Viscosity of heparin in formamide and glycerol

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The viscosity of heparin was examined in pure glycerol, formamide and in mixtures of the two solvents. This study confirms that the equation $\eta_{sp}/c = [\eta]_\infty [1 + k/(c)^{1/2}]$, where $[\eta]_\infty$ is the shielded intrinsic viscosity, adequately describes the concentration dependence of the reduced viscosity of the polyelectrolyte, heparin. The $[\eta]_\infty$ linearly increases with the dielectric constant of the solvent.

INTRODUCTION

Heparin is a mucopolysaccharide composed of partly sulphated units of α-D-glucuronic acid and 2-amino-2-deoxy-D-glucose joined by 1,4 bonds. It is found in high concentration in the mast cells of connective tissue and is important pharmacologically as a blood anticoagulant. Because of its high negative charge, heparin exhibits typically polyelectrolyte behaviour in aqueous solutions[1–3]. As a result, viscosity and other physicochemical measurements are sensitive to changes in ionic strength[1,3,4] and pH[5]. Heparin has been reported to be polydispersed[1,3,6] having a molecular weight range of about 6000 to 35 000 daltons. Stivala and coworkers[1,3] and Laurent[6] have demonstrated that the biological activity of heparin is a function of the molecular weight.

Heparin was reported to behave as a Gaussian coil molecule from small-angle X-ray scattering[7]. It has also been suggested that heparin exhibits flexibility in solution[1], conforming to near random-coil when charges are swamped and rod-like in the absence of added salt, i.e. pure water. It exhibits typical helical polypeptide[8] behaviour when examined by dye-stacking methods from optical rotatory dispersion.

Yuan, Dougherty and Stivala[9] showed that the concentration dependence of the reduced viscosity, η_{sp}/c, of strong polyelectrolytes in salt-free polar solvents can be adequately described by the equation:

$$\eta_{sp}/c = [\eta]_\infty \left[1 + \frac{k}{(c)^{1/2}}\right] \quad (1)$$

as long as the concentration is not too low. The shielded intrinsic viscosity, $[\eta]_\infty$, represents the intrinsic viscosity, $[\eta]$, of the well shielded polyion and it corresponds to the $[\eta]$ that would be obtained in salt solution of sufficient ionic strength to swamp out the charges. The parameter k is an interaction parameter apparently dependent only on the polymer backbone and not on any electrical properties of either the solute or the solvent. It was shown[9] that:

$$k \simeq 5.22 \left(\frac{P}{a^2}\right)^{1/2} \quad (2)$$

where P is the mass per unit length in angstroms and a is the equivalent freely jointed segment length when the backbone of the polyion is represented by a Gaussian coil of N segments.

In an earlier paper[10] Yuan and Stivala examined the dependence of k and $[\eta]_\infty$ on the dielectric constant, D, of various solvents within the range $54.7 \leqslant D \leqslant 93.2$. They found that the $[\eta]_\infty$ of heparin in salt-free polar solvents is a linear function of D in the range examined. The purpose of this paper is to examine the viscosity behaviour of heparin in pure glycerol and formamide and mixtures of the two, having dielectric constants in the range of 42.5 and 107.4, and to correlate the $[\eta]_\infty$ with molecular weights of various heparin fractions.

EXPERIMENTAL

Pure commercial heparin sodium derived from beef lung, obtained from Organon, Inc., West Orange, NJ, Lot No: 22419 having an anticoagulant activity of 164 IU/mg, was used throughout this work. Prior to use, the heparin sodium was dried in a vacuum oven at 55°C for 2 days and stored in a vacuum desiccator. Approximately 10% loss in weight was observed upon drying.

Heparin sodium was fractionated into four fractions using water as the solvent and ethanol as the non-solvent at 4°C according to a method described earlier[3]. The total recovery was better than 96%.

Weight-average molecular weights, $\bar{M}_w$, of the unfractionated and of the fractions of heparin were determined by measuring the intrinsic viscosities in 0.10 M NaCl solutions and using the Mark–Houwink relationships established by Stivala and coworkers[1,3]. Values of $[\eta]$ and $\bar{M}_w$ of the various heparin fractions are contained in *Table 1*. EH–O is the original unfractionated sample.

The formamide used in this work was Fisher reagent grade material, dried according to a modification of the method of Notley and Spiro[11]. Thus, the formamide was passed through a heated column constructed with 3/16 in. pellets of Linde type 3A molecular sieves. The temperature of the column was maintained at 60°C. The eluted formamide has a specific conductance of 1.1064×10^{-3} ohm^{-1} cm^{-1}.

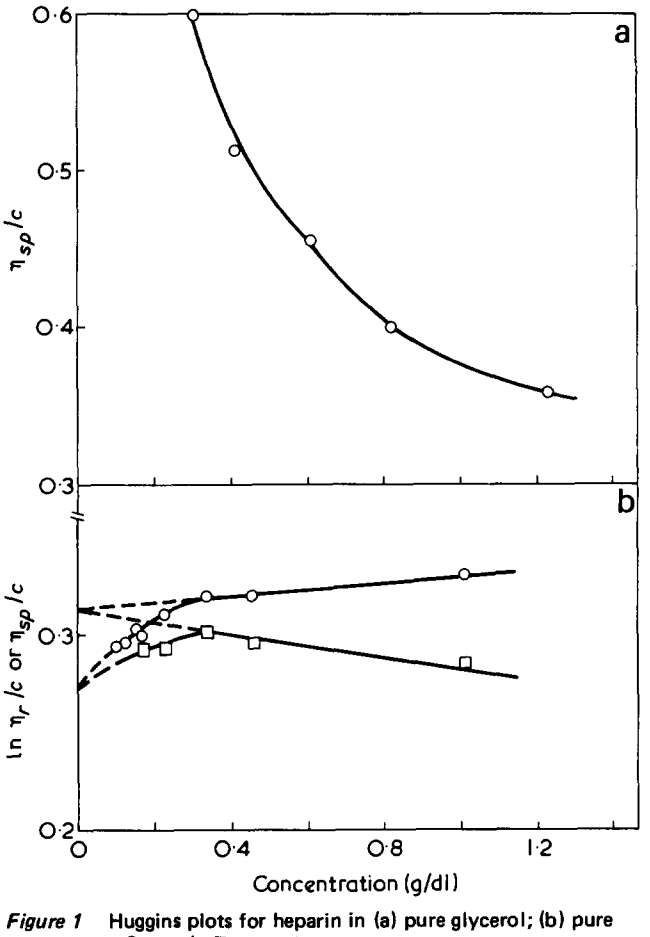

Figure 1 Huggins plots for heparin in (a) pure glycerol; (b) pure formamide. ○, η_{sp}/c; □, $\log\eta_r/c$

Glycerol (Fisher reagent grade, 99.3% pure) was further purified according to the method of Segur and Oberstar[12]. Thus the glycerol was distilled under reduced pressure at 210°C. About 300 ml of the distillate were obtained as the middle 70% fraction for use in this work.

Viscosity measurements were made in Cannon–Ubbelhode semimicro dilution viscometers number 75 to 400. The solvent flow times were usually greater than 200 sec. Measurements were made at 25° ± 0.01°C. Concentrations were in g/dl converted to mol/l to give the shielded intrinsic viscosities in l/mol.

All measurements were made under controlled conditions so as to exclude any moisture, from the immediate environment entering the solutions, by closing all openings to atmosphere with towers of the 'Drierite'.

Solutions of heparin in formamide and glycerol were made by heating the samples to 60°C, with stirring for 1 h followed by cooling to room temperature.

Analysis for total sulphur and nitrogen content was made by Schwarzkopf Microanalytical Laboratory, NY. Elemental analysis on the heparin before and after dissolution in formamide with subsequent recovery showed for pure heparin 11.21% sulphur and 1.81% nitrogen while for the heparin dissolved in formamide with subsequent recovery by precipitation with ethanol showed 8.32% sulphur and 8.13% nitrogen.

Dielectric constants for pure glycerol and formamide were obtained from the literature[13]. The values for the two mixtures of glycerol and formamide were obtained from n.m.r. phase measurements according to the method of Malinowski and Garg[14]. In this method the difference between the phase readings for air and the sample required to obtain a pure absorption signal from a reference sample, hexamethyldisiloxane (HMD), influenced by the sample is measured and the dielectric constant obtained from a calibration plot. The determination was carried out on a Varian EM–390 n.m.r. spectrophotometer at a probe temperature of 25°C as measured from methanol internal shift.

Anticoagulant activity for heparin after dissolution in formamide and subsequent recovery was measured by the Activated Partial Thromboplastin Time *(APTT)* Test. All the materials used were obtained from Hyland Laboratories, Costa Mesa, California, USA. Measurements of clotting time were made on a Becton–Dickinson fibrometer system. The anticoagulant activity obtained was 150 IU/mg, as opposed to 164 IU/mg for the starting material.

RESULTS

Figure 1a shows the plot of reduced viscosity η_{sp}/c, vs. concentration, c, of the heparin in glycerol. It exhibits the usual rapid asymptotic rise of η_{sp}/c with dilution characteristic of polyelectrolytes in salt-free polar solvents. The η_{sp}/c vs. c for heparin in formamide is shown in *Figure 1b*. Notwithstanding the high dielectric constant of formamide, heparin does not exhibit polyelectrolyte behaviour in this solvent. It may be noted from *Figure 1b* that the plot consists of two linear portions. Since the data of heparin in formamide are linear, the intrinsic viscosity $[\eta]$, may be obtained from the specific viscosity, η_{sp}, using the Huggins equation[15]:

$$\frac{\eta_{sp}}{c} = [\eta] + K'[\eta]^2 c \qquad (3)$$

by extrapolation to infinite dilution. Two values of $[\eta]$ may be obtained from the plot in *Figure 1b*, i.e. $[\eta]_a$ and $[\eta]_b$ corresponding to regions of higher and lower concentration, respectively. *Table 1* lists the values of $[\eta]_a$ and $[\eta]_b$ for the various heparin fractions as a function of $\bar{M}_w$.

The reduced viscosities for heparin in 50/50(w/w) glycerol/formamide and 25/75(w/w) glycerol/formamide also show the Huggins dependence on concentration (see *Figure 2*). No breaks in the curve are observed, as in formamide, (see *Figure 1b*).

Figure 3 shows the Malinowski–Garg calibration plot for determining the dielectric constant. Pure formamide, mixtures of ethanol and water, and urea solutions were used as standards. The dielectric constants of the standards were obtained at 25°C, from the paper by Yuan and Stivala[10]. The dielectric constants for 50/50 and 25/75

Table 1 Intrinsic viscosity and molecular weight data for heparin

Sample	$\bar{M}_w$*	$[\eta]_{0.10}$† (dl/g)	$[\eta]_a$ (dl/g)	$[\eta]_b$ (dl/g)
EH – 0	12325	0.184	0.312	0.277
EH – 35	14760	0.220	0.343	0.309
EH – 40	13325	0.199	0.310	0.275
EH – 45	10280	0.154	0.300	0.220‡
EH – 50	8085	0.121	0.268	0.185‡

*Calculated as an average from: $[\eta]_{0.10} = 1.54 \times 10^{-5} (\bar{M}_w)^{1.00}$ (ref 3) and $[\eta]_{0.10} = 1.75 \times 10^{-5} (\bar{M}_w)^{0.98}$ (ref 1).
† Measured in 0.10 M NaCl solutions
‡ Calculated from the Yuan, Dougherty, Stivala equation (ref 10)

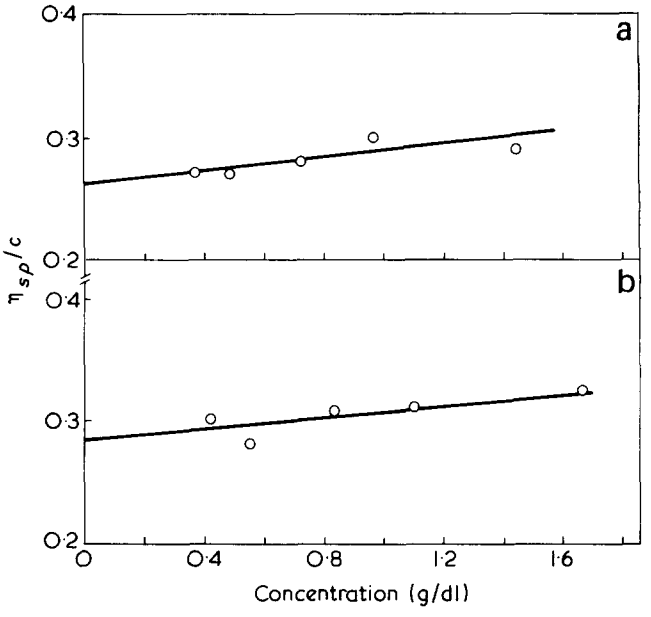

Figure 2 Huggins plots for heparin in glycerol–formamide mixtures. (a) 50% glycerol–50% formamide; (b) 25% glycerol–75% formamide

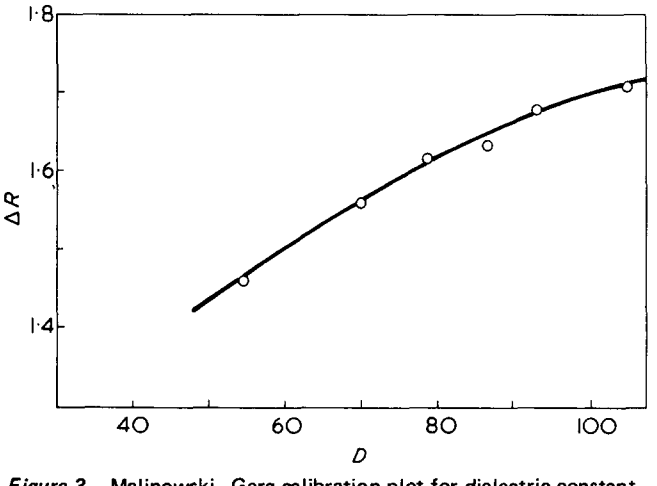

Figure 3 Malinowski–Garg calibration plot for dielectric constant determination at 25°C

(w/w) glycerol/formamide mixtures obtained from *Figure 3* are shown in *Table 2*.

Table 2 lists the values of $[\eta]_\infty$ and k obtained from treating the data according to equation (1), along with the values of dielectric constants of the various solvents.

Figure 4 shows the Huggins plots of four heparin fractions in formamide. The two higher molecular weight samples EH35 and EH40 show the break in the plots similar to the one observed in *Figure 1b*, with the break for both occurring around the same concentration. The two lower molecular weight samples EH–45 and EH–50, however, after an initial Huggins-like dependence show marked polyelectrolyte behaviour. The $[\eta]_b$ for these samples were then obtained from Yuan–Dougherty–Stivala plots[9,10] for the lower concentration range.

DISCUSSION

Heparin behaves as a typical polyelectrolyte in pure glycerol. The value of the interaction parameter k obtained from equation (1) is 5.69×10^{-2}. This value is about 2 times larger than the average value of 2.66×10^{-2} reported by Yuan and Stivala[10] for heparin in various solvents. k is independent of the electrical properties of the solvent or the solute. The higher value of k in glycerol could result from one of two factors (equation 2): (a) increase in the mass per unit length, P, of the molecule or (b) decrease in the persistence length, a, of the heparin in glycerol. Stivala and coworkers[9] have shown that the persistence length as measured from small-angle X-ray scattering is a good approximation for the equivalent freely jointed segmental length for heparin. The mass per unit length of a molecule is, in general, a fixed quantity ignoring any small solvation effects in different solvents. k, then, becomes larger apparently due to a shortening of the persistence length. This in turn implies that heparin is less extended in its solution in glycerol. The electrostatic forces in solvents of low dielectric constants will be larger than those in solvents of high dielectric constants will be larger than those in solvents of high dielectric constants. As a result, the attractive forces between the counterion and the heparin backbone will be enhanced leading to a weaker ionic atmosphere around the

Table 2 $[\eta]_\infty$, D and k for heparin in various solvents

Solvent	D*	$[\eta]_\infty$ (l/mol)	$k \times 10^2$
Glycerol	42.5	164	5.69
Ethanol/water 40/60 (w/w)	54.7	191†	2.65
Ethanol/water 15.5/84.5 (w/w)	69.8	244†	2.64
Water	78.5	290†	2.57
Urea solution, 3.40M	87.7	326†	2.73
Urea solution, 6.00M	93.2	346†	2.60
Glycerol/formamide 50/50 (w/w)	89.0‡	338	–
Glycerol/formamide 25/75 (w/w)	98.5‡	370	–
Formamide	107.4	410§	–

* At 25°C; † taken from ref 10; ‡ obtained from the method of Malinowski and Garg[14]; §converted from $[\eta]_a$(dl/g) (*Table 1*)

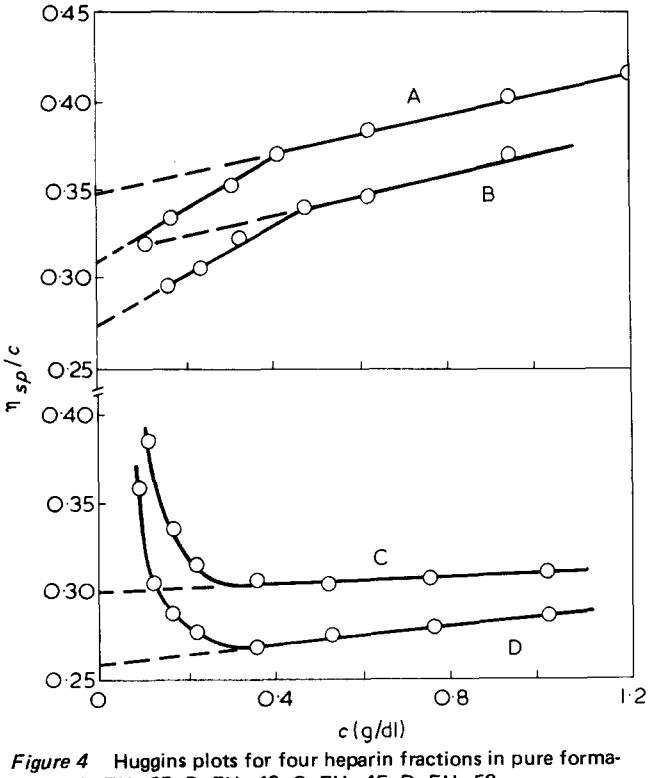

Figure 4 Huggins plots for four heparin fractions in pure formamide. A, EH–35; B, EH–40; C, EH–45; D, EH–50

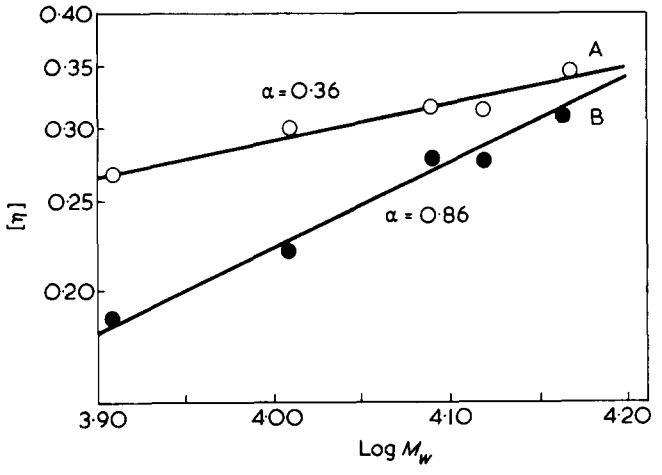

Figure 5 Mark–Houwink plots for heparin in (A) higher concentration range; (B) lower concentration range in pure formamide

polyion. In such a situation it is conceivable that the polyion may lose its extended conformation and become more compact causing a decrease in the persistence length.

Low dielectric constant of the solvent should also have the effect of intensifying the repulsive forces between the charges along the polyion backbone. This will, of course, have the opposite effect on the persistence length. This however, is not in keeping with these and other[10] observations made in this laboratory. Further, as a consequence of Lifson's studies[16], strong repulsive forces along the polyion backbone may actually inhibit the tendency for the polyion to fully extend itself due to twisting of the molecular chain chain[10].

Heparin does not behave as a polyelectrolyte in its solution in formamide. As seen from *Figure 1b*, the reduced viscosity in formamide decreases with decreasing concentrations as it would for a non-charged macromolecule. Moreover, the concentration dependence of the reduced viscosity is not a single linear function of concentration but exhibits a second concentration dependence at lower concentrations of heparin in formamide. Therefore, two intrinsic viscosities may be calculated from the Huggins plot (see *Table 1*). Since the formamide had been rigorously dried, it was considered improbable that the solvent contained sufficient counterions (cations) to effectively swamp the charges in heparin. Nevertheless, from the measured specific conductance of 1.1064×10^{-3} ohm^{-1} cm^{-1} for the formamide used in this work, the approximate ionic concentration was calculated to be equivalent in effect to 0.02 M KCl, i.e. the specific conductance obtained from the formamide is that which would have been obtained for 0.02 M KCl. This was achieved from the data for the variation of the equivalent conductivity, Λ ohm^{-1} cm^2 equivalent^{-1}, of KCl solution in water with concentration and the fact that $\Lambda C = \kappa$, where C is the concentration of the salt in equivalents per ml and κ is the specific conductance in ohm^{-1} cm^{-1}. Liberti and Stivala[3], have previously indicated that this concentration of salt in water is sufficient to swamp out charges in heparin, the minimum salt concentration required being 0.01 M. The very real possibility exists, therefore, that the non-polyelectrolyte behaviour of heparin in formamide may result from the presence 0.02 M ionic species.

The possibility of formamide reacting with heparin was also examined. This was also prompted by the observation that heparin does not readily dissolve in formamide but does so only on heating the sample to 60°C with stirring. A portion of the heparin was dissolved in formamide and the solution allowed to cool to the room temperature. The dissolved heparin was precipitated from its formamide solution with absolute ethanol and the heparin recovered after dissolving the precipitate in water and freeze-drying the solution. Approximately 50% increase in weight occurred in the heparin as result of this treatment and prolonged heating in a vacuum oven at 55°C did not lower this increase. It was concluded that this weight increase was not due to trapped formamide. Analysis for total sulphur and nitrogen contents showed that the ratio of the number of moles of nitrogen to sulphur changed from 0.73 to 4.46 for heparin before and after dissolution with subsequent recovery from formamide, respectively. Moreover, the anticoagulant activity for the recovered heparin was lower than the original heparin, a fact that may be the direct result of the lowered sulphur content of the recovered material. In aqueous solutions, the recovered material exhibited much lower polyelectrolyte behaviour than the original, i.e., the concentration dependence of the reduced viscosity was much flatter than that for the original material pointing to at least a partial swamping of the charges as a result of the dissolution in formamide. It is possible that this observation in conjunction with the earlier one may explain the anomalous and altogether unexpected behaviour of heparin in formamide.

The change in the concentration of the reduced viscosity of heparin in formamide was studied in greater detail using fractions of the native heparin. All the fractions, ranging in molecular weight from about 8000 to 14 000 show a similar behaviour as the unfractionated material except that the lowest two molecular weight fractions show a marked polyelectrolyte behaviour at lower concentrations *(Figure 4)*. From the values listed in *Table 1*, two Mark–Houwink plots, i.e. $[\eta]_a$ vs. $\bar{M}_w$ and $[\eta]_b$ vs. $\bar{M}_w$, were constructed (see *Figure 5*).

The equations for the two straight lines in *Figure 5* are:

$$[\eta]_a = 1.047 \times 10^{-2} \bar{M}_w^{0.36} \quad (4)$$

$$[\eta]_b = 7.131 \times 10^{-5} \bar{M}_w^{0.87} \quad (5)$$

The value of the exponent α, is 0.36 for the curve using $[\eta]_a$ and 0.87 for the curve using $[\eta]_b$. Values of α between 0.5 and 0.9 indicate random coil molecules[17,18] whereas values around 0.25 suggest branched structures[19]. The value of 0.36 points to a considerable deviation from random coil behaviour. It appears, therefore, that heparin may behave as a Gaussian coil molecule in formamide at low concentration. In the solutions of higher concentrations, formamide may hydrogen bond to heparin to give a branched structure. The possibility of heparin aggregation using formamide as a bridge for hydrogen bonding between two or more heparin molecules also exists. In fact, the increased nitrogen content in the heparin after recovery from a formamide solution may result from this sort of behaviour. The observation that $[\eta]_a$ is higher than $[\eta]_b$ in all cases may point to a larger effective volume of the molecule in solutions of higher concentrations, which could result from aggregation. It should be noted here that the two $[\eta]_b$ values obtained from the Yuan–Dougherty–Stivala (YDS) equation (1), fall on the Mark–Houwink plot also. These are, in fact, the shielded intrinsic viscosities for the two samples in the lower concentration range. It is clear that so far as this work is concerned, $[\eta]$ and $[\eta]_\infty$ can be used interchangeably.

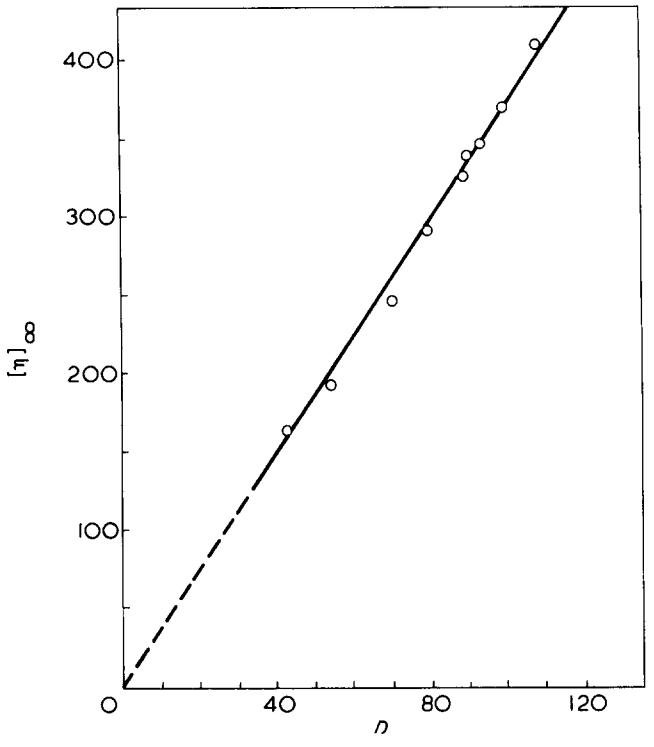

Figure 6 Plot of shielded intrinsic viscosity $[\eta]_\infty$ (l/mol) vs. dielectric constant, D, of solvent at 25°C. $[\eta]_\infty = 3.74D$

Heparin does not behave like a polyelectrolyte in formamide–glycerol mixture. This is presumably because of high formamide concentration. No break of the type occurring in *Figure 1b* for pure formamide is noted in either curve in *Figure 2*. The data obtained from these viscosity plots and from the dielectric constant experiments on the n.m.r. spectrometer fall on a straight line in *Figure 6*, yielding the relationship:

$$[\eta]_\infty = 3.74 \times D \qquad 42.5 \leqslant D \leqslant 107.4 \qquad (6)$$

Yuan[20] reported:

$$[\eta]_\infty = 3.60 \times D \qquad 54.7 \leqslant D \leqslant 93.2 \qquad (7)$$

The two equations are in good agreement, within experimental error. It is to be noted that the work presented in this paper not only confirms the linear dependence of $[\eta]_\infty$ on D observed by Yuan and Stivala[10], but also extends the range of this dependency from a low value of D of 42.5 to a high value of 107.4

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