Structural Basis for the Anticoagulant Activity of Heparin. 1. Relationship to the Number of Charged Groups†

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ABSTRACT: This study was undertaken to provide further information concerning the chemical heterogeneity of heparins and the relationships between the anticoagulant activity (USP assay) and the anionic density of the heparin. A sample of commercial heparin was fractionated into 13 fractions by sequential extraction in a two-phase system of 1-butanol–aqueous NaCl containing excess hexadecylpyridinium chloride. The anionic density distribution was characterized by the fractional distribution of uronate among the fractions. The fractions were characterized by several molar ratios of constituents, molecular weight, charge density, and anticoagulant activity in recalcified sheep plasma. The heparin was broadly distributed among the last 10 fractions; the first three contained impurities which were completely separated from the heparin fractions. The heparin fractions differ systematically in anionic density but are of substantially the same molecular weight. Anticoagulant activity increased markedly with anionic density, ranging from 81 units/mg for the heparin fraction with the lowest anionic density up to a high of 243 units/mg. The relationship between anticoagulant activity and either anionic density or its square is nonlinear. However, in the latter case an initial linear relationship was observed for anticoagulant activities of less than 200 units/mg.

Heparin is a highly charged glycosaminoglycan with multiple biological effects, the most important of these being its actions on the blood coagulation system. However, the relationship between the chemical structure and the amount of such activities is still obscure.

Several investigators have attempted to assess the contributions of various charged substituents to the amount of anticoagulant activity. Chemical modifications of the functional groups have shown that the amount of activity of a given heparin is correlated with the presence of N-sulfation, although appreciable activity is found over a wide range of values for N-sulfation (Cifonelli, 1975; Nagasawa et al., 1977), and complete removal of these groups results in the complete loss of anticoagulant activity (Danishefsky, 1975). It has not been possible to investigate the role of O-sulfation in the same manner because of the difficulty of producing samples varying only in this parameter. However, studies of heparins from different animal sources have shown that heparins can vary widely in amount of O-sulfation and iduronate content, yet have similar anticoagulant activities (Cifonelli, 1975).

As it is commonly isolated, heparin is a complex mixture of different chemical species [e.g., Johnson & Mulloy (1976)], with the relative proportions of different chemical species probably varying from preparation to preparation. One probable consequence of this variability is the common observation that different measures of activity correlate poorly with each other when compared for a variety of samples. This is true whether one is correlating different biological activities, such as anticoagulant and cellular antiproliferative activities (Lippmann & Mathews, 1977), different measures of anticoagulant activity (Barrowcliffe et al., 1977; Teien et al., 1977), or even different measures of the effect of heparin on the antithrombin–thrombin system (Bounameaux et al., 1978).

We have recently shown that glycosaminoglycans can be fractionated according to anionic density by partitioning their hexadecylpyridinium complexes in two-phase solutions of 1-butanol–aqueous NaCl (Jennings & Hurst, 1974; Hurst & Sheng, 1976, 1977; Hurst, 1978). With such fractionation, the separation is completely insensitive to molecular weight for polymer chains in excess of a critical value of 10000–12000 daltons (Hurst & Sheng, 1977). Thus, partition fractionation affords a means of isolating a series of heparin fractions which differ in their charge density but are of substantially the same molecular weight. Such fractions present the possibility of removing molecular weight as a variable and investigating the correlation of various biological activities with differences in chemical structure.

Our objectives in this study were twofold. Firstly, a commercial sample of "crude" heparin from hog mucosa was fractionated by partitioning in order to provide information concerning the distribution of species with different anionic densities. A relatively unpurified sample was chosen since it would contain the widest possible distribution of different species. Several molar ratios of each fraction were determined in order to provide information concerning some of the structural differences among the fractions. Secondly, the anticoagulant activities of the various fractions were measured in order to demonstrate whether they vary with the anionic density of the heparin. Interestingly, although Jorpes & Bergstrom (1937) suggested that the charge of the heparin was important to its anticoagulant activity, the nature of the relationship has not been quantitated.

Materials and Methods

The hog mucosal heparin sample subjected to fractionation was provided by Cohelfred Laboratories, Chicago, IL. It was obtained prior to the bleaching and refractionation stages and still contained some nonheparin glycosaminoglycan. The anticoagulant activity was 143 USP units/mg. A more highly purified preparation (Cohelfred) of 13000 molecular weight

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and 168 USP units/mg and a chondroitin sulfate (Miles Laboratories) of 30 000 molecular weight (Hurst, 1978) were used as molecular weight markers. Hexadecylpyridinium chloride, 1-butanol, glucuronolactone, and glucosamine hydrochloride were obtained and purified as previously described (Hurst et al., 1978). Three heparin preparations, fractionated by affinity chromatography on antithrombin into high-affinity, low-affinity, and very low-affinity fractions (Höök et al., 1976), were provided by Dr. Ulf Lindahl. The three heparins are referred to as HA, LA, and NA heparins, respectively. Though the anticoagulant activities of these samples were not measured, previous preparations of these heparins have been in the range of 250–300 units/mg, 10–30 units/mg, and <10 units/mg, respectively (U. Lindahl, personal communication).

Uronate was determined against a glucuronolactone standard (Blumenkrantz & Asboe-Hansen, 1973), hexosamine against a glucosamine hydrochloride standard (Gatt & Berman, 1966) with modification of hydrolysis to 17 h with 4 N HCl, sulfaminohexose by deamination in 33% acetic acid and reaction with indole against a glucosamine hydrochloride standard (Lagunoff & Warren, 1962), and sulfate by the modified (Hurst & Sheng, 1977) barium rhodizonate method (Terho & Hartiala, 1971) against an NBS-certified sulfuric acid standard. Anticoagulant activities were determined on aqueous solutions of heparin fractions containing a known amount of uronate (UA) using the USP assay in recalcified sheep plasma. The results were converted from USP units/nmol of UA by multiplying by the uronate content of heparin dried over P_2O_5 in vacuo for 30 days, namely, 1.85 μmol/mg. Samples were analyzed by electrophoresis in 0.1 M HCl on Separax (Fuji Film Co.) as previously described (Nakamura et al., 1978).

Preparative scale fractionation was carried out by sequential extraction of eight tubes, each containing 10 mg of heparin in 20 mL of 1-butanol (upper phase) and 20 mL of 0.01 M NaCl (lower phase). The biphasic solution contained 3.0 g of hexadecylpyridinium chloride/100 mL of butanol phase. After removal of the original lower, aqueous phase, the upper butanol phases of each tube were successively extracted with 4 mL of aqueous butanol-saturated solutions of NaCl containing 2 g/L hexadecylpyridinium chloride, as listed in Table I. Equivalent fractions from different tubes were combined, and the heparin was precipitated with 3 volumes of ethanol. A more detailed description of the procedure was published previously (Hurst et al., 1978). Analytical scale fractionation of the three affinity-fractionated heparins was carried out similarly, except that only 1 μmol (as uronate) of each was fractionated.

Molecular weight distributions of the individual fractions were obtained by gel filtration on a column of 6% agarose (A-0.5m; Bio-Rad Laboratories) with bed dimensions of 0.8 × 60 cm. The column is adjustable to eliminate dead volume at the column head and is equipped with an injection valve (Pierce Chemical Co.) and a 0.15-mL sample loop. A 0.15-mL aliquot of ca. 1 to 2 mg of heparin/mL of sample was injected with this valve, and the column was eluted with 0.2 M NaCl at a rate of 12 mL/h using a syringe pump (Harvard Apparatus Model 44). Fractions of 1 mL were collected and lyophilized, and the uronate content was determined after reconstitution to 0.5 mL with deionized water.

Results

Properties of Heparin Fractions. Table I lists the distribution of uronate among the various fractions together with several molar ratios measured for the individual fractions. The fractions are identified by the NaCl concentrations at which they were extracted. The uronate is broadly distributed among the 13 fractions, which indicates a high degree of compositional variability in the original sample. The majority of the material, 67%, is concentrated in the six fractions extracted between 0.140 and 0.165 M NaCl. The fractions differ in anionic density, as shown by the steady increase in the sulfate/uronate ratio. The sulfaminohexose/uronate ratio, which measures the extent of N-sulfation, increases from fractions 3 through 7. In contrast, its value is approximately constant in the remaining fractions. With the exception of the 0.130 M fraction, the hexosamine/uronate ratio is approximately constant for all the fractions.

Table I: Distribution and Properties of Heparin Fractions[a]

no.	[NaCl] at which extracted (M)	% of total UA in fraction	molar ratios SO_4/UA	molar ratios SAH/UA	molar ratios Hex/UA	USP (units/mg)	Z^2
1	0.100	0.7	0.85				
2	0.110	1.9	0.85				
3	0.120	4.1	0.91	0.42			
4	0.130	5.7	1.21	0.57	1.03	81	4.88
5	0.140	11.3	1.36	0.84	0.83	126	5.55
6	0.145	10.4	1.39	0.87	0.77	156	5.69
7	0.150	10.7	1.41	0.93	0.80	174	5.81
8	0.155	10.0	1.42	0.98	0.79	188	5.93
9	0.160	13.2	1.54	0.99	0.73	210	6.45
10	0.165	11.5	1.57	0.95	0.79	210	6.60
11	0.170	8.2	1.62	1.03	0.75	221	6.84
12	0.175	5.6	1.82	1.03	0.74	243	7.95
13	0.200	6.8	1.93	1.01	0.76	197	8.58

[a] SO_4 = sulfate; SAH = sulfaminohexose; Hex = hexosamine; UA = uronate.

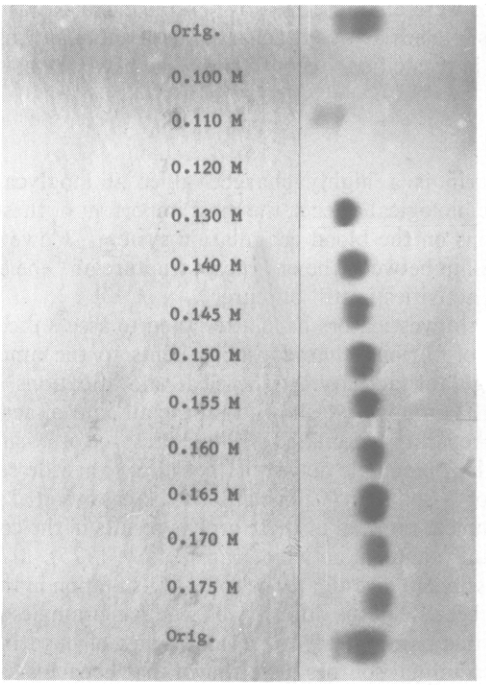

FIGURE 1: Electrophoresis of heparin fractions and the original sample in 0.1 M HCl on the Separax brand of cellulose acetate. The fractions are identified by the NaCl concentration at which they were extracted.

Confirmation that the fractions differ in anionic density is graphically provided by the electrophoretic patterns in 0.1 M HCl shown in Figure 1. Figure 1 also demonstrates that nonheparin contaminants are removed from the heparin fractions by partition fractionation. Three bands are evident in the original material. The faint band which migrates well behind the two main bands is extracted mainly into the 0.110

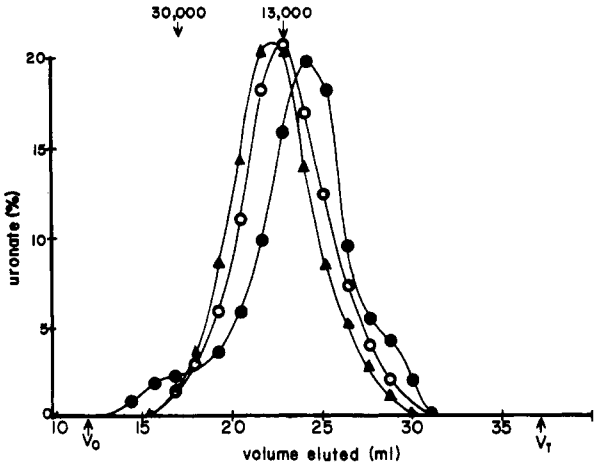

FIGURE 2: Representative gel filtration separations of heparin fractions obtained on 6% agarose. Results are expressed as the percentage of the total uronate eluted in each fraction as a function of the volume eluted. The void volume (V_0) and the total volume (V_t) are indicated on the abscissa. The elution volumes observed with a 30000 molecular weight chondroitin sulfate sample and a 13000 molecular weight heparin sample are also indicated by arrows at the top of the diagram. (●) 0.140 M fraction; (△) 0.165 M fraction; (○) 0.170 M fraction.

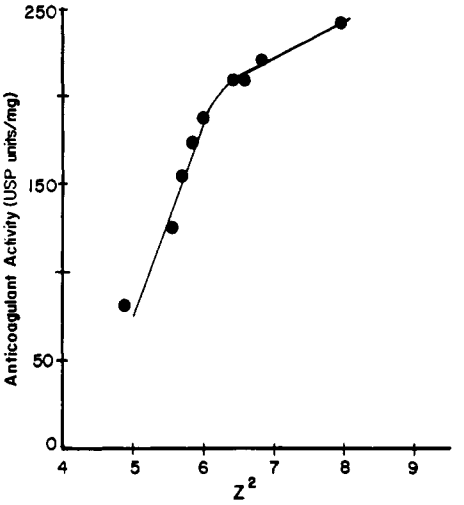

FIGURE 3: Dependence of the anticoagulant activity (USP assay) of heparin fractions upon Z^2, where Z is the number of chemically measured anionic groups per uronate moiety.

M fraction. The electrophoretic mobility and composition suggest that this material is not heparin, but probably represents a dermatan sulfate contaminant. The 0.120 M fraction is too dilute to yield a detectable band in this photograph; however, electrophoresis of a more concentrated sample has shown the material to migrate as a band with a mobility slightly less than that of the 0.130 M fraction. The 0.130 M fraction contains most of the material present in the slower of the two main bands evident in the original sample. The remaining fractions span the range of mobilities exhibited in the main band of the original material, and their steadily increasing mobility demonstrates the increase in anionic density also shown by the increasing values for the SO_4/UA ratio listed in Table I. The anionic density, Z, which is defined as the number of anionic groups per uronate moiety, is calculated as $Z = 1 + (SO_4/UA)$, where SO_4/UA represents the molar ratio of sulfate to uronate. The parameter Z represents the number of charged groups per repeating unit and is equivalent to the charge density if all groups are ionized. Values of Z^2 are also listed in Table I.

The data listed above demonstrate a difference in anionic density among the fractions. Gel filtration of the fractions (Figure 2) illustrates some molecular weight differences in the fractions extracted below 0.150 M but shows that above this fraction all fractions have virtually the same molecular weight distribution and average molecular weight. Such a result is not unexpected, since studies with fractionated chondroitin sulfate had previously shown that the partition properties of glycosaminoglycans were independent of molecular weight only for chains in excess of 10000–12000 daltons (Hurst & Sheng, 1977). Thus, any chains smaller than this critical size would be extracted at lower salt concentrations than would be predicted from the anionic density alone. Nevertheless, within the resolution afforded by this column, the average molecular weights and molecular weight distributions of the fractions extracted at 0.150 M and above were identical with each other and with the distribution obtained with a more highly purified commercial heparin sample with an average molecular weight of 13000 (not shown). The magnitude of the molecular weight differences of the lower fractions is illustrated by the distribution afforded by the 0.140 M fraction, which is similar to that found for the 0.130 M fraction. If linearity is assumed, then the average molecular weight of the 0.140 M fraction is 10600 ± 1000. The results also point out an interesting relationship between the anionic density and molecular weight. The fractions which have the lowest anionic density also have the lowest average molecular weight. Thus, molecular weight and anionic density of heparins are not completely independent, with smaller chains having a lower anionic density.

Correlation of Anionic Density with Anticoagulant Activity. The data in the "USP" column in Table I list the anticoagulant activities of each of the fractions. With the exception of fraction 13, the anticoagulant activities steadily increase with increasing sulfation. However, a simple linear relationshp is not evident. A second possibility is that anticoagulant activity is proportional to Z^2. Such a relationship would hold if the variations in anticoagulant activity were due only to differences in the strength of the electrostatic field, and hence the electrostatic free energy, surrounding the heparin polyion. Since the electrostatic free energy of a polyion is proportional to the square of the charge density (Tanford, 1961), if Z, the number of anionic groups on the polyion, can be equated to charge, parameters which are proportional to the electrostatic free energy will similarly be proportional to Z^2.

Figure 3 shows a plot of the anticoagulant activities of the individual heparin fractions as a function of Z^2. The anticoagulant activity is linearly related to Z^2 between $Z^2 = 5$ and $Z^2 = 6$. However, a simple linear relationship will not describe these data over the entire range of anionic densities, and the relationship is obviously more complex. The activity of the 0.2 M fraction is not shown on this figure, but its activity (Table I) is well below the value anticipated from the figure. The first five points describe the initial linear portion. A least-squares analysis showed an intercept of −414 ± 78 and a slope of 100 ± 14, with a correlation coefficient of 0.95. The change in slope near $Z^2 = 6$ is slightly more than fourfold. Thus, if anticoagulant activity does reflect factors related to the overall charge density of the heparin, then the nature of these factors abruptly changes when Z^2 assumes a value of ~6.

Properties of Affinity-Fractionated Heparins. One possible alternate explanation for Figure 3 is that fractionation by anionic density is accompanied by fractionation according to antithrombin affinity. Were such fractionation to occur, marked differences in anticoagulant activity among the fractions would result. In an effort to determine whether such is the case, we determined the anionic density distributions

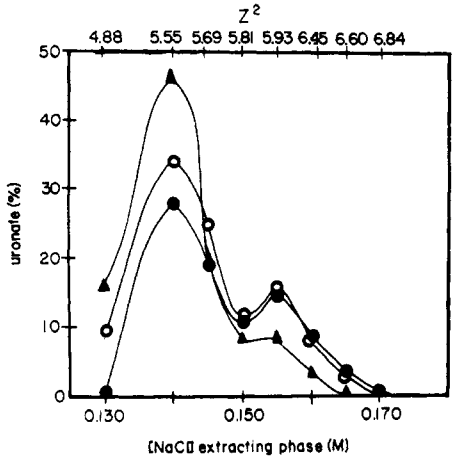

FIGURE 4: Partition fractionation of affinity-fractionated heparin samples. Results are expressed as the percentage of the total uronate extracted in a given fraction as a function of the NaCl concentration in the extracting phase. Such fractionation is a demonstration of the range of variation in anionic density found in each sample. Values of Z^2 are also listed on the top axis. (●) HA heparin; (▲) LA heparin; (○) NA heparin.

of heparins with high, low, and very low antithrombin-binding affinities by partition fractionation. The results of this experiment are shown in Figure 4 as plots of the distribution of uronate for each heparin as a function of the salt concentration in the extracting solution. The corresponding values of Z^2 are listed on the top axis. These distributions should not be quantitatively compared with the distribution listed in Table I because these samples were obtained from a different manufacturer and were subjected to extensive purification prior to affinity chromatography (Höök et al., 1976). The distributions in Figure 4 are distinctly sharper, indicating less heterogeneity, as might be expected from samples which had undergone such purification.

Although some differences in these distributions are evident, it is also evident that HA heparins exist within a similar range of sulfation as do the LA and HA heparins. HA heparin shows somewhat less low anionic density material than do LA or NA heparin. However, there is no marked correlation, thus suggesting that fractionation according to antithrombin affinity did not occur to an extensive degree as a result of fractionation according to anionic density.

Discussion

Although anticoagulant activity of the commercial heparin is related to its anionic density, a simple straight-line relationship to Z^2 was not observed. Instead, a more complex relationship was seen. The existence of such an empirical relationship does not establish a causal relationship. However, our data do show that this relationship does not arise primarily from differences in molecular weight or ability to bind to antithrombin. Our data do not permit total separation of molecular weight and anionic density effects. The fractions separated at or above 0.15 M NaCl do not differ in molecular weight, but below this fraction (fractions 4–6) a difference of 2400 in molecular weight is evident. This difference in molecular weight could lead to some differences in anticoagulant activity (Laurent, 1961; Johnson & Mulloy, 1976; Laurent et al., 1978; Lane et al., 1978) for these fractions; however, molecular weight is clearly not a variable for fractions 7–13. The similarities in anionic density distributions of HA, LA, and NA heparins demonstrate that fractionations by anionic density and by the ability to bind antithrombin are essentially independent of each other. As a consequence, the differences in anticoagulant activity shown in Figure 3 can not be mainly due to a concomitant fractionation according to the ability to bind antithrombin.

We conclude that the empirical relationship shown in Figure 3 is not an artifact due to other factors and that the anticoagulant activity is a function of the anionic density by some as yet unknown mechanism. We attempted to determine whether electrostatics is the main determinant of the relationship by plotting the anticoagulant activity against Z^2. Although an initial linear portion was observed, the plot was distinctly nonlinear. However, it is by no means clear that linearity with respect to Z^2 should be expected. Steric effects, "shielding", or other interactions between segments of the heparin chain would all lead to a nonlinear plot against Z^2. Therefore, we conclude that the theoretical interpretation awaits further data. However, as an empirical correlation, it offers a means of comparing the properties of different heparins in terms of (1) their anionic density distributions and (2) the anticoagulant activities of the individual fractions. We are currently investigating different hog mucosal and beef lung heparins, and preliminary results show that different heparins have quite different anionic density distributions.

Interestingly, HA heparin, which has a high anticoagulant activity, has very nearly the same anionic density distribution as do LA and NA heparins, neither of which exhibits appreciable anticoagulant activity. For LA and NA heparins, then, a knowledge of anionic density gives little information concerning anticoagulant activity. Clearly, while anionic density may be a necessary condition for anticoagulation, it is not a sufficient condition.

Similar conclusions concerning the role of antithrombin binding have also been reached. It is clear that binding of heparins to antithrombin is a prerequisite for high anticoagulant activity (Höök et al.; Lam et al., 1976). The binding of heparin to antithrombin has been shown to arise from oligosaccharide sequences, probably representing a dodecasaccharide, within the heparin chain (Hopwood et al., 1976; Laurent et al., 1978; Rosenberg et al., 1978). However, when such oligosaccharide sequences were isolated, they were found to have little anticoagulant activity, though they bound tightly to antithrombin (Laurent et al., 1978). Thus, information which specifies the amount of anticoagulant activity must reside in the structure of the heparin chain outside the "binding oligosaccharide" region. We speculate that the anionic density of the remainder of the chain is related to the amount of induced activity. Thus, at least two, approximately independent structural variables appear to govern the activity of heparin. The ability to bind to antithrombin appears to act as a "master on–off switch", whereas the anionic density appears to act as a "rheostat".

It is by no means clear that these are the sole structural variables which can confer anticoagulant activity. One other variable which has been extensively studied is molecular weight. However, Laurent et al. (1978) have related the dependence of anticoagulant activity upon molecular weight to a variation in the fraction of high-affinity heparin which occurs with a chain length over the molecular weight range of 5600–36 000. Moreover, our own data (Figure 2) demonstrate that low anionic density (and hence low activity) and low molecular weight are correlated. Thus, molecular weight may well reflect a combination of these other two variables. Additionally, the effect of heparin in modifying the activity of antithrombin toward thrombin and factor VIII, or any other factor, cannot be the sole mechanism by which anticoagulation occurs. This is clearly illustrated by dermatan sulfate, which

can exhibit an anticoagulant effect of one-third that of heparin on a weight basis at concentrations which do not produce a detectable effect upon the activity of antithrombin (Teien et al., 1976). Yet, the anticoagulant activity of dermatan sulfate, when plotted against physical parameters related to binding of acridine orange, fell on the same line defined by the heparin fractions used in this study (Menter et al., 1979). Moreover, LA heparin is not devoid of anticoagulant activity. Thus, it is clear that further work will be needed to clarify the complex role of anticoagulant activity in relation to anionic density and the relation to specific coagulation factors and inhibitors.

Interestingly, the last (0.2 M NaCl) fraction has an anticoagulant activity well below that predicted from its anionic density alone. This fraction contains the most highly sulfated of the heparins. However, at least one unsulfated uronate residue, either glucuronic acid (Rosenberg et al., 1978) or iduronic acid (U. Lindahl, personal communication), in the "antithrombin-binding oligosaccharide" appears to be required for binding to antithrombin. Thus, sulfation of all the uronate residues would prevent binding to antithrombin, which would virtually eliminate the anticoagulant activity of such highly sulfated heparins. While our data by no means prove this to be the case, such a phenomenon does provide a possible explanation for the lower anticoagulant activity of the last fraction.

Partition fractionation may provide an important new tool for isolating and characterizing heparins as well as for investigating the molecular basis for heparin action. Its advantage is that it separates according to chemical differences virtually unaffected by differences in molecular weight (Hurst & Sheng, 1977; Hurst, 1978). Thus, it serves as the basis for describing and reducing chemical heterogeneity of heparin samples from different sources by making it possible to control anionic density as a variable. Moreover, with such samples it is possible to more clearly relate various measures of the biological activities of heparin to defined chemical properties and to each other. Also, partition fractionation readily and completely removed nonheparin contaminants. Thus, partition fractionation offers a new means for isolating and characterizing heparins and relating the properties of different heparins.

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