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COMMUNICATIONS

Simple metachromatic assay methods for heparin and protamine

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Over many years methods have been described for estimating heparin and other acidic mucopolysaccharides by their metachromatic reaction with basic dyes such as toluidine blue (Walton & Ricketts, 1954) and azure A (Jaques & Wollin, 1967). The heparin-dye complexes are however very sparingly soluble; this is an advantage when the dyes are used as stains in histology and electrophoresis, but has led to considerable complications if quantitative estimations in solution are required (Walton & Ricketts, 1954). For some years we have used a simple procedure which ignores rather than circumvents the supposed hazard of precipitation, but which nevertheless is reproducible enough to have become our standard 'chemical' means of estimating heparin concentrations (not its anticoagulant activity) whenever it is applicable. It is of course not specific for heparin and may be used for estimating chondroitin, heparan and dermatan sulphate with appropriate calibration. It requires solutions of low ionic strength free of materials which can bind competitively with heparin or with dye.

It was thought that a similar method might be developed for protamine, a polycation, using in this case an acidic dye. Trials with various dyes encountered severe precipitation problems, but eventually *p*-benzylanilinoazobenzenesulphonic acid (BAABSA) was found to give satisfactory results.

Heparin was kindly donated by Paines and Byrne Ltd. Shark chondroitin sulphate and protamine (salmine and clupeine sulphates) were obtained from

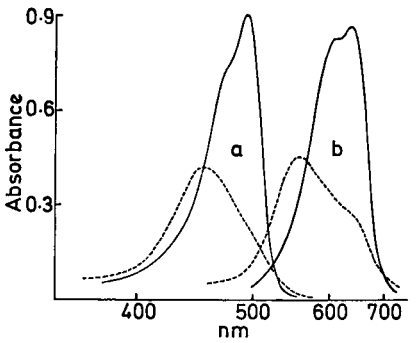

FIG. 1. Absorbance of (a) acridine orange, 0·001% w/v, pH 4, (b) brilliant cresyl blue, 0·0016% w/v, pH 4. ——— without heparin - - - - with small excess of heparin. Concentrations are approximate.

* Correspondence.

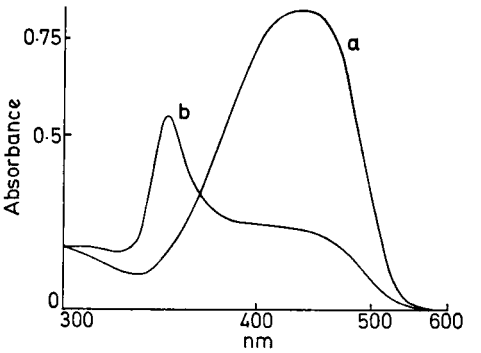

FIG. 2. Absorbance of *p*-benzylanilinoazobenzenesulphonic acid, 0·0013% w/v, pH 8. (a) without protamine, (b) with small excess of protamine. The concentration is approximate.

Koch-Light Laboratories Ltd. Acridine orange (AO) was obtained from BDH Ltd, and brilliant cresyl blue (BCB) from the Aldrich Chemical Company Inc. Benzylanilinoazobenzenesulphonic acid (BAABSA) was initially a very old sample from BDH Ltd. A further batch was prepared by standard condensation of *N*-benzylaniline with diazotized sulphanilic acid, recrystallized until the absorption spectrum (Fig. 2) was constant. It is currently listed by Pfaltz and Bauer Inc.

It can be seen from Figs 1 and 2 that the greatest spectral change upon interaction of polyion with dye occurs in each case at the dye maximum or towards the long wavelength side of it, and these regions were chosen for the assay method; increase in polyion

Table 1. *Solution data and measuring wavelengths for the three dyes.*

Dye	Concn. (% w/v)	Solvent	λ (nm)	Abs. dye : H_2O 1 : 1 v/v
AO	0·002	0·01 M phthalate pH 4	492	0·87
BCB	0·0035	"	640	0·93
BAABSA	0·0035	0·01 M tris, pH 8	440	1·08

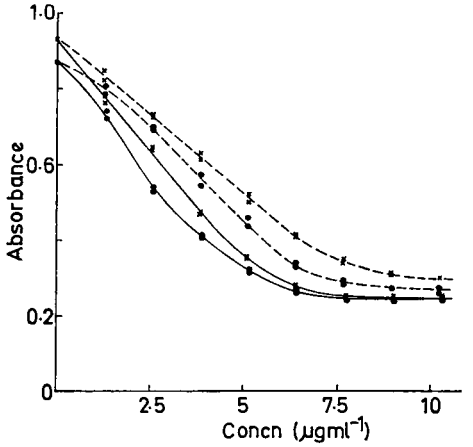

FIG. 3. Absorbance changes of: ● acridine orange at 492 nm, × brilliant cresyl blue at 640 nm with ——— heparin, – – – – – chrondroitin sulphate.

concentration is therefore indicated by decrease in absorbance. The general procedure was in every case the same: one volume of dye solution was mixed with one volume of heparin or protamine and the absorbance measured immediately in 1 cm path length cuvettes. It was necessary to rinse the cuvettes with 2% NaCl solution (followed by distilled water) between readings to remove adsorbed and precipitated dye.

Solution data and measuring wavelengths for the three dyes are given in Table 1.

Care must be taken to ensure complete solution of the dyes; solutions may be kept for 1 week at room temperature. Fig. 3 gives calibration curves for heparin and chondroitin sulphate using AO and BCB. Fig. 4 gives a protamine calibration curve using BAABSA. It may be seen that heparin, chondroitin and protamine are conveniently estimated at concentrations up to 5, 6 and 12 μg ml^{-1} respectively in the final solution (i.e. at double those concentrations

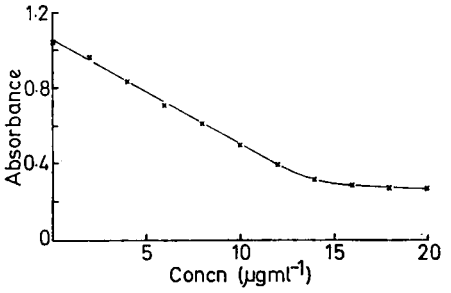

FIG. 4. Absorbance changes of *p*-benzylanilinoazobenzenesulphonic acid at 440 nm with added protamine.

before addition of dye). The working parts of the curves are more nearly linear with BCB and BAABSA than with AO.

Other sulphated mucopolysaccharides, e.g. those isolated from urines, may be estimated using AO or BCB. The method cannot be used in solutions of ionic strength much above 0·05, and appropriate calibration curves should be made above about 0·01. With BAABSA, polylysine was the only material found which gave spectral changes indistinguishable from those with protamine, but other highly basic proteins such as histones and platelet factor 4 may behave similarly. Polybrene gave a much less pronounced effect. Serum proteins interfered with protamine estimation in concentrations above about 10 μg ml^{-1}, and, as with the heparin estimation, the ionic strength had to be very low.

Such restrictions make these methods difficult or impossible to apply to many biological and biochemical systems, but for purposes like monitoring eluates from columns, or determining concentrations in vials for pharmaceutical use, they have in our hands proved much more convenient and often more precise than, for example, the carbazole or the Sakaguchi methods.

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