The multiple complexes formed by the interaction of platelet factor 4 with heparin

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1. The anisotropy of the fluorescence of dansyl (5-dimethylaminonaphthalene-1-sulphonyl) groups covalently attached to human platelet factor 4 was used to detect the macromolecular compounds formed when the factor was mixed with heparin. At low heparin/protein ratios a very-high-molecular-weight compound (1) was formed that dissociated to give a smaller compound (2) when excess heparin was added. 2. A large complex was also detected as a precipitate that formed at high protein concentrations in chloride buffer. It contained 15.7% (w/w) polysaccharide, equivalent to four or five heparin tetrasaccharide units per protein tetramer. In this complex, more than one molecule of protein binds to each heparin molecule of molecular weight greater than about 6×10^3 . 3. The stability of these complexes varied with pH, salt concentration and the chain length of the heparin. The limit complexes found in excess of the larger heparins consisted of only one heparin molecule per protein tetramer, and the failure to observe complexes with four heparin molecules/protein tetramer is discussed.

Platelet factor 4 is one of the proteins from the α -granules of platelets, which can neutralize the anticoagulant effects of the polysaccharide heparin (Kaplan *et al.*, 1979; Rucinski *et al.*, 1979). It is a tetramer of identical subunits each of mol.wt. 7800 (Kurachi, 1978). The protein occurs in the α -granules as a complex with a chondroitin 4-sulphate proteoglycan (Barber *et al.*, 1972). The platelet factor 4 from this complex can be transferred to more highly sulphated polysaccharides, such as heparin, with the release of the chondroitin 4-sulphate proteoglycan (Busch *et al.*, 1980). In the present paper we report a study of the complexes formed between platelet factor 4 and heparin. The anisotropy of the fluorescence of dansyl-(platelet factor 4) was used to detect these complexes and to obtain information on the changes in the size of different macromolecular intermediates that formed from dilute solutions of protein and at different concentrations of heparin. We suggest that the complexes we detect are a reflection of the oligomeric structure of the protein and the presence on the polysaccharide of multiple binding sites.

Abbreviation used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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Experimental and results

Human platelet factor 4

This was prepared from outdated human platelet concentrates by affinity chromatography on heparin-agarose (Ginsberg *et al.*, 1979) and by gel-filtration at high and low ionic strength (Moore *et al.*, 1975).

The protein-heparin complex was labelled with dansyl chloride by incubating a solution (10 mg of protein/ml and 5 mg of heparin/ml in 0.05 M-sodium phosphate buffer, pH 9) with a 5-fold excess of dansyl chloride over the molarity of the protein subunits for 1 h at room temperature. We used the complex rather than free protein for the labelling to ensure that any basic residues important for the interaction with heparin would be protected from modification. The labelled protein was separated from the excess dye by filtering the reaction mixture through a column (1.5 cm $\times$ 33 cm) of Sephadex G-50 in 0.1 M-Tris/acetate, pH 7.5. The labelled protein was then separated from the heparin by filtering the solution through a column of (1.1 cm $\times$ 4.5 cm) of heparin-agarose (Iverius, 1971). Any denatured protein and free heparin were eluted by washing the column with 50 ml of 0.1 M-Tris/acetate buffer, pH 7.5, containing 0.6 M-NaCl. The labelled platelet factor 4 was then eluted with a

similar buffer containing 2M-NaCl. On one analytical column we found that the dansylated protein eluted as a single peak and at the same high molarity of NaCl (1.5M) as the unmodified protein.

Platelet factor 4 protein concentrations were determined by using a modification of the Lowry method (Miller, 1959), with lysozyme as the standard protein. Platelet factor 4 concentrations were obtained as 1.47 times the equivalent concentrations of lysozyme: this factor was determined by assaying weighed portions of dried pure platelet factor 4. The concentration of dansyl groups in the protein was obtained using $4300 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for the molar absorption coefficient of the dye at 340nm (Laurence, 1957). The preparations of labelled protein used in the present experiments contained from 0.5 to 1 mol of dye/mol of protein tetramer.

Heparin

Pig mucosal heparin from Sigma, St. Louis, MO, U.S.A. and from Paines and Byrne Ltd., Greenford, Middx., U.K. was used. This latter preparation was fractionated by molecular weight by the method of Laurent *et al.*, 1978. After dialysis against water for 24 h and freeze-drying, 750 mg of heparin in 2 ml of 0.3M-NaCl was filtered through a column (2.6 cm $\times$ 76 cm) of Sephadex G-100 equilibrated with 0.3M-NaCl. The profile of elution detected by uronic acid measurement was divided into nine equal fractions (27.5 ml). Corresponding fractions from four such separations were pooled, dialysed against water and freeze-dried. The K_{av} of each fraction was calculated as described by Laurent & Killander (1964). The weight-average molecular weights ($\bar{M}_w$) of fractions 1, 4 and 8 were kindly determined by Dr. J. M. Creeth of this Department by the equilibrium-sedimentation technique using eqn. 5.27 of Eisenberg (1976). The weighed samples of heparin were dialysed against 0.1M-phosphate, pH 7, containing 0.9M-NaCl. The progress of sedimentation was monitored in a Beckman model E ultracentrifuge with interference optics. The partial specific volumes ($\bar{v}$) were determined with an Anton Parr K.G. model DMA 02C instrument. A plot of the log(molecular weight) of the known fractions against K_{av} was used to estimate the molecular weights of the intermediate fractions.

Sulphate was determined as described by Terho & Hartiala (1971), and uronic acid was measured by the biphenyl-3-ol method of Blumenkranz & Absoe-Hansen (1973), with glucuronolactone as standard. Fractionated heparin was also assayed by the carbazole method as modified by Bitter & Muir (1962). Tris and sodium acetate buffers were made by adjusting 0.1M-acetic acid with the named base. For buffers at pH 9, 0.1M-glycine was adjusted to

pH with NaOH. Where necessary, NaCl or Na_2SO_4 were included to obtain the required ionic strength.

Analysis of an insoluble complex of heparin and platelet factor 4

This complex could only be dissolved in 2M-NaCl, and because this high salt concentration interfered with the analytical methods, we used a difference method of analysis; solutions (2 ml) of platelet factor 4 (0.5 mg/ml) and various concentrations of heparin in 0.1M-Tris/acetate, pH 7, and 0.4M-NaCl were prepared. The protein and uronic acid concentrations were determined. Mixtures of the protein with three heparin concentrations were made and left for 15 h in the cold-room until precipitation was complete. The precipitate was spun down at 17000g and its composition calculated as the difference between the concentrations of heparin and protein before mixing and those in the supernatants after mixing.

Molecular weight of platelet factor 4 in excess heparin

A column (1 cm $\times$ 70 cm) of Sephacryl 200 was calibrated for molecular weights of proteins by using as standards: pig heart lactate dehydrogenase (mol.wt. 144 000), bovine serum albumin (monomer 67 000 and trace of dimer), human platelet factor 4 (32 000), chymotrypsinogen A (25 000), myoglobin (17 500) and horse cytochrome c (12 000) in 0.4M-NaCl/Tris/acetate, pH 7.5. Heparin fraction 5 (mol.wt. 12 000) was eluted as if it were a protein of mol.wt. 50 000. When platelet factor 4 (0.8 mg/ml) and heparin fraction 5 (0.8 mg/ml) were filtered through the column in the same buffer containing heparin fraction 5 (0.01 mg/ml), the protein-heparin complex was eluted as if it were a protein of mol.wt. 80 000.

Fluorescence anisotropy measurements

The instrument described by Freyssinet *et al.* (1978) was used with excitation at 366 nm from a Mg-Xe source and emission selected by a Kodak-Wratten filter 58 (maximum transmission at 530 nm). For some experiments we used a machine that differed in that the exciting light from an Xe arc was selected by a double monochromator set at 335 nm (20 nm band pass) and the emitted light selected by a wedge interference filter monochromator set at 530 nm (band pass 18 nm). No differences were observed in results from the two instruments. In all titration experiments a solution of heparin was continuously added from a motor-driven Agla syringe to a stirred solution (2.5 ml) of dansyl-(platelet factor 4) at 25°C. No change in anisotropy was observed when a volume of buffer

(up to 0.3 ml) was added instead of the heparin. The ratio (R) of the intensity of fluorescence transmitted by polarizing films with axes horizontal and vertical was continuously monitored by an analogue divider and stored on digital tape (Lodola *et al.*, 1978). The anisotropy of the emission was calculated as $1.5(1-R)/(1+2R)$. In some experiments we also measured the intensity of horizontally emitted light separately. The intensity of the fluorescence of *N*-dansylbutylamine decreased rapidly at acidities below pH 4.3 and we thus restricted our interpretations to results obtained above this pH. The equilibrium constant for curves in which there was a hyperbolic relation between anisotropy change and added heparin were evaluated as described by Stinson & Holbrook (1973). We interpret the changes in anisotropy of dansyl fluorescence as reflecting the aggregation state of all the protein molecules (both unlabelled and labelled with the fluorescent indicator dye) in solution, since we obtain superimposable curves whether we used all labelled protein or an equal concentration of protein made from a 1:1 mixture of labelled and unlabelled preparations (Fig. 1).

Changes in anisotropy of fluorescence when heparin is added to dansyl-(platelet factor 4)

This factor is not soluble at low ionic strength when not complexed to a polysaccharide carrier, and thus, for our experiments, we initially used 0.1 M-Tris/acetate buffer containing 0.4 M-NaCl. The trace of fluorescence anisotropy change obtained when

Fig. 1. Effect of added unlabelled platelet factor 4 on the anisotropy change when heparin binds to the dansylated factor

Heparin fraction 4 (mol.wt. 15 000) was added at 1 μ g/min to 2.5 ml of 0.1 M-Tris/acetate buffer, pH 5.0, containing 0.133 M- Na_2SO_4 and (a) 32 μ g/ml dansyl-(platelet factor 4) or (b) 16 μ g of unlabelled plus 16 μ g of labelled platelet factor/ml. Curve (b) is displaced upwards by 0.025 unit as it would otherwise overlap curve (a).

heparin was added to dansyl-(platelet factor 4) in this chloride-containing buffer had the unusual form shown in Fig. 2. We interpret this curve to indicate that the labelled factor first forms soluble high-molecular-weight complexes that, in the presence of excess heparin, rearrange to give a smaller complex that is still larger than the original platelet factor 4. The anisotropy changes could be exactly reversed by slowly titrating unlabelled platelet factor 4 into the solution obtained at the end of the experiment shown in Fig. 2. We conclude that we are observing reversible equilibria rather than the results of degradation by traces of an undetected hydrolytic enzyme. In the present paper we shall refer to the material at the maximum anisotropy as 'complex 1' and that present in excess heparin as 'complex 2'.

An insoluble complex of platelet factor 4 and heparin

No light-scattering precipitate was formed during the titration shown as Fig. 2. However, when we added heparin to solutions of either dansylated or native platelet factor that were more concentrated than about 0.1 mg/ml and contained in 0.1 M-Tris/acetate, pH 7.5, containing 0.4 M-NaCl, we observed a white opalescence that was most pronounced at the protein/heparin weight ratio corresponding to the maximum anisotropy for dansyl-(platelet factor 4) at the lower protein concentrations (where no turbidity was seen).

Fig. 2. Changes in anisotropy of fluorescence of dansyl-(platelet factor 4) with the addition of heparin. Unfractionated heparin was added at 4 μ g/min to 2.5 ml of 0.1 M-Tris/acetate buffer, pH 7.5, containing 0.4 M-NaCl and 36 μ g of dansyl-(platelet factor 4)/ml. Arrows indicate the ratios of protein/heparin at which precipitates were taken for the analyses shown in Table 1 (but at about 0.4 mg of protein/ml).

Table 1. *Composition of a precipitate formed between high concentrations of platelet factor 4 and heparin*
For further details see the Experimental and results section. *n* is the number of experiments.

Sample (A, B and C approximate to the initial compositions marked A, B and C on Fig. 2)	Compositions of initial solutions		Compositions of supernatants		Deduced composition of precipitates from 1 ml of initial solution			
	Platelet factor 4* (nmol/ml)	Heparin (nmol of uronate/ml)	Platelet factor 4* (nmol/ml)	Heparin (nmol of uronate/ml)	Platelet factor 4 (nmol)	Heparin (nmol of uronate)	Ratio†	Proportion of heparin (%, w/w)
A (<i>n</i> = 2)	13.4	55	7.7	5	5.7	50	4.4	14.3
B (<i>n</i> = 5) ± s.d.	13.0	107	2.9	7	10.1 ± 0.7	100 ± 3	4.9	15.7 ± 0.04
C (<i>n</i> = 2)	8.7	155	7.5	143.5	1.2	11.5	4.5	15.0
Dansyl-(platelet factor 4) (B/C)‡ (<i>n</i> = 1)	5.1	115	2.2	85	2.9	20	5.1	16.4

* Taking platelet factor 4 to be a tetrameric protein mol.wt. 32 000

† Ratio heparin tetrasaccharide (mol.wt. 1212)/mol of platelet factor 4.

‡ 'B/C' indicates that this experiment was carried out at a ratio of protein to heparin between B and C (Fig. 2).

The composition of the insoluble protein-heparin complex was determined at protein/heparin weight ratios equivalent to the arrows labelled A, B and C in Fig. 2 and is shown in Table 1. We note that, for A and B, where the heparin is limiting and within the experimental limits of a difference method, there is no unbound heparin. We also observed that the deduced composition of the precipitate did not vary whether we used native or dansyl-(platelet factor 4) or whether we used slightly limiting or slight excess heparin. In view of the possibility of formation of a precipitate in the anisotropy titrations, we restricted these experiments to protein concentrations below 0.1 mg/ml and sometimes checked for light scatter by independently recording the fluorescence emitted through the horizontal polarizing film.

Dependence of heparin binding to platelet factor 4 on ionic strength

The fluorescence anisotropy of the dansyl-factor in solutions of 0.4–2 M-NaCl was constant. At lower concentrations of salt, the anisotropy increased as expected from the known insolubility of the free factor at low ionic strength (Moore *et al.*, 1975). When the labelled factor was titrated with heparin in solutions of increasing concentration of NaCl, the extent to which complex 1 formed was first decreased. At about 0.8 M-NaCl there is little evidence for complex 1, whereas complex 2 is still easily detected. At above 1.0 M-NaCl, neither complex is seen at these protein concentrations. There was no difference in titrations at 0.4 M-NaCl when 3 mM-CaCl₂ was included in the buffer. The titrations at above 0.6 M-NaCl represent the equilibrium situation (that is, if addition was stopped at any point on the curve, there was no time-dependent change in anisotropy). Qualitatively similar behaviour was obtained when NaCl was replaced by the same ionic strength of Na₂SO₄, except that, at *I*

0.5 and pH 5 or higher, true equilibrium was maintained in experiments that lasted 5000 s (Fig. 3). The 0.1 M-Na₂SO₄ trace in Fig. 3 is the only curve reported in the present paper where equilibrium is not quite attained.

Effect of pH on the binding of heparin to platelet factor 4

Fig. 4 shows titrations of the factor with heparin at *I* 0.7 in buffers containing Na₂SO₄ from pH 5 to 10.5. At low pH, complex 1 forms with least added heparin. Both complexes 1 and 2 are destroyed as the pH is increased, but it is possible to select conditions of pH (10.5) and ionic strength where complex 2 but not complex 1 is seen. When the experiments were repeated in buffer containing Na₂SO₄ at *I* 1.6, hyperbolic formation of complex 2 was observed and the dissociation constants for heparin were 9×10^{-9} M (pH 5), 4.4×10^{-8} M (pH 7.5), 1.3×10^{-7} M (pH 9.5), and no detectable binding at pH 10.5. For these experiments, fractionated heparin of molecular weight 15 000 was used. The decrease in ionic interaction between acidic sugars and basic-protein lysine residues would explain the weaker binding at high pH (Deuel *et al.*, 1977; Handin & Cohen, 1976).

Interaction of platelet factor 4 with heparins of more defined molecular weights

One explanation for the shape of titration curves such as Fig. 1 might be that, in a heterogeneous population of heparin molecules, initially all molecules combined with the protein, but, as excess heparin was added, the protein was able to select the smaller but tighter binding species in the mixture. We therefore fractionated the heparin according to molecular weight and obtained fractions whose analyses are shown in Table 2. Fraction 1, the largest, had a low degree of sulphation and high

Fig. 3. *Effect of ionic strength on the interaction of heparin with platelet factor 4*

The anisotropy changes are shown for titrations similar to Fig. 1(a), when Na_2SO_4 was at the molarity shown against each curve. The inset Figure is a plot on logarithmic paper of (s/s_0) [where s is peak anisotropy and s_0 is anisotropy in $0.67\text{ M-Na}_2\text{SO}_4$] against $I^{1/2}$. The slope of the curve further strengthens the conclusion that binding mainly depends on ionic interactions.

Fig. 4. *Effect of pH on the interaction of platelet factor 4 and heparin*

The anisotropy changes were measured as in Fig. 1(a) with heparin fraction 4 (mol.wt. 15 000; added at $0.5\text{ }\mu\text{g/min}$) and with buffers of the pH values shown against each curve (see the Experimental and results section for buffer compositions) containing $0.2\text{ M-Na}_2\text{SO}_4$. The platelet factor 4 concentration was $24\text{ }\mu\text{g/ml}$.

partial specific volume (Ehrlich & Stivala, 1973), and was not considered as typical of heparin.

The form of the titration curves obtained when heparins of narrower molecular-weight distribution were titrated into dansyl-(platelet factor 4) is shown in Fig. 5. In contrast with the above suggestion, we observed that complex 1 was most evident in the fractions of highest average molecular weight: thus

size heterogeneity is not a likely explanation for the shape of the titration curves. Heparins of mol.wts. 10 000–21 000 all clearly showed complex 1, yet the weight of heparin needed for maximum formation of complex 1 did not vary within our experimental error. In contrast, the weight of heparin needed to form complex 2 did vary with molecular weight. The point at which complex 1 is all converted into

Table 2. *Analysis of heparin fractionated according to weight-average molecular weight*
 Sulphate and uronic acid contents are expressed as percentages of dry weight. For details of assays and heparin fractionation, see the text.

Fraction no.	Weight (% of the total recovered)	K_{av} on Sephadex G-100	$10^{-3} \times \bar{M}_w$	Sulphate	Uronic acid	
					Biphenyl-3-ol method	Carbazole method
1	5.3	0.044	25*	30.5	25.2	34.6
2	13.4	0.104	20.8	32.0	24.2	33.0
3	16.6	0.165	17.6	35.0	25.6	35.6
4	19.8	0.226	15.0*	35.0	25.6	34.2
5	16.6	0.286	12.0	38.0	25.6	36.4
6	12.0	0.347	9.8	36.5	25.4	35.4
7	10.2	0.407	7.8	38.0	25.9	34.4
8	4.4	0.476	6.0*	39.0	24.0	35.2
9	1.5	0.564	4.0	—	26.4	—

* These values were determined from the ultracentrifuge as described in the text. Intermediate values were extrapolated from a plot of log (molecular weight) against K_{av} .

complex 2 cannot be determined with great precision, but for fractions 2–5 is 0.73, 0.76, 1.0 and 0.95 mol of heparin/mol of protein tetramer. These values are consistent with a 1:1, 2:2 (etc.) molar ratio in the complex. Heparin fractions with mol.wts. below 10000 have values significantly greater than 1:1 (e.g. for heparin with mol.wt. 7800 the ratio was 2.1:1), although the weak binding of these small heparins made this estimation less reliable than for the larger heparins. The 1:1 stoichiometry for a 15000-mol.wt. heparin can also be seen in Fig. 3, where at 0.4M- Na_2SO_4 , complex-1 formation is largely suppressed and complex 2 is still formed. The initial and final tangents to that curve intercept at 0.9mol of heparin/mol of tetrameric platelet factor 4.

Molecular weight of complex 2

The previous experiment establishes that, in excess of heparin of mol.wts. above about 10000 and below 23000, platelet factor 4 forms a soluble complex 2 in which there are equal numbers of heparin and protein molecules. We thus needed to estimate the molecular weight of this complex with sufficient precision to distinguish 1:1 compounds from 2:2 compounds and larger. On a gel-filtration column that was calibrated with proteins of known molecular weight we observed that free platelet factor 4 eluted as expected for a protein of mol.wt. 32000, that fraction-5 heparin (mol.wt. 12000) eluted from the column at the same position as a protein of mol.wt. 50000 and that complex 2 formed from these components filtered through the column (equilibrated with excess heparin) as a protein of mol.wt. 80000. As expected, the pure polysaccharide was eluted from the column at a much lower volume than a protein of the same molecular weight

(Squire, 1964). Complex 2 behaves as would be expected for a particle that was a loose association of one molecule of 32000-mol.wt. protein and one molecule of 12000-mol.wt. heparin. The elution position was very well separated from that of protein with mol.wt. 160000, and thus the complex 2 does not behave as a 2:2 (or larger) association of protein and heparin.

Discussion

In the present paper we partially characterize the macromolecular complexes formed between heparin and tetrameric platelet factor 4. In general we assume that the fluorescence anisotropy of a macromolecule labelled with a fluorescent dye will increase as the rate of rotation in solution decreases and the molecular weight increases. The increase in anisotropy with molecular weight will not be linear and will eventually reach a limit set by the intrinsic mobility of the dye. It is possible with some dyes for the anisotropy to decrease as size increases. This is most likely when internal energy transfer of electronic excitation energy between many dye molecules attached to the same macromolecule is possible. To avoid this possibility we kept the average degree of substitution of protein with dye below 1 mol of dye/mol of protein and used a dye, dansyl chloride, where the Stoke's shift between excitation wavelength and emission wavelength is large (200 nm). It was also necessary that changes in the size of dye-labelled proteins exactly reported the changes in size of all the proteins in the system. To ensure that dye did not attach to protein surfaces that interact with heparin, the labelling reaction was carried out in the presence of excess heparin, where these surfaces would not be exposed to dye.

Secondly we showed that the same fluorescence anisotropy changes were observed whether we used all dye-labelled protein, or a 1:1 mixture of labelled and unlabelled material (Fig. 1).

The results demonstrate that at least two complexes can form between heparin and platelet factor 4. When the concentration of heparin is limiting low, a complex 1 of very high anisotropy forms. As the concentration of heparin is increased, this complex is converted into complex 2 of lower anisotropy, but still of higher anisotropy than the starting, tetrameric, protein. The weights of the larger heparin molecules that are needed to form complex 2 suggests that they are 1:1 complexes of heparin and platelet factor 4. We did consider that in the final complex the protein might have dissociated to give monomers; however, a 1:1 complex of protein monomer (7800) and heparin (12000) would be about 30% protein by weight in contrast with the 65% protein by weight in a 1:1 complex of tetrameric protein (32000) and heparin (12000).

This 1:1 result was unexpected and needs explanation. Although at the end of the titrations there is present a large excess of heparin molecules over protein tetramers, we only observe the binding of one, not four, heparin molecules per tetramer. Since the protein is composed of identical subunits, this suggests that the multiple protein-binding sites of the 'longer' heparins (demonstrated in complex 1; see below) are used to wrap around the protein and thus satisfy the binding interaction of all four subunits in each molecule. Such an interaction would be expected to be favoured as it is of lower entropy than the association of four heparin molecules with one protein molecule. On this basis the same class of ionic binding sites could provide an explanation for

high-molecular-weight complex 1 and the lower-molecular-weight complex 2. This interpretation also predicts that heparin molecules with a length that does not allow them to encircle the protein [a length that can be estimated to be equivalent to mol.wt. 10000 from the crystallographic information on both the protein (Kurachi, 1978) and the heparin (Atkins & Nieduszynski, 1976)] would bind more than one heparin molecule per protein tetramer in excess heparin. Fig. 5 shows that the smaller heparins bind to platelet factor 4 with stoichiometries much greater than 1:1.

We observed that, in the presence of excess protein, all the heparin (unfractionated for size) was complexed by the protein (Table 1, samples A and B). We interpret this to mean that all heparin molecules carry binding sites for platelet factor 4. This conclusion agrees with that of Niewiarowski *et al.* (1979). This is to be contrasted with the large fraction of heparin molecules that lack a tight binding site for another protein, antithrombin III (Jordan *et al.*, 1979; Lindahl *et al.*, 1979). Both the rapid decrease in binding at high pH and the dependence of binding on ionic strength would be consistent with much of the binding interaction reflecting the acidic sulphate and carboxy groups of the heparin and the basic residues of the protein.

Both in the insoluble complex of heparin and platelet factor 4 and in the soluble complex 1 there is evidence that more than one molecule of protein can bind to one molecule of the longer heparins. Thus a heparin molecule of mol.wt. 15000 binds about three molecules of protein tetramer in complex 1. Since the overall structure of heparin can be represented as tetrasaccharides substituted by five sulphate groups (Taylor *et al.*, 1973), the length of heparin that can

Fig. 5. Effect of changing the average chain length of heparin on its interaction with platelet factor 4. Heparins of the average molecular weights listed in Table 2 were added at $0.5 \mu\text{g/ml}$ to dansyl-(platelet factor 4) ($23.6 \mu\text{g/ml}$) in $0.133 \text{ M-Na}_2\text{SO}_4$ and $0.1 \text{ M-Tris/acetate pH } 5$. Curve 2, heparin mol.wt. 20800; curve 3, 17600; curve 4, 15000; curve 5, 12000; curve 6, 9800; curve 7, 7800; curve 8, 6000. The curve numbers correspond to the fraction numbers in Table 2.

bind one protein tetramer approximates to four of these tetrasaccharide units. Interestingly it is only heparin molecules with a molecular weight that is about twice this value (i.e. fraction 7; Fig. 5) that are easily able to form the high-molecular-weight complex 1. This suggests that, in this complex, the platelet factor 4 molecules interact with two (or more in the longer heparin molecules) sites on the heparin to form the high-molecular-weight aggregate.

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