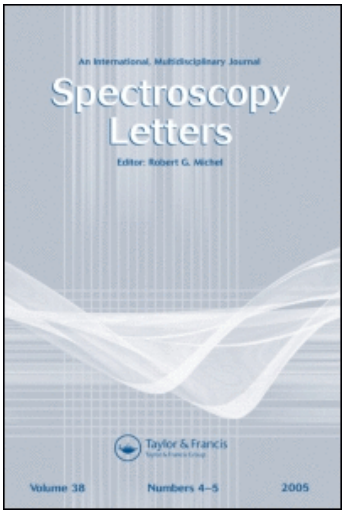

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Mechanism of Methylene Blue Action and Interference in the Heparin Assay

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MECHANISM OF METHYLENE BLUE ACTION AND INTERFERENCE IN THE HEPARIN ASSAY

Key words: Methylene blue, Heparin, Binding assay, Spectrophotometry, Interference

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ABSTRACT

Methylene blue dye binding heparin is not affected by *p*H among 2 and 12. Dye binding requires a macromolecular form with both carboxyl and sulfate-rich groups. The carboxyl groups without sulfate groups give no responses. The binding behavior is attributed to electrostatic attractions and Van der waals forces. Assay interference by base, SDS, and other compounds is explained in terms of the effect upon the equilibria between methylene blue and heparin.

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INTRODUCTION

Heparin is a heterodisperse sulfated glycosaminoglycan. It consists of repeating disaccharide units of duronic/glucuronic acid and glucosamine residues, in which the glucosamine residues are either N-sulfated or N-acetylated (free amino group are rare). In addition, the uronic acid residues are sometimes 2-O-sulfated and the glucosamine residues can carry 6-O- and 3-O-sulfate groups[1,2]. Owing to the presence of the sulfate and carboxyl groups, heparin has a high anionic charge density. Based on this ability, heparin is known to interact with a large number of cationic dye[3-5].

Methylene blue (MB)[6,7] has been used as a staining reagent in the determination of heparin. The method is based on the different absorption spectra of the dye and dye–heparin complexes. The heparin determination method of dye–heparin assay is popular because it is rapid, sensitively, and relatively inexpensive, but little effort has been made to investigate the mechanism of MB dye binding, or the identities of the functional groups involved. Powell et al.[5] suggested that the mechanism was thought to be a conformation change caused by an interaction between the positive charge of dye and the negative charge of heparin (dye binding was due to electrostatic attractions of the heparin sulfate and carboxyl groups with dye group). We test this model by examining the spectroscopy and chemistry of the dye and assay responses to various reagents. Our objectives are to establish the requirements for, and factors influencing binding of MB.

EXPERIMENTAL

Apparatus

A Shimadzu UV-240 spectrophotometer (Shimadzu, Tokyo, Japan) is used for recording absorption spectra. A Model 721 spectrophotometer

from Shanghai, China is used for measuring the absorbance at a given wavelength, using a 1-cm path length. A *p*H–HJ90B model portable acidity meter (Beijing hangtian computer company, China) is used for the *p*H measurements.

Reagents

Heparin, sodium salt, ≥ 160 IU/mg, is obtained from Shanghai, China and used without further purification. All calculations reported by heparin are in terms of a molecular weight of 12,000 Da[8]. The aqueous heparin solution (5.21×10^{-5} mol/L) is prepared by dissolving 0.05 g heparin reagent in 80 mL deionized water. This stock solution of heparin is pipetted 2 mL into 100 mL volumetric flask, and then diluted to the mark with water. This stock solution is stable for several weeks when kept in the dark at 4 °C.

The MB (Structure of MB is shown in Fig. 1.) is purchased from Shanghai, China. The MB stock solution (2.67×10^{-3} mol/L) is prepared by dissolving 0.5 g dye in 500 mL deionized water. The operating solution of MB is prepared by diluting 5 mL stock solution with water into 30 mL.

All other reagents are of analytical or guaranteed reagent grade.

Procedure

Spectra and absorbance measurement are taken after 2 hrs and before 2.5 hrs to eliminate temporal variation in dye response. The effect of *p*H on the MB and assay is examined at various *p*H values obtained by adding appropriate quantities of H_2SO_4 or NaOH. The mixtures are diluted to a certain volume with water and mixed either by inversion or vortexing. All runs are thermostated at 20 °C, and are performed in triplicate.

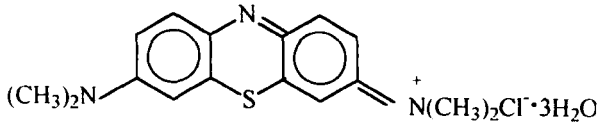

Fig. 1. Structure of methylene blue (MB) dye.

RESULTS AND DISCUSSION

According to the model of Powell et al.[5], carboxyl groups will also bind cationic dye. We find, however, that treatment of protein in conditions negative modification of carboxyl groups residues[9] has no significant effect upon dye response (treated with NaOH, *p*H 8.5, 1 hr, 20 °C). We therefore investigate factors influencing dye binding with the aim of identifying the responsible sulfate groups or sulfate with carboxyl groups.

*p*H and Dye Spectra

Figure 2 show that MB dye exists four species. However, a series of dye solutions varying in *p*H (1.7 ~ 12) shows no isobestic points in the spectral region studied, 400 ~ 800 nm. Had only two dye forms been present, two or more isobestic points would have been seen[10]. We therefore set out to determine the number and identity of MB dye species by measuring visible spectra as we titrate the dye reagent in the absence of heparin. We reason that modifications of the conjugated system would change the dye absorbance maximum.

The dye absorbance spectrum shows maxima at 663 nm and 614 nm corresponding to monomer and dimer of the dye, respectively[6]. Our data are in agreement with previous studies of a similar cationic dye, Toluidine

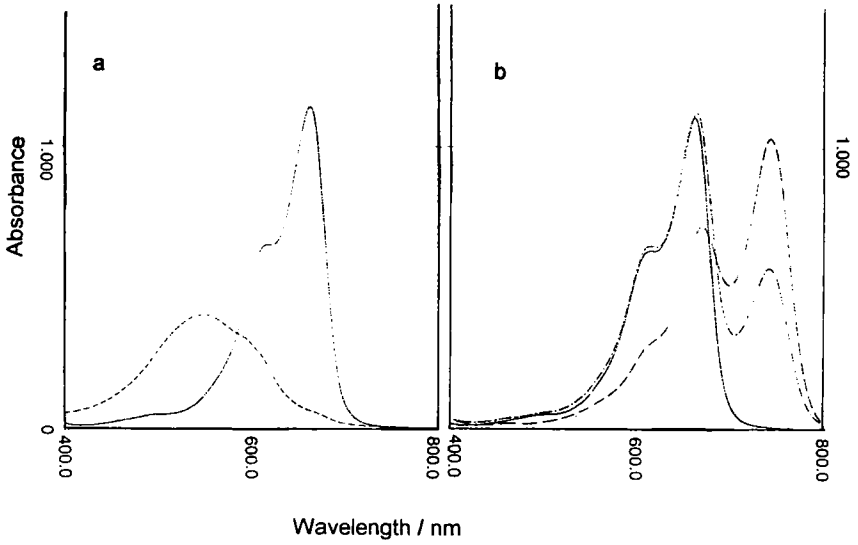

Fig. 2. Dye spectra. (—) Dye reagent MB treated with: (a) (---)NaOH, *p*H 13; (b) (-·-) H_2SO_4, *p*H 0; (–-) H_2SO_4, more than *p*H 0.

blue[11]. Stepwise additions of sodium hydroxide decreased absorbance at 663 nm and 614 nm, then replaced the 663 nm and 614 nm peak with a new peak at 570 nm and then shift to 545 nm (see Fig. 2a). The dye structure requires that the 570 nm absorbing species be unprotonated dye species. Addition of concentrated sulfuric acid to the reagent led to replacement of the peak at 663 nm and 614 nm with maximum at 743 nm. The red shift and hyperchromic effect observed in Fig. 2b can be explained by the conjugation theory[12].

Dye–Heparin Spectra

We next wish to establish which of the four dye species binds with heparin. Addition of heparin to the dye reagent results in a marked

decrease of the 663 nm and 614 nm absorption, a new shoulder peak at 570 nm (see Fig. 3.). Subtraction of the dye blank results in a single absorption maximum at 570 nm (whose magnitude is a function of the heparin concentration) as well as negative minima at 663 and 614 nm, while decreasing in absorbance at 663 nm is also in proportion to heparin concentration. (the assay at 663 nm is superior due to the considerations of linearity and sensitivity.)

Heparin has three O-sulfate group, two N-sulfate group, and two carboxyl groups per tetrasaccharide unit. The O-sulfate and N-sulfate groups are completely dissociated, even below *p*H 3.0. In this region, therefore, MB binds only to the O-sulfate and N-sulfate groups. But the carboxyl group is weakly acidic, and the *p*Ka of D-glucuronic acid in heparin is 3.6. On increasing the *p*H above 3.0, the carboxyl groups gradually dissociate and combine with MB, and reaching a constant level above *p*H 5.0, where dissociation of the carboxyl groups is complete. Accordingly, the carboxyl group dissociates at high *p*H values, so heparin is subjected to bind to dye at various *p*H values[13].

Yutaka et al.[11] reported that metachromasia was the property shown by certain pure dyestuffs in coloring certain tissue elements in a different color, usually of a shorter wavelength absorption maximum, than most other tissue. Although the exact mechanism has not yet been elucidated, the following explanation is generally accepted: For the first stage of metachromasia, a certain minimum surface density of negative charge is a primary requirement. The positively charged dye molecules are then attracted by the negative charge, and come to close enough to aggregate. Under such conditions, π-electrons (including chromophore and auxochrome) of the dye interact with each other, so that a hypochromism and then a hypsochromism occurs. When the metachromatic reaction is

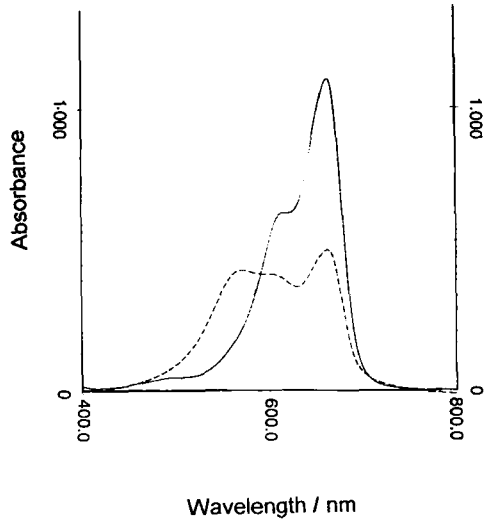

Fig. 3. Dye–heparin spectra. (—) MB; (---) MB–heparin.

saturated, above 100 molecules of MB are attracted around 1 molecule of heparin.

Functional and Structural Requirements of Heparin for Dye Binding

Although the assay's metachromasia has been demonstrated, no studies have been made of the binding requirements of MB. To identify the heparin functional groups responsible for dye binding, we test the response of the MB assay to a variety of reagents. The greatest response is given by ammonium hypersulfate ($(NH_4)_2S_2O_8$), followed by sodium dodecyl sulfate (SDS) (see Fig. 4.). When SDS is added to heparin–dye complex, the inhibition on dye becomes slightly larger than that of heparin alone but is less than the expected extent. This indicates that SDS and heparin interact

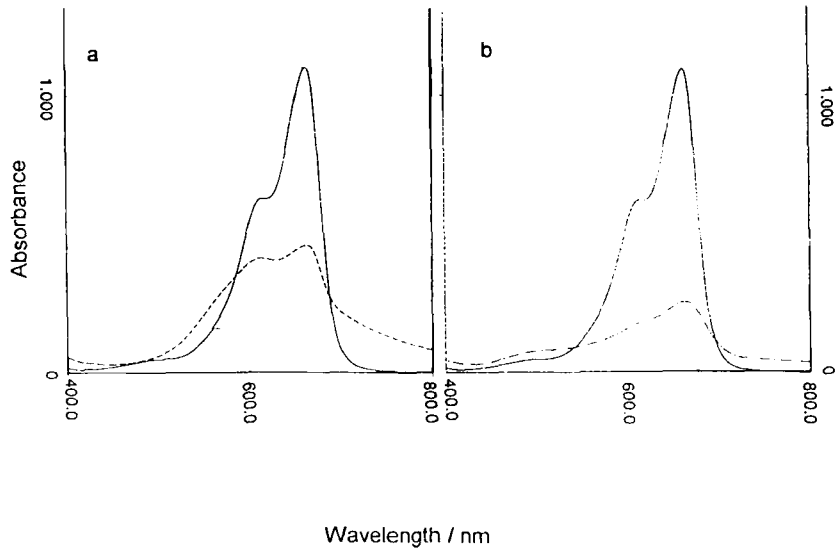

Fig. 4. Dye spectra. (—) Dye reagent MB treated with: (a) (---) SDS, 0.05%; (b) (---) Ammonium hypersulfate $(NH_4)_2S_2O_8$, 0.1N.

with the same site on the dye and compete against each other. We also assay various inorganic salts (see Tab.1.). None of these compounds gives a significant color response. The dye activate anions by forming '1 : 1 ' binding complex, dye molecular can not come to close enough to aggregate, So metachromasia could not appear. Furthermore, it is demonstrated by dye lack of response to a wide range of inorganic anions that nonelectrostatic interactions must play a role in MB dye binding.

We conclude that a compound must have both a macromolecular form and a sulfate-rich group in order to bind with more cationic dye MB. Our data also suggest that the MB dye will be most responsive to sulfate-rich or sulfate with carboxyl-rich macromolecular compounds.

Table 1. Effect of Various Reagents on MB–heparin Complex and MB dye.[a]

Substance	Absorbance at 663 nm	
	Blank(MB)	heparin-MB Complex
Distilled water	1.20	0.48
Na_2SO_4	1.20	1.20
$(NH_4)_2SO_4$	1.20	1.20
Na_2SO_3	1.20	1.15
NaAC	1.20	1.00
SBBT	1.20	1.20
PBS	1.20	1.20
NaCl	1.20	0.95
$(NH_4)_2S_2O_8$	0.37	0.32
SDS	0.47	0.48
P-TA	1.20	0.88

a: MB concentration is: $3.72X10^{-5}$ mol/L, heparin concentration is: $2.60X10^{-7}$ mol/L, 1. Distilled water; 2. 0.2M Na_2SO_4 ; 3. 0.2M $(NH_4)_2SO_4$; 4. 0.2M Na_2SO_3 ; 5. 0.2M sodium acetate (NaAc); 6. 0.2M sodium barbital (SBBT); 7. 0.2M PBS; 8. 0.2M NaCl; 9. 0.1M $(NH_4)_2S_2O_8$; 10. 0.05% sodium dodecyl sulfate (SDS); 11. 0.1% P-Toluenesulfonic acid (P-TA) (The above values are obtained when 1mL of each substance is assayed in the standard assay)

Binding Forces

The binding properties of MB may best be explained by the binding energies involved. While the positively charged dye may well experience electrostatic interactions with negatively charged functional groups of heparin[14], however, this mechanism is not convincing in this study, because the difference response between heparin and inorganic anions demonstrates the importance of nonelectrostatic interactions.

However, the binding forces of heparin to the dye seem to be weak because the metachromatic reaction of heparin is easy diminished by the addition of the salt or heating.

Interferences

Discrepancies may arise from nonheparin interferences that produce heparin overestimation, underestimation, and a reduction of the linear response range (see Table 1.). These sources of interference are best explained from the knowledge of the cationic dye species involved. In the absence of interfering components, the heparin assay measures the amount of dye (663 nm) present as compared to a dye reagent–solvent blank. The response must be due to dye bound to heparin. The blank–subtracted 663 nm absorption decreasing is proportional to the concentration of bound dye.

Overestimation of heparin will occur when a nonheparin produces a 663 nm absorption decreasing. This interference is seen in the case of ammonium hypersulfate ($(NH_4)_2S_2O_8$), sodium dodecyl sulfate (SDS), and strongly alkaline samples. The response of the heparin assay to basic samples permits its use in the study of basic strength. Although the heparin assay has been used in the presence of basic heparin buffers, prior removing strongly alkaline samples may improve sensitivity and linearity. SDS gives the same color reaction as heparin, possibly through a direct binding process. Dye–ammonium hypersulfate mixture giving a response has absorbance maxima at 663 nm, with no peaks and shoulders observed at 570 nm.

Underestimation of heparin may result from factors reducing dye binding. For controlling the acidity and the ion strength, buffer and salt must

be added to the solution. The concentrations of the anions are usually 100 ~ 1,000 higher than that of the dye species, so this decreases assay response to heparin, and most likely due to competition with the dye for heparin. The net result of these opposing effects is difficult to predict. Competitive effects leading to heparin underestimation have been demonstrated with inorganic anions. Competition effects of known added reagents may generally be compensated through their inclusion in the standard calibration.

While the potential interference problems of over- and underestimation due to additives may generally be avoided using proper control, an important side effect is a reduction in the assay's linear response range. Assay response to heparin is due to the equilibrium shift away from the undetected anion. Since the linear assay response must therefore be due to the net conversion of cationic dye to polyanion heparin, the concentration of dye in the mixture ultimately limits linear range.

While many workers consider the choice of dye–heparin assay method due to its sensitivity, speed, and inexpensive, its limitations must also be considered. Reagents affecting the dye equilibria can cause interference. We hope that this more mechanistic understanding of the dye reagent system will allow anticipation of other possible interference and promote a more effective use of the assay.

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