple and requires little beyond that which is typically available in any laboratory. We

have stressed the limitations set on the method by the strong interacting tendencies of heparin. By either eliminating interfering components, or incorporating them into standardization procedures, such problems can be circumvented enough to ensure the utility of this procedure.

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