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THIN-LAYER GEL CHROMATOGRAPHY OF DYED PROTEINS

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SUMMARY

The thin-layer gel chromatographic behaviour of a number of proteins dyed with Procion Brilliant Orange has been studied. Spectrophotometric studies show that the number of dye molecules bound to each protein molecule is normally small. The chromatographic behaviour of the proteins is thus hardly affected by dyeing. The dyeing procedure is simple and the technique may have several advantages over conventional methods in which proteins are stained after chromatography.

INTRODUCTION

Thin-layer gel chromatography (TLG) is a simple and convenient technique for the analysis of protein mixtures, which permits the simultaneous comparison of several samples, and the use of very small sample volumes[1]. It is frequently employed in the estimation of molecular weights[2], and recent work has shown that such experiments are feasible in the presence of denaturing agents such as urea[3] and guanidine hydrochloride[4,5] as well as in conventional buffers.

TLG separations of proteins are normally monitored by taking a paper "print" of the gel layer at the conclusion of the chromatography and locating the protein zones using an appropriate staining technique[6]. Recent work has shown, however, that pre-staining methods may be of value in chromatographic and other separations. Remazol Brilliant Blue R (ref. 7) and related dyes[8] have been used to stain proteins before polyacrylamide gel electrophoresis. Many polysaccharides can be dyed using the Procion series of triazine dyes, and the coloured products can be analysed by cellulose acetate[9,10] or polyacrylamide gel[11] electrophoresis, and column[12] or thin-layer[13] gel chromatography. Procion dyes also form covalent bonds with proteins[14], and the present paper shows that the procedure is appropriate for staining proteins before their separations by TLG.

MATERIALS AND METHODS

Sephadex G-25 (coarse grade), G-100 (superfine) and G-200 (superfine) were obtained from Pharmacia (London, Great Britain), and Procion Brilliant Orange

2RS (molecular weight 781) was purchased from Dylon International (London, Great Britain). Before use the dye was desalted using an Amicon (High Wycombe, Great Britain) Model 52 Ultrafiltration cell and a UM05 filter, and dried in a rotary evaporator. The following proteins were used without further purification: hen egg-white lysozyme and yeast alcohol dehydrogenase obtained from Boehringer (London, Great Britain), cytochrome *c*, β-lactoglobulin, carbonic anhydrase, and bovine hemoglobin from BDH (Poole, Great Britain) bovine trypsin and *B. subtilis* α-amylase from Koch-Light Labs. (Colnbrook, Great Britain), bovine trypsinogen and ovalbumin from Sigma (London, Great Britain), human γ-globulin and apoferritin from Miles Research Labs. (Stoke Pages, Breat Gritain), and iron-free human transferrin from Hoechst Pharmaceuticals (London, Great Britain). Buffer solutions were made up in glass-distilled water and all reagents were AnalaR or equivalent grade.

Proteins were dissolved at concentrations of 5–10 mg/ml in pH 7.6 phosphate buffer containing 0.85% sodium chloride and mixed with equal volumes of a solution of Procion Brilliant Orange (10 mg/ml) in the same buffer. The dyeing reaction was normally allowed to proceed overnight, although it was substantially completed within 2 h. In experiments in which dye-binding ratios were determined, the protein–dye conjugates were freed from excess dye by passing the reaction mixtures through short columns of Sephadex G-25. Some of the free dye became irreversibly bound to the Sephadex during this step and each column was used once only. In other experiments the column gel filtration step was omitted and the dye–protein mixture was applied directly to the TLG plate.

Dye-binding ratios were estimated by determining the absorbance of the protein–dye conjugate at 280 nm, where both protein and dye moieties absorb light, and at 494 nm, when only the dye has a significant absorbance. An SP-600 UV spectrophotometer (Pye-Unicam, Cambridge, Great Britain) was used in all spectroscopic measurements.

TLG was performed on 1-mm thick layers of Sephadex gels using 10 × 20-cm glass plates. After overnight pre-equilibration of the gels, 5-μl samples were applied using a micropipette. Orange dextran (molecular weight, 2×10^6) was used as a void-volume marker[15]. At the end of the chromatography (about 3 h) a paper print of the gel layer was made using a sheet of Whatman No. 1 paper, which was subsequently dried in warm air. In experiments where undyed proteins were studied, the paper prints were stained using bromophenol blue. The prints were examined in a Chromoscan densitometer (Joyce-Loebl, Gateshead, Great Britain) using the reflectance mode, a 0.5-mm wide entrance slit and no filter.

RESULTS AND DISCUSSION

Procion Brilliant Orange 2RS was found to have extinction coefficients of 846.1 and 615.1 at 494 nm and 280 nm, respectively. The former value is much higher than that cited in earlier work[13], probably because commercial samples of the dye contain considerable quantities of inorganic salts, which are removed by ultrafiltration. Removal of these salts is not, however, essential before preparing protein–dye conjugates for chromatography.

Table I shows that the gel-chromatographic properties of several of the dyed proteins do not differ significantly from those of the undyed molecules. In this table

TABLE I

MIGRATION RATES OF DYED AND UNDYED PROTEINS

Results are expressed by comparing the migration distance of the protein zone with that of orange dextran; each result shows the mean value and standard deviation of six replicate experiments.

Protein	*Sephadex G-100*		*Sephadex G-200*	
	Dyed	*Undyed*	*Dyed*	*Undyed*
Human γ-globulin	—	—	0.87 ± 0.02	0.87 ± 0.02
Human serum albumin	0.84 ± 0.01	0.83 ± 0.01	0.65 ± 0.01	0.64 ± 0.01
Ovalbumin	0.72 ± 0.01	0.72 ± 0.01	0.52 ± 0.01	0.52 ± 0.01
β-Lactoglobulin	0.63 ± 0.01	0.63 ± 0.01	0.46 ± 0.02	0.47 ± 0.01
Trypsin	0.46 ± 0.02	0.46 ± 0.02	—	—

the migration rates of the proteins are compared with that of the void-volume marker, orange dextran. The finding that dyeing of the proteins has no significant effect on their apparent molecular weight indicates that, in the given reaction conditions, only a small number of dye molecules are bound to each protein molecule. This is confirmed by the results in Table II, which shows that in none of the proteins studied does the resultant molecular-weight increase exceed 3.5%, a figure within the estimated precision of the method. When a similar technique was employed in the determination of the molecular weights of dextrans[13], dye-binding caused rather greater increases in molecular weight (approx. 12% when the revised value of $E^{1\%}_{1\,cm}$ (494 nm) for the dye is used), but a smooth relationship between chromatographic behaviour and molecular weight persisted, presumably because all the dextran molecules were affected similarly.

TABLE II

BINDING OF PROCION BRILLIANT ORANGE 2RS TO PROTEINS

Protein	*Molecular weight*	*Ref.*	*Moles of dye bound /mole protein**	*Increase in molecular weight (%)*
Human γ-globulin	150,000	16	2.82 (3)	1.6
Human serum albumin	65,000	17	2.85 (3)	3.5
α-Amylase	45,000	18	1.87 (2)	3.5
Ovalbumin	44,000	19	0.36 (<1)	<1.8
β-Lactoglobulin	35,000	20	1.10 (1)	2.2
Trypsin	23,500	21	0.96 (1)	3.3

* Figures in parentheses indicate numbers of moles of dye bound, rounded off to the nearest integer.

In all the TLG experiments the dyed proteins migrated as compact, easily visible zones. When no attempt was made to remove excess Procion Brilliant Orange from the reaction mixture, the free dye migrated very slowly as an intensely coloured spot. This free dye zone was used as an internal standard in expressing the migration rates of the dyed proteins. When R_D, the migration distance of the dyed protein divided by that of the free dye, was plotted against log (molecular weight) the curves shown in Figs. 1 and 2 were obtained from experiments on Sephadex G-100 and G-200, respectively. The figures indicate that the method is suitable for the estimation

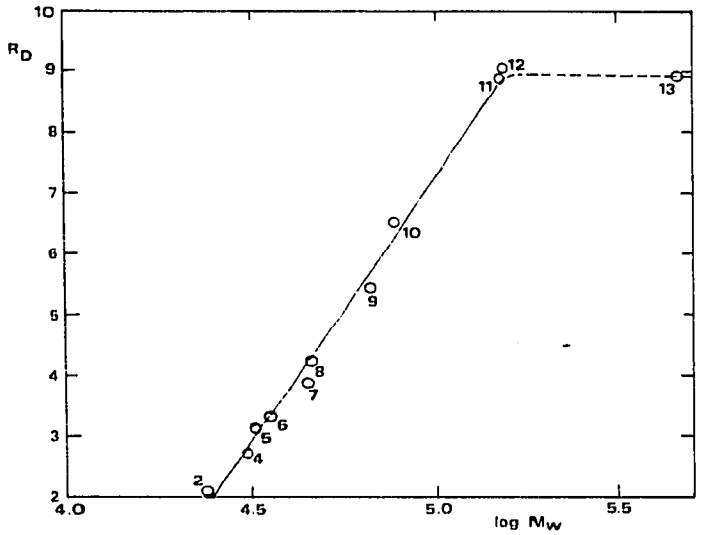

Fig. 1. Thin-layer gel chromatographic behaviour of dyed proteins on Sephadex G-100 superfine. R_D is plotted against the log (molecular weight) of the native protein. 1, Cytochrome *c*; 2, trypsin; 3, trypsinogen; 4, carbonic anhydrase; 5, hemoglobin; 6, β-lactoglobulin; 7, ovalbumin; 8, α-amylase; 9, human serum albumin; 10, transferrin; 11, γ-globulin; 12, alcohol dehydrogenase; 13, apoferritin. Hemoglobin behaves as though its molecular weight is 32,000 (ref. 22).

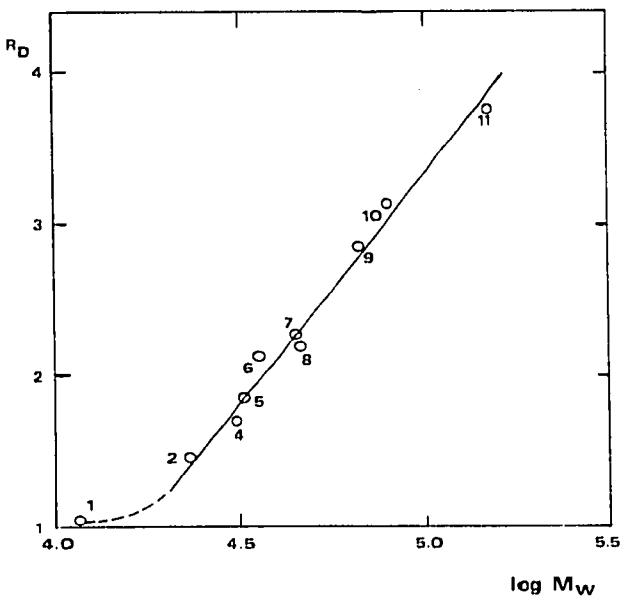

Fig. 2. Thin-layer gel chromatographic behaviour of dyed proteins on Sephadex G-200 superfine. For identification of proteins, see legend of Fig. 1.

of protein molecular weights, at least in the range 20,000–150,000. Several three- and four-component mixtures of proteins have also been investigated; in each case the proteins were found to migrate at the same rate as when studied individually.

Staining proteins by a simple procedure before their analysis by TLG has a number of advantages. With coloured solutes, the quality of the sample application process is easily checked and the progress of the chromatography can be followed throughout. Experiments may thus be halted as soon as satisfactory separations are observed, with no danger of solutes migrating too far. Preliminary studies suggest that, after dye-binding, chromatography in denaturing solvents such as 5 *M* guanidine hydrochloride is also feasible. When unstained proteins are investigated in this solvent, the presence of guanidine hydrochloride frequently makes it difficult to precipitate the protein zones on the paper print[5]; the use of pre-stained proteins removes this difficulty. Dilute solutions of proteins (< 1 mg/ml) can be readily visualised on the TLG plates and paper prints, and extra sensitivity might be gained by utilising the fluorescence properties of the dye[22]. The method may be unsuitable for the study of certain proteins. Instances of proteins behaving abnormally during gel-filtration experiments are well known[23], and in the present work lysozyme was found to precipitate from solution when the dye was added to it. In general, however, it seems likely that the method will be widely applicable.

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