# Heparinic Acids: Determination of Equivalent Weights and Sulfate to Carboxyl Ratios

Abstract. *By means of an automatic titration apparatus, a polyelectrolyte of biological origin, heparin, from two animal sources, was found to have an average equivalent weight of 170.0, a sulfate content of 34.44 percent, and a sulfate to carboxyl ratio of 2.59.*

The chemical structure of heparin is not yet completely known (*1*). Hydrolysis of heparin yields mainly, but not entirely (*2*), D-glucuronic acid (*3*), D-glucosamine, and sulfuric acid. While titration studies by Wilander (*4*) and chemical analyses by Wolfrom *et al.* (*5*) suggested that no free amino groups are present in the molecule, later studies by Gibbons and Wolfrom (*6*) indicated that 94 percent of the nitrogen in their heparin preparation was incorporated in sulfamino groups of the hexosamine portion, and the remainder consisted of unsubstituted amino nitrogen.

Although it is clear that the sugar components of heparin consist mainly of alternating glucuronic acid and glucosamine units, the amount of sulfate ester per heparin unit is still questionable. The work of Wolfrom *et al.* (*5*) indicated a tetrasaccharide unit containing five sulfate groups (either *O*-sulfate or *N*-sulfate) and two carboxyl groups. The molecular weight of this tetrasaccharide unit would be 1075, the sulfate-carboxyl ratio 2.50, and the equivalent weight 145.9. The polysaccharide structure of Gibbons and Wolfrom (*6*), containing 6 percent unsubstituted amino groups, would give an equivalent weight of 150.8 and a sulfate-carboxyl ratio of 2.88. Hoffman and Meyer (*7*) suggested a disaccharide unit containing three sulfate groups and one carboxyl group; this would give an equivalent weight of 143.8 and a ratio of 3.00. Helbert and Marini (*8*) have recently suggested a unit consisting of 20 disaccharides $(C_{12}H_{19}O_{16}NS_2)_{20}$, with 40 ionizable sulfate and 20 ionizable carboxyl groups, leading to an equivalent weight of 165.8 and a ratio of 2.00.

One reason for these contradictory results may be the difficulty in obtaining uniform heparin preparations. The effect of purification procedures on the chemical structure of the molecule is still unknown. Although heparin extracts from lung and intestinal mucosa generally contain less protein than other mucopolysaccharides such as the chondroitin sulfates, amino acid residues have been shown to be present even in highly purified heparin (*2*). It has been suggested (*9*) that different tissues may produce different kinds of heparin. Barlow *et al.* (*10*) tried to compare the macromolecular properties and the biological activity of different heparin preparations by measuring the molecular weight (by light-scattering and sedimentation techniques). Different molecular weight values were found for different samples, but they were unable to correlate the differences with changes in bioactivity. The best way to classify heparin is still on a physiological basis, expressing purity in terms of anticoagulant activity. One source of confusion in all this work may be that many of the studies of heparin appearing in the literature have been done on materials of diverse or unknown origin.

We have recently constructed a sensitive automatic titration apparatus based largely upon a design originally conceived and applied by H. Majer of CIBA Research Laboratories, Basle, Switzerland, which appears to be particularly useful in rapidly resolving and measuring diverse titratable groups in macromolecular polyelectrolytes, such as mucopolysaccharides, and possibly mucoproteins. The apparatus consists of two 10-milliliter piston burettes containing 0.1*N* NaOH and 0.1*N* HCl, respectively, driven by synchronous motors. Small liquid samples magnetically stirred at constant temperature (25°C) under nitrogen are titrated by use of a combined glass-calomel electrode (type M 455-NS/2 from Polymetron Ltd., Zurich, Switzerland) connected to a direct-reading *p*H meter (Leeds and Northrup No. 7664 in the present apparatus) and a recorder driven by its own synchronous motor for instantaneous measurement of *p*H changes in the titration vessel.

The accuracy and sensitivity of the apparatus was first determined with mixtures of standard inorganic acids. For the measurement of titratable groups of a typical polyelectrolyte of biological origin, heparin was selected because of its previous chemical characterizations and ready availability. There is little information in the literature regarding titration of such polyelectrolytes and in all cases the titrations were point to point (*4, 8, 11*), thus offering less precision at inflection points.

In view of the unestablished relationship between the bioactivity and the chemical or physical properties of heparin, it seemed worthwhile to determine the sulfate-carboxyl ratio and equivalent weight of heparin preparations of different bioactivity.

Six different heparin samples, prepared from pig intestinal mucosa and containing 106, 140, 155, 159, 172, and 182 anticoagulant units per milligram, respectively, plus a seventh sample prepared from beef lung with 123 units per milligram, were used (*12*). All samples contained the normal amount of uronic acid (orcinol and carbazole methods) and less than 0.5 percent protein (Lowry method).

To ensure complete conversion of sodium heparinate to heparinic acid, each sample was passed through a column of approximately 50-fold excess Dowex-50 W ($H^+$ form, ×4, 200 to 400 mesh) immediately before titration. The possibility of holdup of hep-

Table 1. Analysis of porcine* (intestinal mucosa) heparins of varying bioactivity. Most values represent the mean of two to five determinations followed by the standard error of the mean.

| Bioassay (unit/mg) | Eq. wt. | Sulfate (%) | Ratios | |
|---|---|---|---|---|
| | | | Sulfate × 100 / sulfate + carboxyl | Sulfate / carboxyl |
| 106 | 177.8 ± 3.4 | 32.80 | 71.95 ± 0.29 | 2.57 ± 0.04 |
| 123 | 159.7 ± 2.8 | 37.21 | 73.29 ± 0.07 | 2.74 ± 0.01 |
| 140 | 171.8 | 34.59 | 73.30 | 2.74 |
| 155 | 164.6 ± 3.3 | 35.38 | 71.84 ± 0.27 | 2.55 ± 0.03 |
| 159 | 175.0 ± 2.0 | 33.48 | 72.28 ± 0.29 | 2.61 ± 0.04 |
| 172 | 169.2 ± 0.1 | 33.82 | 70.58 ± 0.34 | 2.40 ± 0.04 |
| 182 | 171.6 | 33.78 | 71.49 ± 0.60 | 2.51 ± 0.08 |
| Av. | 170.0 | 34.44 | 72.10 | 2.59 |

* Except one sample (123 units per milligram) obtained from beef lung.

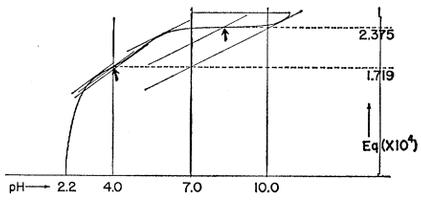


Fig. 1. Original titration chart obtained with 41.6 mg of heparinic acid prepared from sodium heparinate (159 units per milligram) and titrated with 0.1048*N* NaOH. Superimposed are *p*H values on the horizontal axis and equivalents of base represented by vertical distances above the starting line. Tangential lines constructed to determine sulfate and carboxyl end points (indicated by arrows) are also shown.

arin on the column was eliminated by subsequent passage of a tenfold excess, over heparin, of 4*N* HCl. The neutralized effluent gave negative orcinol and anthrone reactions.

Helbert and Marini (*8*) recently found a 2.7 percent increase in acid titratable groups of heparinic acid prepared from dialyzed sodium heparinate upon standing at 60°C for 1½ hours. To determine the extent of autohydrolysis resulting from the ion-exchange procedure we dialyzed two different heparin preparations for extended periods through cellophane (*13*) membranes. This initial dialysis removed about one-fifth of the original heparin. The 80 percent remaining in the retentate was passed through Dowex-50 W resin, and again dialyzed at 25°C. Titration of the second dialysate after one hour (when dilute sulfuric acid would have come to equilibrium) indicated loss of only about 2 percent of the total acid groups present, presumably in the form of macromolecular subunits or free sulfuric acid resulting either from autohydrolysis or from exchanged inorganic sulfate. Since the diffusion of 20 percent of the heparin during the initial dialysis indicated the probable presence of a mixture of heparins of different molecular weights, much as shown earlier by Barlow *et al.* (*10*), we decided to conduct our titrations on the original undialyzed material to avoid possible alterations in biological activity resulting from such a separation.

Each heparin, made up to about 0.5 percent in water, was passed through a cation exchange column as previously described. Equal portions containing about 60 mg of heparinic acid were immediately diluted to 20 ml with water and titrated with 0.1*N* NaOH. After passing the second equivalent point (*p*H range 11 to 13) the NaOH burette was turned off and the excess base immediately back-titrated with 0.1*N* HCl to *p*H 7. A typical titration curve is shown in Fig. 1.

Heparin is very hygroscopic, and after 3 to 10 minutes exposure to air after lyophilization, or vacuum storage over $P_2O_5$, or both, may increase up to 10 percent in weight. To avoid this problem the heparin solution was quantitatively transferred, immediately after titration, into a tared, lightweight lyophilization flask equipped with a high-vacuum teflon stopper. The solution was then lyophilized for 48 hours at a pressure of approximately 0.3 mm Hg, and the dried heparin weighed by difference.

The dry weight of each titrated heparin sample, obtained after titration and lyophilization, was used for the calculation of equivalent weight, corrections being made for the weight of additional NaCl formed during back-titration and for conversion of the heparinic acid to the sodium form. This weight was then divided by the total equivalents of base used for neutralization to give the equivalent weight (acid equivalent) of the heparinic acid. As shown in Table 1, the equivalent weights of the different heparin preparations averaged 170.0, including the material giving the lowest value, which was derived from beef lung. There was no significant correlation between bioactivity and equivalent weight. Figure 1 shows that the continuous titration procedure provides a sharp inflection point at which sulfate groups are titrated to R-$SO_3Na$, so that by midtangential construction, values for the total titratable $-SO_3H$ groups may be calculated. Individual values, given in Table 1, averaged 34.44 percent for sulfate ($-SO_3H$) and 2.59 for sulfate to carboxyl ratio. Again there was no notable relationship between the sulfate content, sulfate to carboxyl ratio, and the bioactivity. These ratios may be uncertain because of possible interference in the sulfate titrations by carboxyl groups. Improvement in the accuracy of the sulfate end point may be possible by such techniques as use of a first derivative circuit in the recorder or alteration of the dielectric constant of the solvent (*14*).

The large fraction of dialyzable heparin in our samples would also suggest that in the future, attempts should be made to correlate biological activity with parameters, such as equivalent weight or sulfate to carboxyl ratio, in more physically homogeneous material. However, the technique described herein provides perhaps the most accurate values for commercial heparin so far reported.

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## DNA Contents of Chromosome Ph[1] and Chromosome 21 in Human Chronic Granulocytic Leukemia

Abstract. *The Philadelphia[1] chromosome, characteristic of chronic granulocytic leukemia, contained 61±1 percent as much DNA as a number 21 (or 22) chromosome from which it is believed to be derived. The remaining 39 percent represents 0.5 percent of a diploid chromosome complement, approximately 2 × 10⁷ nucleotide pairs; the method used was not sensitive enough to detect whether it had been translocated to other chromosomes.*

Chronic granulocytic leukemia (CGL), the first of the neoplastic diseases shown to be associated with a constant abnormal karyotype, is unusual among human disorders associ-