Analysis of Dye Binding Sites in Mast Cell Granules†

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ABSTRACT: The phenomenon of metachromasia has been used to characterize the anionic sites on the mast cell granule. Between 2.0 and 2.5 of the 3.0–3.5 sites present per disaccharide repeat unit of mast cell granule heparin are available *in situ*. The hypochromism of Acridine Orange bound to mast cell granules is suppressed with an apparent p*K* of 4.28, indicating that the effect is dependent on a carboxyl group. The change in the center of gravity of the absorbance spectrum of Methylene Blue on binding to mast cell granules more closely resembles that of Methylene Blue on binding to heparin than to *N*-acetylheparin, chondroitin 4-sulfate, poly(glutamate–tyrosine) copolymer, polyglutamate, or bovine serum albumin. On the basis of these observations, it is proposed that the ionic complex of heparin and protein in the granule involves one anionic site of heparin, probably an *O*-sulfate, leaving the remaining anionic sites, the *N*-sulfate, the uronic carboxyl and a variable *O*-sulfate, available for binding small molecular weight cations such as dyes and histamine.

Heparin is a major constituent of the mast cell granule (Lagunoff *et al.*, 1964). The obvious hypothesis that this highly negatively charged macromolecule is the site of histamine binding within the cell (Amann and Werle, 1956; Barlow, 1964; Keller, 1958; Kobayashi, 1962; Lagunoff *et al.*, 1964; MacIntosh, 1956; Parrot *et al.*, 1960; Sanyal and West, 1956; Werle and Amann, 1956) has been challenged and the alternative of protein binding proposed (Uvnäs *et al.*, 1970). Little is known of the character of the complex between mucopolysaccharide and protein in the granule other than its dissociability at high salt concentrations. This effect of salt on granule integrity is the basis for the hypothesis that the granule is an ionic complex (Lagunoff, 1966). The suggestion that protein rather than heparin is the binding site for histamine depends strongly on the assumption that heparin carboxyl groups are involved in ionic interaction with protein (Uvnäs *et al.*, 1970) and therefore are unavailable for histamine binding. Knowledge of the disposition of the several negatively charged groups of heparin is clearly critical for any meaningful analysis of granule structure and histamine binding.

In the work reported here, the metachromasia of Acridine Orange and Methylene Blue on binding to heparin and to mast cell granules has been utilized to explore the disposition of the negatively charged groups of heparin in the intact mast cell granule.

Materials and Methods

Mast cells were obtained from adult male CD rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) as previously described (Lagunoff, 1972a). In some instances the mast cells were disrupted by sonication (Lagunoff *et al.*, 1964), but standardly, a modification of the procedure of Uvnäs *et al.* (1970) for lysis of mast cells in distilled water was used. Mast cells isolated by centrifugation through 35% albumin (Pathocyte IV, Miles Laboratories) were rinsed free of albumin in modified Ringer's solution, washed once in ice-cold distilled water, and resuspended in ice-cold distilled water, usually 2.0 ml for mast cells from 10 rats. The cells were repeatedly mixed vigorously on a Vortex Jr. Mixer® (Scientific Products Inc.) for 15-sec intervals, chilling the suspension for 15–30 sec between mixing, until more than 90% of the mast cells were disrupted as determined by light microscopic observation. The granules were then collected by differential centrifugation (Lagunoff *et al.*, 1964).

Sodium heparinate (grade I) was purchased from Sigma Chemical Corp. Chondroitin sulfate (Type A, 4 sulfate/6 sulfate = 80:20), poly(L-glutamic acid–L-tyrosine) copolymer (1:1) (Poly(G-T)), and bovine serum albumin were purchased from Miles Laboratories. Sodium poly-L-glutamate was purchased from Schwarz/Mann Laboratories. *N*-Acetylheparin was prepared by mild acid hydrolysis of heparin, which exposed more than 90% of the amine groups and subsequent acetylation with acetic anhydride (Lagunoff and Warren, 1962). Rat mast cell heparin was extracted from sonicated granules with 2.0 M KCl (Lagunoff, 1966): the solubilized heparin was precipitated with cetylpyridinium chloride after dilution to 0.5 M KCl; cetylpyridinium chloride was removed by washing the precipitate with ethanol saturated with NaCl leaving a precipitate of sodium heparinate (Scott, 1960). Acridine Orange and Methylene Blue were purchased from National Aniline Division, Allied Chemical and Dye Corp.

Uronic acid was measured by the method of Bitter and Muir (1962), *N*-sulfated hexosamine by the method of Lagunoff and Warren (1962), free amine by the method of Habeeb (1966), and total sulfate by the method of Terho and Hartiala (1971).

Metachromatic titrations were performed essentially according to the procedure of Stone and Bradley (1967); 0.2 ml of Acridine Orange stock solution, nominally 0.2 mM in 0.001 M cacodylate buffer (pH 6.7), was added to 2.0 ml of the same buffer in a quartz cuvet and the absorbance read in a Gilford 2200 spectrophotometer at 492 nm.[1] Alternatively the absorbance spectrum was scanned in a Cary 15 spectrophotometer between 525 and 390 nm. Aliquots of a solution of polyanion or a suspension of mast cell granules in water were added with thorough mixing, and the absorbance at 492 nm was determined or the spectrum between 525 and 390 nm recorded (Figure 1a and b). The number of hypochromic binding sites per polymer repeat unit was determined from a plot of absorbance *vs.* volume of polyanion solution added. The intersection of the

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[1] Dye concentrations were calculated using the value $\epsilon_{492} = 5.6 \times 10^4 - (4.3 \times 10^3 \times \text{absorbance})$ for Acridine Orange and $\epsilon_{660} = 7.6 \times 10^4$ for Methylene Blue.

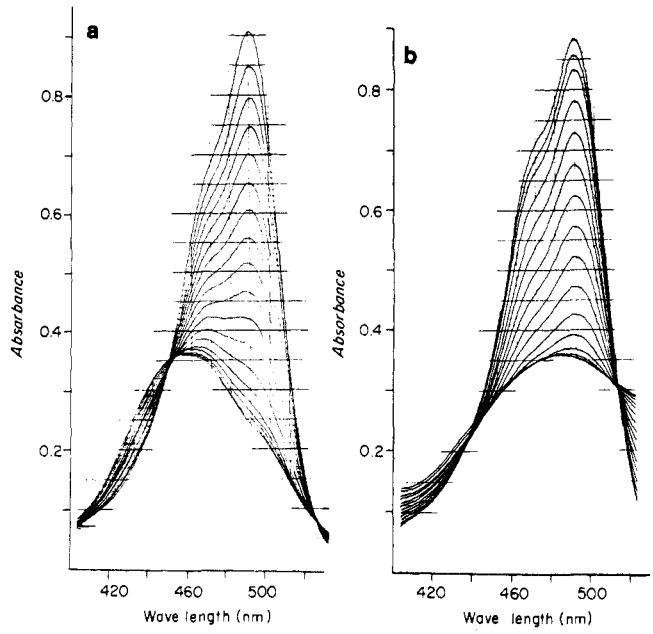

FIGURE 1: (a) Acridine Orange binding by heparin determined from metachromasia. Heparin solution (50 μg/ml) was added in 10-μl aliquots to an Acridine Orange solution (pH 6.7) with an initial absorbance of 0.905. The spectrum was recorded after each addition. (b) Acridine Orange binding by mast cell granules determined from metachromasia. Mast cell granule suspension (141 μg of glucuronolactone/ml) was diluted $\frac{1}{5}$ and added in 10-μl aliquots to 2.2 ml of Acridine Orange solution (pH 6.7) with initial absorbance of 0.882. The spectrum was recorded after each addition.

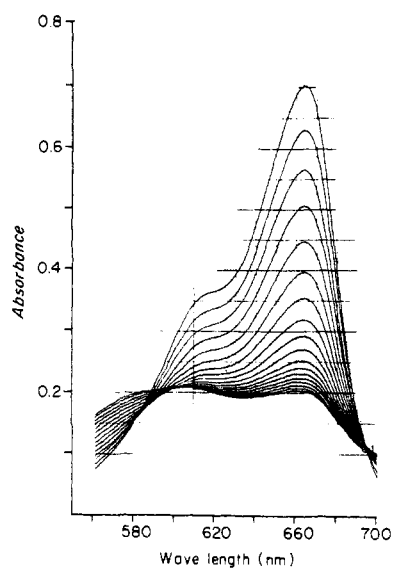

FIGURE 2: Metachromatic spectra of Methylene Blue bound to mast cell granules. Mast cell granule suspension (190 μg of glucuronolactone/ml, 2.57 groups/disaccharide) was diluted $\frac{1}{5}$ and added in 5-μl aliquots to 2.1 ml of Methylene Blue, initial absorbance of 0.902. The spectrum was scanned after each addition.

two limbs was taken as the titration end point (Stone, 1967). Uronic acid content was used as the basis for the calculations of granule heparin concentration, since no other uronic acid containing substances are present in the granule. For measurements of the metachromasia of Methylene Blue, 0.1 ml of stock solution of dye, nominally 0.2 mM, was added to 2.0 ml of buffer and the spectrum scanned from 700 to 500 nm[1] (Figures 2 and 3). The metachromatic spectral shift was assessed in terms of the center of gravity (cg) of the absorbance spectrum. This value was calculated according to Stone (1967) from the equation $cg = \int \epsilon\sigma d\sigma / \int \epsilon d\sigma$, where ϵ is the extinction coefficient in mole^{-1} of dye per cm and σ is the frequency in wave numbers. A Hewlett—Packard 9830 calculator was used for the integration at 10-nm intervals. The values of cg and the oscillator strength, $f = 4.32 \times 10^{-9} \int \epsilon d\sigma$, obtained for Methylene Blue by this method agreed reasonably with those reported by Stone (1967) (Table I). Routinely I have calculated cg for spectra between 700 and 500 nm, whereas Stone utilized the range 730–460 nm. In the case of mast cell granules, apparent absorbance values were corrected for light scattering.

Analysis of H^+ dissociation constants by titrating hypochromic binding sites over a range of pH was feasible only with heparin. However, it proved possible to determine an apparent pK (pK_a) for each of the polymers and whole granules as well, by adding 1 equiv of polymer in aqueous solution to 2.0 ml of unbuffered aqueous Acridine Orange solution adjusted to pH 7.0 and measuring the reversal of hypochromism on progressive acidification of the solution with 5-μl aliquots of 6 N HCl (Figure 4). pK_a values were determined by linear regression analysis using the Katchalsky–Spitnik equation (Katchalsky and Spitnik, 1947), $pH_j = pK_a - n \log [(1 - \alpha_j)/\alpha_j]$, where α_j is the proportion of dissociated carboxyl groups at pH_j and is calculated as $[Abs(pH\ 2.0) - Abs(pH_j)]/[Abs(pH\ 2.0) - Abs(pH_j)]$; pH_j = initial pH after the addition of polymer (Figure 5).

Results

Results of analyses of heparin, *N*-acetylheparin, and chondroitin sulfate are presented in Table II. The number of negatively charged sites per dimer determined for these substances by the hypochromic binding titration agreed reasonably with the sum of sulfate and carboxyl groups. Similarly, the results of the titrations were in agreement with the known number of carboxyl groups in polyglutamate and poly(G-T). The addition of albumin to an Acridine Orange solution caused no change in the absorbance spectrum of Acridine Orange.

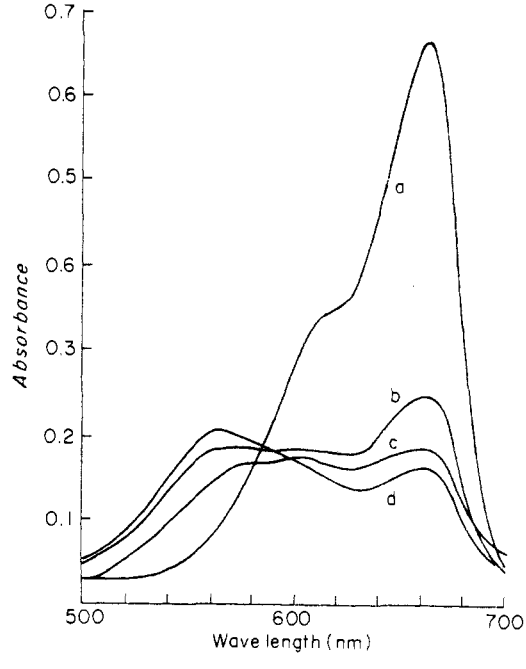

FIGURE 3: Spectral absorbance curves of (a) Methylene Blue, (b) 2 equiv of heparin added to Methylene Blue, (c) 2 equiv of mast cell granules added to Methylene Blue, and (d) 8 equiv of heparin added to Methylene Blue. The absorbance values for (c) are corrected for light scattering by the granules. The curves have been redrawn from values determined at 10-nm intervals.

TABLE I: cg and f for Methylene Blue.[a]

Source of Data	Program	λ range (nm)	cg (cm^{-1} × 10^{-3})	f
Stone (1967)	Devoe	460–730	16.06	6.50
Stone (1967)	Lagunoff	500–700	15.95	6.30
Lagunoff	Lagunoff	500–700	15.99	6.22

[a] cg (center of gravity of the absorbance spectrum) and f (oscillator strength) were calculated from absorbance spectra by numerical integration of the equations given in the text.

Heparin prepared from rat mast cell granules yielded essentially the same number of hypochromic binding sites as the commercial heparin derived from hog intestinal mucosa (Table II). In contrast, when the hypochromic binding sites on mast cell granules were calculated, only 2.4 sites per mole of uronic acid were found (Table II).

Although only one binding site of two per dimer in *N*-acetylheparin and chondroitin sulfate is a carboxyl group, acidification to pH 2.0 virtually completely reversed the hypochromism of both of these polymers. In the case of heparin, only one binding site per repeat dimer was titrated between pH 7.0 and 2.0, that site had a pK_a of 4.15 (Figure 5). Mast cell granules lost all their hypochromic effect at pH 2.0; the titration yielded a pK_a of 4.28 (Figure 5). This value was close to that for heparin, significantly higher than that for *N*-acetylheparin or chondroitin sulfate, and lower than that for glutamate containing polymers (Table III).

The centers of gravity of the absorbance spectra of Methylene Blue for polymer anionic site/dye (P/D) ratios from 1 to 8 were determined for dye bound to mast cell granules, heparin, *N*-acetylheparin, chondroitin sulfate, polyglutamate and poly(G-T) (Figure 6).

Discussion

A shift in the absorbance spectrum of certain dyes on binding to mast cell granules was observed by Ehrlich (1877) and has remained the hallmark of the cell. This phenomenon of metachromasia has been studied extensively by physical chemists and histochemists, the former are interested in elucidating the mechanism, the latter in identifying the cellular constituents responsible for the metachromatic shift. It is now widely held that the metachromatic spectral shift and the associated hypochromism are induced when dye binds to a polyanion and depend on the association of dye molecules with one another on binding in appropriate proximity (Bergeron and Singer, 1958; Phillips, 1970; Stone, 1969; Sylvén, 1954).

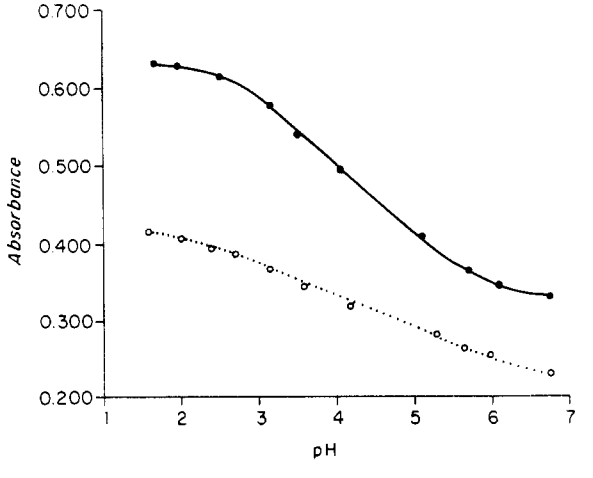

FIGURE 4: Effect of pH on the absorbance at 492 nm of mast cell granules and Acridine Orange and heparin and Acridine Orange. One equivalent of mast cell granules or heparin was added to 2.2 ml of Acridine Orange solution at pH 6.75 and the solutions were acidified with 5-μl serial additions of 0.1 N HCl. (●——●) Mast cell granules; (○ - - - ○) heparin.

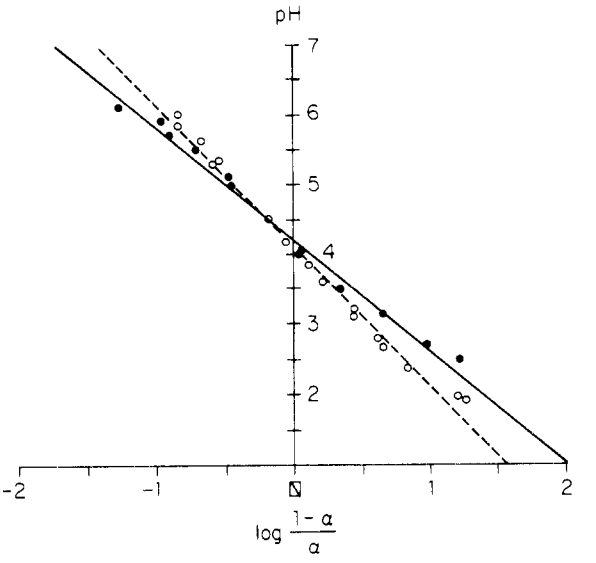

FIGURE 5: Katchalsky–Spitnik plots for titrations of Acridine Orange hypochromism. Log $[(1 - \alpha)/\alpha]$ was calculated as described in the text. The straight lines were determined by least-squares method for linear regression. (●——●) Heparin; (○ - - - ○) mast cell granules.

The bulk of tissue histamine is stored in the mast cell. Heparin, also present in mast cells, has been shown to have a substantial affinity for histamine (Kobayashi, 1962), and it has been accordingly proposed that mast cell histamine is bound intracellularly to heparin. With the development of methods for the isolation of mast cell granules, it became feasible to directly examine the intracellular binding sites for histamine. Histamine and heparin have been demonstrated to be largely, if not exclusively, localized in the mast cell granules and the maximum binding capacity estimated to be 0.72 mol of histamine/mol of heparin disaccharide for granules at pH 6.5 (Lagunoff, 1966). This value is well within the histamine binding capacity of heparin of 1 mol/mol of disaccharide (Kobayashi, 1962). A value of 1 μmol of histamine bound/mg of dry weight of granules obtained by Uvnäs *et al.* (1970) is also consistent with these values. Uvnäs and his coworkers (1970) determined the pK_a of the histamine binding site of the granule to be between 4 and 5. Based on this observation and the finding that the granules possessed a large number of titratable carboxyl groups, Uvnäs *et al.* (1970) proposed that histamine binds to the carboxyl groups of granule protein, rather than to negative sites of heparin. From their amino acid analysis of total granule protein (Bergqvist *et al.*, 1971), it can be calculated that approximately 80% of the side-chain carboxyl groups would be required to provide sufficient sites to account for the measured histamine binding by the granules; the value found by Uvnäs *et al.* (1970) on direct titration of granules meets the requirement for free protein carboxyl groups.

In their interpretation, Uvnäs and coworkers (1970) assumed that the negatively charged groups and specifically the uronic carboxyls of heparin interact with the positively charged groups of protein so that they are unavailable for binding histamine. Strong occlusion of all the anionic groups of heparin is

TABLE II: Sites Per Repeat Unit.[a]

Polyanion	Anionic Sites per Repeat Unit: Expected	Anionic Sites per Repeat Unit: Determined by Chemical Analysis	Acridine Orange[b] Binding Sites per Repeat Unit
Heparin (Sigma)	3.0–3.5	3.3	3.31 ± 0.09 (7)
Heparin (rat mast cell)	3.0–3.5		3.36 ± (2)
N-Acetylheparin	2.0–2.5	2.3	2.57 ± 0.04 (4)
Mast cell granules			2.53 ± 0.08 (8)[c]
Chondroitin sulfate	2.0	1.6	2.01 ± 0.06 (3)
Polyglutamate	1.0		0.87 ± 0.14 (4)
Poly(G-T)	1.0		0.92 ± 0.06 (4)

[a] Expected values for anionic sites were based on the known structures of the macromolecules. Values for chemical analysis were calculated from the sums of uronic acid and sulfate determinations. [b] Acridine Orange binding sites were determined by metachromatic titration and are recorded as the mean ± S.E.; the number in parentheses indicates the number of times the assay was repeated. [c] Acridine Orange binding sites per repeat unit for mast cell granules were calculated using uronic acid content of the granules as the basis for the calculation of repeat units.

TABLE III: Apparent pK Values for Hypochromic Binding Sites.[a]

Polyanion	pK_a	n
Heparin	4.15 ± 0.08	1.98 ± 0.12
N-Acetylheparin	3.35 ± 0.10	1.56 ± 0.20
CS	3.08 ± 0.17	1.36 ± 0.15
Polyglutamate	5.14 ± 0.35	1.96 ± 0.50
Poly(G-T)	4.54 ± 0.20	1.69 ± 0.24
Mast cell granules	4.28 ± 0.09	1.43 ± 0.16

[a] The pK_a values were determined from the pH value at log $[(1 - \alpha)/\alpha] = 0$; n is the slope of the line determined from the Katchalsky–Spitnik plot. pK_a and n were obtained by least-squares analysis of linear regression ± the 95% confidence intervals.

difficult to reconcile with the intense metachromasia of mast cell granules without the additional assumption that granule protein is responsible for the metachromasia. Metachromasia of cationic dyes has not been demonstrated with any naturally occurring protein.[2] In order to test directly the possibility that granule protein might provide negatively charged sites for metachromatic binding of dyes to mast cell granules, albumin was examined and found devoid of metachromatic binding sites. The choice of albumin was based on the fact that there are 92 glutamic and aspartic residues per molecule of 566 amino acid residues (Peters and Hawn, 1967), yielding a frequency of 16.3 negatively charged side chains per 100 residues which closely matches the 16 acidic side chains per 100 residues for total mast cell granule proteins found by Bergqvist *et al.* (1971). Furthermore, on the basis of its net negative charge at pH 6.7, albumin would be more likely to exhibit metachromatic binding than the proteins of mast cell granules which carry a net positive charge at that pH. A more extreme test of the possibility of a metachromatic protein–dye interaction was conducted with two synthetic polypeptide polymers, polyglutamate and poly(G-T). Methylene Blue exhibits metachromasia on binding to both polymers; poly(G-T) has a greater effect than polyglutamate (Figure 6), but neither polypeptide is as strong an inducer of metachromasia as heparin or mast cell granules at low P/D ratios. At a high P/D ratio, the value for cg for poly(G-T) approaches that obtained with mast cell granules. The carboxyl frequency of 1 per 2 residues in poly(G-T) far exceeds that of the granule proteins.

Since the granules bind approximately 2.5 mol of Acridine Orange/mol of uronic acid (mol wt of heparin disaccharide with 2.4 sulfate groups per disaccharide = 600) and the ratio of heparin to protein in the granules is about 1:2, for the total granule proteins to account for Acridine Orange binding would require 20–25 carboxyl residues per 100 amino acid residues (average mol wt = 125); since Berqvist *et al.* (1971) find only 16 acidic residues per 100, aside from the difficulty of providing a metachromatic array of carboxyl groups, there does not appear to be a sufficient number of acidic amino acid residues in mast cell granule proteins to account for the observed metachromatic dye binding.

Heparin has one hexuronic carboxyl group and one glucosamine *N*-sulfate group per disaccharide (Perlin *et al.*, 1971). The other negatively charged sites are contributed by *O*-sulfate groups which are probably located on C-6 of glucosamine and C-2 of the hexuronic acid; the former sulfate is believed to occur more regularly than the latter which is considered to comprise the variable sulfate component (Lindahl and Axelsson, 1971; Linker and Hovingh, 1972). A pK_a of 4.28 for the hypochromic dye binding sites in the granule implicates carboxyl groups in the granule as at least one class of the Acridine Orange binding sites, as in the case for heparin. The value for the pK_a cannot be taken as a reliable estimate of the intrinsic

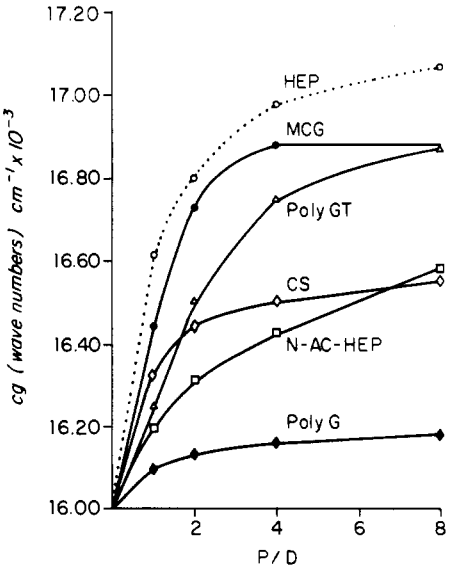

FIGURE 6: Plot of cg *vs.* P/D. cg values for Methylene Blue absorbance spectra were calculated from spectral data as described in the text. Polymer anionic sites to dye ratios (P/D) were calculated from titration of polymer with Acridine Orange and the initial absorbance of the Methylene Blue solution.

[2] The metachromasia of crystal violet with amyloid is explicable on the basis of the presence of heparan sulfate (Pras *et al.*, 1971) rather than on the basis of any peculiar properties of amyloid protein.

pK of the carboxyl group, since the affinity of the dye for the heparin site will have a significant influence on the pK_a.

The essentially complete reversal of the hypochromism of Acridine Orange bound to mast cell granules at pH 2.0 contrasts with the persistence of the hypochromic effect of heparin at that pH. This observation strongly mitigates against the possibility that the difference between the number of hypochromic binding sites and the number of sites calculated on the basis of uronic acid content is attributable to inaccessibility to dye of an appropriate fraction (1/3.4) of granule heparin. If such a fraction were inaccessible, then all the potential binding sites per disaccharide of the accessible granule heparin would be available, and the titration curve of granule dye binding sites would be expected to be the same as that of heparin. The complete reversal of hypochromism at pH 2.0 does not indicate that all the dye binding sites are necessarily carboxyl groups, since, as in the case of *N*-acetylheparin, the neutralization of the carboxyl group can abolish the hypochromic effect even when sulfate groups are still available for binding.

Any distinction between the *N*-sulfate and *O*-sulfate groups of mast cell granule heparin to be drawn from the present observations depends on the assumption that the differences between the metachromatic properties of heparin and *N*-acetylheparin are predominantly attributable to the *N*-sulfate group of heparin. The magnitude of induced metachromasia measured as Δcg for the absorbance spectrum of Methylene Blue is significantly greater with heparin and with granules than with *N*-acetylheparin. The difference in Δcg between heparin and *N*-acetylheparin, however, could be a function of either the higher charge density of heparin or the specific *N*-sulfate group; but since according to the interpretation of the observation, mast cell granule heparin *in situ* has one of its sulfate groups unavailable, it seems most likely that the large Δcg for mast cell granules relative to *N*-acetylheparin is assignable to a free *N*-sulfate group. A further test of this hypothesis would be provided by an examination of Δcg for heparins low in *O*-sulfate.

If the analysis offered of the influence of the *N*-sulfate group on metachromasia is correct, then it follows that *O*-sulfate is responsible for the ionic binding of granule heparin to granule proteins, leaving the *N*-sulfate, the uronic carboxyl and a variable *O*-sulfate available for binding Acridine Orange, Methylene Blue, and, by analogy, histamine. At pH's below the pK of the imidazole N, the *N*-sulfate and uronic carboxyl groups could provide a divalent anionic binding site for histamine as Stone and Moss (1964) have previously proposed. In their model, histamine fit snugly between the *N*-sulfate and the preceding uronic carboxyl in a hydrophobic groove of the helical polymer. More recent models for the structure of heparin retain the possibility of fitting histamine between the *N*-sulfate and uronic carboxyl groups (Hirano, 1972; Nieduszynski and Atkins, 1973; Perlin *et al.*, 1972), although apparently with less likelihood for hydrophobic interaction. Results of preliminary studies of histamine binding to mast cell granules are in general accord with this proposal (Lagunoff, 1972b).

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References

Amann, R., and Werle, E. 1956), *Klin. Wochenschr. 34,* 207.
Barlow, G. H. (1964), *Biochim. Biophys. Acta 83,* 120.
Bergeron, J. A., and Singer, M. (1958), *J. Biophys. Biochem. Cytol. 4,* 433.
Bergqvist, U., Samuelsson, G., and Uvnäs, B. (1971), *Acta Physiol. Scand. 83,* 362.
Bitter, T., and Muir, H. M. (1962), *Anal. Biochem. 4,* 330.
Ehrlich, P. (1877), *Arch. Mikroskop. Anat. Entwicklungsmech. 13,* 263.
Habeeb, A. F. S. A. (1966), *Anal. Biochem. 14,* 328.
Hirano, S. (1972), *Int. J. Biochem. 3,* 677.
Katchalsky, A., and Spitnik, P. (1947), *J. Polymer Sci. 2,* 432.
Keller, R. (1958), *Arzneim-Forsch. 8,* 390.
Kobayashi, Y. (1962), *Arch. Biochem. Biophys. 96,* 20.
Lagunoff, D. (1966), *in* Mechanisms of Release of Biogenic Amines, von Euler, U. S., Rosell, S., and Uvnäs, B., Ed., Oxford, Pergamon Press, p 79.
Lagunoff, D. (1972a) *Biochem. Pharmacol. 21,* 1889.
Lagunoff, D. (1972b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31,* 531.
Lagunoff, D., Phillips, M. T., Iseri, O. A., and Benditt, E. P. (1964), *Lab. Invest. 13,* 1331.
Lagunoff, D., and Warren, G. (1962), *Arch. Biochem. Biophys. 99,* 396.
Lindahl, U., and Axelsson, O. (1971), *J. Biol. Chem. 246,* 74.
Linker, A., and Hovingh, P. (1972), *Biochemistry 11,* 563.
MacIntosh, F. C. (1956), *in* Ciba Symposium on Histamine, Wolstenholme, G. E. W., and O'Connor, C. M., Ed., Boston, Mass., Little, Brown and Co., p 20.
Nieduszynski, I. A., and Atkins, E. D. T. (1973), *Biochem. J. 135,* 729.
Parrot, J.-L., Nicot, G., and Laborde, C. (1960), *C. R. Soc. Biol. 154,* 1426.
Perlin, A. S., Mackie, D. M., and Dietrich, G. P. (1971), *Carbohydr. Res. 18,* 185.
Perlin, A. S., Ng Ying Kin, N. M. K., Bhattacharjee, S. S., and Johnson, L. F. (1972), *Can. J. Chem. 50,* 2437.
Peters, T., Jr., and Hawn, C. (1967), *J. Biol. Chem. 242,* 1566.
Phillips, G. O. (1970), *in* Chemistry and Molecular Biology of the Intracellular Matrix, Vol. 2, Balasz, E. A., Ed., New York, N. Y., Academic Press, p 1033.
Pras, M., Nevo, Z., Schubert, M., Rotman. J., and Matalon, R. (1971), *J. Histochem. Cytochem. 19,* 443.
Sanyal, R. K., and West, G. B. (1956), *Nature (London) 178,* 1293.
Scott, J. E. (1960), *Methods Biochem. Anal. 8,* 145.
Stone, A. L. (1967), *Biochim. Biophys. Acta 148,* 193.
Stone, A. L. (1969), *in* Structure and Stability of Biological Macromolecules, Biological Macromolecule Series, Vol. 2, Timasheff, S. M., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 353.
Stone, A. L., and Bradley, D. F. (1967), *Biochim. Biophys. Acta 148,* 172.
Stone, A. L., and Moss, H. (1964), *Biochim. Biophys. Acta 136,* 56.
Sylvén, B. (1954), *Quart. J. Microsc. Sci. 95,* 327.
Terho, T. T., and Hartiala, K. (1971), *Anal. Biochem. 41,* 471.
Uvnäs, B., Åborg, C.-H., and Bergendorff, A. (1970), *Acta Physiol. Scand., Suppl. No. 336,* 1.
Werle, E., and Amann, R. (1956), *Klin. Wochenschr. 34,* 624.