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AFFINITY CHROMATOGRAPHY ON IMMOBILIZED TRIAZINE DYES

POST-IMMOBILIZATION CHEMICAL MODIFICATION OF TRIAZINE DYES

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SUMMARY

Sepharose-immobilized Procion dyes were subjected to simple chemical modifications in an attempt to assess the significance of certain parts of the dye structure in the dye-enzyme interaction, and whether these modifications would alter the chromatographic properties of the adsorbents towards several model enzymes such as pig heart lactate dehydrogenase, yeast glucose-6-phosphate dehydrogenase and yeast hexokinase. In general terms, cleavage or hydrogenation of the azo-linkages by treatment with sodium dithionite or borohydride, respectively, resulted in weakened enzyme–dye interactions. This is a consequence of the removal of sulphonated polycyclic parts from the dye structure which presumably interact with the enzymes. However, this effect has proved useful in dye chromatography since, for modified dye gels, non-specific proteins appear in the buffer washings in higher recoveries, and the enzymes bound were eluted at lower eluent concentrations and with higher enzyme recoveries than for the native dye-gels.

INTRODUCTION

Reactive triazine dyes are currently becoming established as useful preparative and analytical tools available to the biochemist[1–3]. These sulphonated polyaromatic triazine molecules have proved to be effective in the purification of NAD^+-dependent dehydrogenases, kinases, glycolytic enzymes, blood proteins and a number of other enzymes and proteins[1–5].

The ability of triazine dyes to bind to a wide variety of enzymes could not at first be explained. Inevitably, in addition to the purely empirical data derived from actual purifications, many direct studies of the interaction of various proteins with the dye ligands have been made. Electrophoretic[6,7], enzyme inhibition[4,8–16], absorption spectral difference[17–20], induced circular dichroism[21,22], X-ray crystallographic[23], affinity labelling[24–26] and chromatographic[4,27] techniques have been employed. In particular, in an attempt to define the structural requirements of the enzymes for the

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binding of dyes, a variety of cellular enzymes have been tested for their ability to bind a number of Cibacron Blue F3G-A analogues[10,14,28]. However, to my knowledge, no detailed studies have been reported on the use of chemically modified immobilized triazine dyes as a means of investigating the mode of binding to enzymes or the use of such modified dyes in enzyme purification. It was briefly reported[29] that reduction of immobilized Procion Red HE-3B with 0.2 *M* sodium dithionite in alkaline solution is accompanied by marked spectral changes in the dye and an alteration in chromatographic properties towards yeast glucose-6-phosphate dehydrogenase and *Escherichia coli* IMP dehydrogenase.

On the basis of that report I proposed a series of experiments to yield information on (i) the functional groups or parts of the dye structure which appear to be important in the binding of the enzyme, and (ii) how to improve the chromatographic behaviour of immobilized dyes in terms of increased enzyme recovery under mild elution conditions, and of increased specific activity of the eluted enzyme.

In the present work I have employed three well-documented enzymes: an NAD^+-dependent oxidoreductase, pig heart lactate dehydrogenase; an $NADP^+$-dependent dehydrogenase, yeast glucose-6-phosphate dehydrogenase and a typical ATP-dependent enzyme, yeast hexokinase. The Sepharose-immobilized native and chemically modified triazine dyes tested were: Procion Blue H-B, Blue HE-RD, Green HE-4BD, Green H-4G, Yellow H-5G and Red HE-3B.

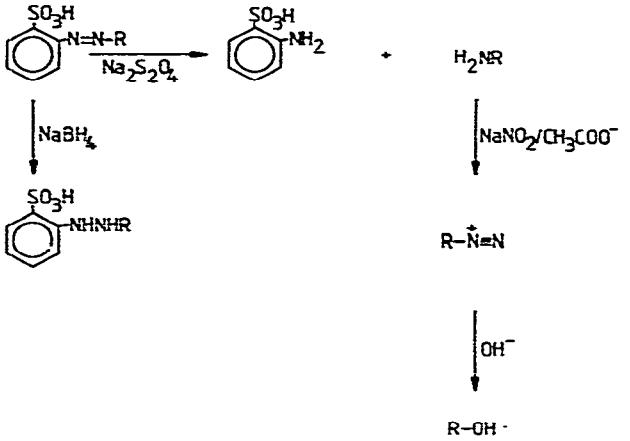

Fig. 1. Anticipated effects of the post-immobilization chemical modification of triazine dyes. R represents the remaining structure of a Procion dye immobilized to Sepharose.

The post-immobilization chemical modification of the triazine dyes involved (i) treatment of the immobilized native dye with sodium dithionite, (ii) treatment of the dithionite-reduced dye with sodium nitrite in acetic acid followed by sodium hydroxide and (iii) treatment of the native dye with sodium or lithium borohydride. Fig. 1 illustrates the anticipated effect of these facile chemical manipulations on the structure of a typical Procion dye.

The chromatographic behaviour of the chemically modified and native immobilized triazine dyes was studied in terms of their ability to bind the three enzymes and bovine serum albumin.

EXPERIMENTAL

Materials

Pig heart lactate dehydrogenase (220 units per mg) was obtained from Boehringer (Lewes, Great Britain), whilst yeast glucose-6-phosphate dehydrogenase (315 units per mg) and yeast hexokinase (310 units per mg) were from Sigma (London, Great Britain). ATP disodium salt and NADH were from Boehringer, $NADP^+$ and glucose-6-phosphate from Sigma and sodium pyruvate, bovine serum albumin (BSA, fraction V) and all other chemicals were from BDH (Poole, Great Britain).

The triazine dyes were a gift from I.C.I. Organic Division (Blackley, Manchester, Great Britain). Dyes are referred to in this paper by their commercial names (I.C.I.). Procion Blue H-B (I.C.I.) is chemically identical to Cibacron Blue F3G-A (Ciba-Geigy).

Sepharose 4B was purchased from Pharmacia (G.B.) (Hounslow, Great Britain).

Enzyme assays

Enzyme assays were performed at 25°C and 340 nm, unless stated otherwise. The reaction mixture contained the following in a total assay volume of 1 ml:

(i) Pig heart lactate dehydrogenase (LDH): potassium phosphate buffer, 50 μmol, pH 7.0; sodium pyruvate, 0.73 μmol; NADH, 0.2 μmol and LDH, 0–0.05 units. One unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 μmol NADH per min at 25°C.

(ii) Yeast glucose-6-phosphate dehydrogenase (G6PDH): Tris–HCl buffer, 30 μmol, pH 7.5; glucose-6-phosphate, 2.0 μmol; $NADP^+$, 0.3 μmol; $MgCl_2$, 6 μmol and G6PDH, 0–0.05 units. One unite of enzyme activity was defined as the amount of enzyme required to produce 1 μmol NADPH per min at 25°C.

(iii) Yeast hexokinase: Tris–HCl buffer, 0.1 mmol, pH 7.5; D-glucose, 5 μmol; ATP, 3 μmol; $NADP^+$, 0.7 μmol; $MgCl_2$, 10 μmol; yeast glucose-6-phosphate dehydrogenase, 3 units and yeast hexokinase, 0–0.03 units. One unit of enzyme activity was defined as in (ii).

All enzyme assays were initiated by adding the enzyme to be assayed and the following molar extinction coefficients (ε_m, $1 \cdot mol^{-1} \cdot cm^{-1}$) were used[30]: NADH, 6220; $NADP^+$, 18,000 and ATP, 15,400.

Chromatographic procedures

All chromatographic procedures were performed on an analytical scale (0.5 g moist weight gel) and at 25°C. Sepharose-immobilized triazine dyes were prepared as described before[4] with the following dye concentrations (μmol dye per g moist weight gel): Procion Blue H-B, 0.8; Blue HE-RD, 1.7; Green HE-4BD, 2.5; Green H-4G, 2.6; Yellow H-5G, 2.7; Red HE-3B, 1.0.

Pig heart lactate dehydrogenase. A sample containing pig heart lactate dehydrogenase, 150 units, and bovine serum albumin, 5.0 mg, in a total volume of 0.4 ml was dialysed overnight at 0–4°C against 1 l of 30 m*M* potassium phosphate buffer, pH 7.0. A sample of the dialysed enzyme (50 μl; 8.8 units LDH; 0.43 mg BSA) was applied to a column (3.2 × 0.45 cm) containing Sepharose-bound triazine dye (native or modified; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) equili-

brated with the same phosphate buffer. Non-adsorbed protein was immediately washed off the column with buffer (10–12 ml) and elution was subsequently effected with a linear gradient of NADH (0–20 μM; total volume 20 ml). Fractions (1.5 ml) were collected at a flow-rate of 23 ml/h and assayed for enzyme activity. Bovine serum albumin was determined from its absorbance at 280 nm, and the progress of the gradient determined by absorbance at 340 nm (Fig. 2).

Yeast glucose-6-phosphate dehydrogenase. A sample containing yeast glucose-6-phosphate dehydrogenase, 70 units, and BSA; 5.0 mg, in a total volume of 0.5 ml was dialysed overnight at 0–4°C against 500 ml of 30 mM Tris–HCl buffer, pH 7.5, containing 1.5 mM $MgCl_2$. A sample of the dialysed enzyme (100 μl; 3.6 units G6PDH; 0.82 mg BSA) was applied to a column (3.2 × 0.45 cm) containing Sepharose-bound triazine dye (native or modified; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) equilibrated with the same buffer. Non-adsorbed protein was immediately washed off the column with buffer (10–12 ml) and elution was subsequently effected with a linear gradient of $NADP^+$ (typically 0–0.1 mM or as specified; total volume 20 ml). Fractions (1.5 ml) were collected at a flow-rate of 23 ml/h and assayed for enzyme activity. Bovine serum albumin was determined from its absorbance at 280 nm, and the progress of the gradient determined by absorbance at 260 nm.

Yeast hexokinase. A sample containing yeast hexokinase, 31 units, and BSA, 5.0 mg, in a total volume of 0.5 ml was dialysed overnight at 0–4°C against 500 ml of 30 mM Tris–HCl buffer, pH 7.5, containing 10 mM $MgCl_2$. A sample of the dialysed enzyme (100 μl; 4.2 units hexokinase; 0.66 mg BSA) was applied to a column (3.2 × 0.45 cm) containing Sepharose-bound triazine dye (native or modified; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) equilibrated with the same buffer. Non-adsorbed protein was immediately washed off the column with buffer (10–12 ml) and elution was subsequently effected either by omitting the Mg^{2+} from the irrigating buffer or with a pulse of ATP (20 mM; total volume 5 ml). Fractions (1.4 ml) were collected at a flow-rate of 23 ml/h and assayed for enzyme activity. Bovine serum albumin was determined from its absorbance at 280 nm, and the eluting nucleotide (ATP) determined by absorbance at 260 nm (Fig. 3).

Post-immobilization chemical modification of Sepharose-bound triazine dyes

Cleavage of azo linkages of immobilized triazine dyes (treatment with sodium dithionite). A Sepharose-bound dye column (3.2 × 0.45 cm; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) was washed with distilled water (5 ml) and then a solution (*ca.* 10 ml) of sodium dithionite (29 mM; 5 mg/ml) in 1% sodium carbonate was slowly passed through at 24°C until a constant colour was observed. The column was then washed with at least 10 ml of 1% sodium carbonate, to remove the excess of sodium dithionite, followed by 10 ml of distilled water. The gel was then ready for equilibration in the buffer to be used in the subsequent studies.

Replacement of aryl-amino groups with phenolic hydroxyl groups of immobilized triazine dyes via diazonium formation. A sodium dithionite-reduced dye column was equilibrated with 0.5 M acetic acid and the gel transferred to a glass vial. The vial was placed in an ice-bath and when the temperature of the gel slurry (*ca.* 2 ml; 0.5 M acetic acid) had fallen to 0°C a total of 140 mg (2 mmol) sodium nitrate was added in two equal portions over a period of 20 min under gentle stirring. The gel suspension

was left under gentle stirring at 0°C for another 10 min and then washed with 1 m*M* NaOH (20 ml) at 24°C followed by distilled water (20 ml).

Reduction of the azo linkages of immobilized triazine dyes with sodium or lithium borohydride. A Sepharose-bound bye column (3.2 × 0.45 cm; 0.5 g moist weight gel; 0.8–2.7 μmol dye per g moist weight gel) was washed with distilled water (5 ml) and 1% sodium bicarbonate until the pH of the washings was 8.0. The gel was then transferred to a glass vial. To the gel slurry (*ca.* 2.0 ml in 1% sodium bicarbonate) was introduced, at 24°C, a total of 40 mg (1 mmol) sodium borohydride or lithium borohydride (1.7 mmol) in portions of 10 mg over a period of 1 h, under gentle stirring. The gel was then washed with water (100 ml) on a sintered funnel under mild suction.

RESULTS AND DISCUSSION

Post-immobilization chemical modification of triazine dyes by treatment with sodium dithionite has been briefly reported[29]. The author reports that a marked spectral change accompanied the treatment of immobilized Procion Red HE-3B by sodium dithionite in alkaline solution. Indeed, Table I reveals the changes in colour and λ_{max} that occurred to Sepharose-immobilized and free triazine dyes when treated with several different reagents (see Experimental section) (Fig. 1). Sodium dithionite is known to be a strong reducing agent for azo-linkages[31] capable of cleaving an azo bond to (aryl) amino groups, thereby disrupting or cleaving part(s) of the dye structure (Fig. 1). The colour changes observed were in agreement both with the structure of the dyes[2,32,33] and the known chemical properties of sodium dithionite. For example, since Procion Blue H-B contains no azo bonds one would expect that sodium dithionite should have no permanent effect on this dye, as was indeed observed. An amber shade developed temporarily but was soon re-oxidized in air to the original blue. On the other hand, Procion Blue HE-RD was converted into a greenish gel. In this case sodium dithionite presumably removed an azo-bonded aromatic moiety. Furthermore, Procion Green HE-4BD was reduced to a colourless gel on treatment with sodium dithionite. This suggests a serious disruption of the dye structure and consequent loss of the chromophore. This dye consists of several polysulphonated aromatic moieties linked together via azo bonds. It is obvious that cleavage of the linking azo bonds would decimate the dye and leave only a small part of the original structure immobilized. The effect of sodium dithionite was not as dramatic on immobilized Procion Red HE-3B where only a terminal benzosulphonyl group was removed, resulting in an orange-yellow shade. Procion Green H-4G changed from brilliant green to turquoise on treatment with sodium dithionite. Treatment of sodium dithionite-reduced immobilized triazine dyes with acetic acid and sodium nitrite in order to convert arylamino groups into diazonium groups, and subsequent treatment with alkali, leads to an overall replacement of the arylamino groups with phenolic hydroxyl groups[31]. In terms of colour changes, the above treatment had no effect since a hydroxyl group is unlikely to change significantly the overall chromophoric properties of the triazine dye.

Treatment of immobilized triazine dyes with sodium or lithium borohydride would lead to hydrogenation of the azo bonds (Ar–N = N–Ar) to the corresponding hydrazino analogues (Ar–NHNH–Ar)[31]. For Procion dyes with large and complex

TABLE I

SHADES OF NATIVE AND CHEMICALLY MODIFIED SEPHAROSE-IMMOBILIZED TRIAZINE DYES

The shades referred to are gels equilibrated at neutral pH or in distilled water. The absorption maxima (nm) are of the free dye in distilled water.

*Treatment with**	*Blue H-B*	*Blue HE-RD*	*Green HE-4BD*	*Green H-4G*	*Yellow H-5G*	*Red HE-3B*
(Native)	Blue (620)	Dark blue (620)	Dark green (630)	Brilliant green (675)	Brilliant yellow (410)	Brilliant red (530)
$Na_2S_2O_4$	Amber (490, amber)	Grey-green	Colourless (520, pale pink)	Turquoise	Brilliant yellow (< 340, colourless)	Orange-yellow (420, orange-yellow)
$Na_2S_2O_4/HNO_2/NaOH$	–	Brown-green	Colourless	–	Brilliant yellow	Orange-yellow
$NaBH_4$ or $LiBH_4$	Pale violet	Dark blue	Very dark green	Brilliant green	Brilliant yellow	Orange-yellow

* See Experimental section and Fig. 1.

chromophores such as Blue HE-RD, Green HE-4BD and Green H-4G the reduction of the azo bonds had a moderate effect on the shades (Table I). In contrast, dyes belonging to the azo-class such as Procion Red HE-3B are profoundly affected on reduction with sodium borohydride, confirming that the azo linkages contribute significantly to the chromophoric properties of these dyes. In general, either cleavage at the azo bonds by sodium dithionite or reduction by sodium borohydride would lead to some loss of the chromophoric properties of the immobilized dye because of the elimination of the azo bonds and the removal of (poly) aromatic moieties of the dye structure. Accordingly, we observed (Table I) a hypsochromic shift of the shades. However, Procion Yellow H-5G maintained its normal shade when subjected to these chemical treatments. This dye contains a single azo bond[33] which forms the chromophore along with a sulphonated aromatic and a heterocyclic moiety; therefore, cleavage of this bond should alter the dye's shade. Perhaps the immobilized dye is less susceptible to modification than the free one since the free dye was decolourized on treatment with sodium dithionite and displayed no absorption in the 340-nm region.

Chemical modification of immobilized triazine dyes also alters their chromatographic properties towards a number of enzymes. Table II illustrates the binding properties of pig heart lactate dehydrogenase for immobilized native and chemically modified Procion dyes under identical experimental conditions. It is evident in all cases that chemical treatment of the immobilized Procion dyes with sodium dithionite resulted in less strong binding of the enzyme than with the native dye, as judged by the lower concentrations of eluent required to desorb peak activity of the enzyme. As a consequence of the weaker binding, higher recoveries of enzyme were obtained. Further treatment of sodium dithionite-treated immobilized triazine dyes with HNO_2 followed by NaOH, in order to replace $-NH_2$ groups by –OH groups, resulted in adsorbents exhibiting slightly weaker binding strengths than the sodium dithionite-treated gels (Table II). It seems that the dramatic changes which occurred in the chromatographic behaviour of immobilized triazine dyes after chemical treatment were due to the elimination of (poly)sulphonated (poly)cyclic part(s) from the native molecules rather than to simple group substitutions such as replacement of $-NH_2$ with –OH. Interestingly, chemically modified dye-gels exhibited, in general, higher recovery of bovine serum albumin than the native gels, suggesting a decrease in non-specific interactions for the chemically treated gels. These observations are logical since removal of some structural parts from the native dye molecule containing hydrophobic (aromatic or other cyclic moieties) and ionic (sulphonic acid) species should lead to some weakening of the dye-macromolecule interaction. In this context, it is known that both hydrophobic[17,34] and ionic[35] interactions occur in dye–macromolecule binding. Furthermore, dyes and complementary enzymes interact with a fair degree of specificity[4,25,26] for the nucleotide binding site, thus, disruption of the native dye structure should, in general, lead to weakened dye–enzyme interaction.

Reduction of the azo linkages of immobilized triazine dyes with either sodium or lithium borohydride resulted in gels with slightly higher (Green HE-4BD and Green H-4G) or slightly lower (Blue H-B, Blue HE-RD, Yellow H-5G and Red HE-3B) binding strengths than the native gels for pig heart lactate dehydrogenase (Table II). These observations suggest that the azo linkages are probably not as significant as other structural parts of the dye chromophore in dye–enzyme binding. Similar results were obtained with yeast glucose-6-phosphate dehydrogenase (Table III). Chemical

TABLE II

BINDING STRENGTH OF PIG HEART LACTATE DEHYDROGENASE (LDH) TO NATIVE AND CHEMICALLY MODIFIED IMMOBILIZED TRIAZINE DYES

Columns (0.5 g moist gel) were equilibrated in 30 m*M* potassium phosphate buffer, pH 7.0. A sample (50 µl) of dialysed LDH (8.8 units) and bovine serum albumin (BSA) (0.43 mg) was applied to each column. Non-adsorbed proteins were washed off with buffer and elution of the bound LDH was effected with a linear gradient of NADH (0–20 µ*M*; total volume 20 ml).

*Immobilized dye treated with**	*LDH activity (%) eluted in the buffer washings and bound and subsequently desorbed*											
	Blue H-B		*Blue HE-RD*		*Green HE-4BD*		*Green H-4G*		*Yellow H-5G*		*Red HE-3B*	
	Eluted	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*
(Native)	29	47	0	72	0	71	0	80	0	76	0	68
	(–)	[2.1]**	(24)***	[243]	(73)	[11.4]	(93)	[1.6]	(116)	[5.6]	(70)	[2.7]
$Na_2S_2O_4$	68	9	0	89	0	90	47	47	26	65	46	46
	(–)	[1.4]	(15)	[16.1]	(106)	[1.3]	(116)	[1.0]	(120)	[1.2]	(114)	[1.2]
$Na_2S_2O_4/HNO_2/NaOH$		–	0	77	48	23		–	57	27	21	47
			(29)	[10.2]	(108)	[1.0]			(103)	[0.5]	(80)	[0.9]
$NaBH_4$ or $LiBH_4$	67	17	0	14	0	45	14	51	0	84	70	7
	(105)	[1.4]	(29)	[34.1]	(33)	[15.9]	(109)	[2.7]	(112)	[3.2]	(102)	[1.3]

* See Experimental section and Fig. 1.

** Concentration (µ*M*) of NADH required to elute LDH with maximal activity on a linear gradient of NADH.

*** Bovine serum albumin (%) in buffer washings.

TABLE III

BINDING STRENGTH OF YEAST GLUCOSE-6-PHOSPHATE DEHYDROGENASE TO NATIVE AND CHEMICALLY MODIFIED IMMOBILIZED TRIAZINE DYES

Columns (0.5 g moist gel) were equilibrated in 30 m*M* Tris–HCl buffer, pH 7.5, containing 1.5 m*M* $MgCl_2$. A sample (100 μl) of dialysed enzyme (3.6 units) and bovine serum albumin (0.82 mg) was applied to each column. Non-adsorbed proteins were washed off with buffer and elution of the enzyme was effected with a linear gradient of $NADP^+$.

*Immobilized dye treated with**	*G6PDH (%) eluted in buffer washings and bound and subsequently desorbed*							
	Blue H-B		*Green H-4G*		*Yellow H-5G*		*Red HE-3B*	
	Eluted	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*	*Eluted*	*Bound*
(Native)	0	101	0	0	0	110	0	105
	(101)**	[0.11]***	(93)	[−]	(112)	[0.12]	(68)	[0.60]
$Na_2S_2O_4$	0	104	0	77	0	120	0	35
	(93)	[0.01]	(49)	[0.70]	(118)	[0.05]	(110)	[0.01]
$NaBH_4$ or $LiBH_4$	0	102	0	62	0	107	0	90
	(104)	[0.03]	(109)	[0.90]	(108)	[0.05]	(105)	[0.09]

* See Experimental section and Fig. 1.

** Bovine serum albumin (%) in buffer washings.

*** Concentration (m*M*) of $NADP^+$ required to elute glucose-6-phosphate dehydrogenase with maximal activity on a linear gradient of $NADP^+$.

modification of the immobilized triazine dyes, in all cases, was accompanied with weaker binding of the enzyme than with the native adsorbents.

The different binding properties of the native and chemically modified immobilized triazine dyes may prove to be useful in practical dye chromatography. Fig. 2 illustrates the elution profiles of pig heart lactate dehydrogenase from native and chemically modified immobilized Procion Green HE-4BD under identical experimental conditions. The enzyme was quantitatively adsorbed to the native gel (Fig. 2a), while bovine serum albumin was recovered (73%) in the buffer washings. The enzyme was recovered (71%) with maximum enzyme activity at 11.4 μM on a linear gradient of NADH. The binding of the enzyme to sodium dithionite-treated gel was weaker than to the native gel. Fig. 2b shows that leaking of the enzyme occurs in the early fractions before the application of eluent. Furthermore, higher recoveries were obtained for both BSA (106%) in the buffer washings and for LDH (90%) at 1.3 μM on a linear gradient of NADH, than with the native gel. The latter results (see also Table II) compare favourably to those of native dye gels. Replacement of aryl-amino group(s) with phenolic hydroxyl group(s) resulted in an adsorbent exhibiting very weak binding properties for lactate dehydrogenase. In this case the enzyme appeared with 71% recovery in the early fractions prior to application of the eluent (Fig. 2c). Finally, reduction of the azo linkages of immobilized Procion Green HE-4BD with borohydride yielded a gel with a higher binding strength than the native gel for lactate dehydrogenase. It might be that reduction of the double bonds increases the flexibility of the dye, allowing orientation to a more favourable position for binding with the macromolecule. In this case (Fig. 2d) the enzyme was recovered (45%) with peak activity at 15.9 μM of eluent (NADH).

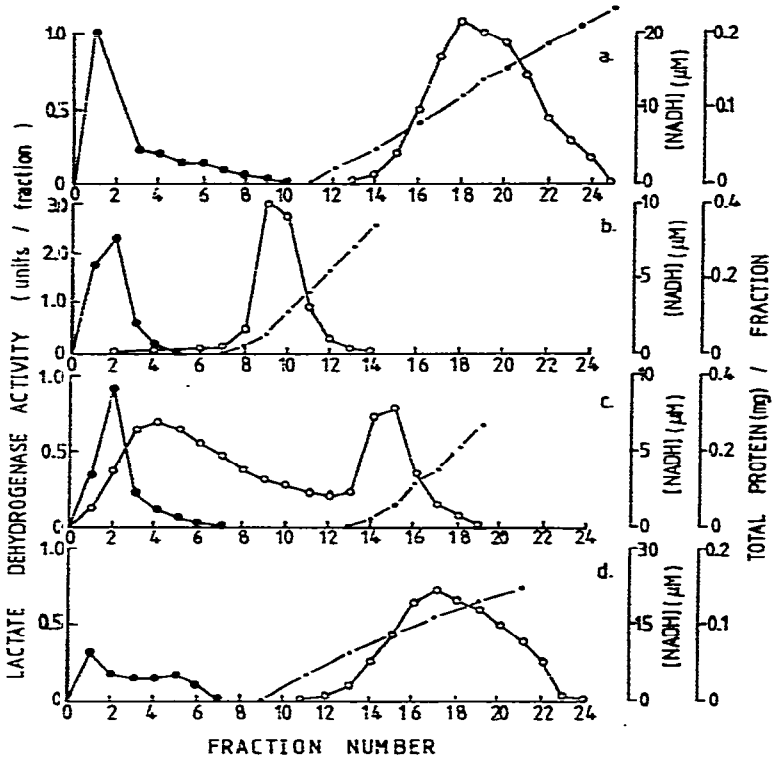

Fig. 2. Elution of pig heart lactate dehydrogenase from Sepharose-bound native and chemically modified Procion Green HE-4BD. A sample (50 μl) containing dialysed enzyme (8.8 units) and bovine serum albumin (0.43 mg) was applied to each column (3.2 × 0.43 cm) of immobilized dye (0.5 g moist gel; 2.5 μmol dye per g moist gel) equilibrated in 30 m*M* potassium phosphate buffer, pH 7.0. Non-adsorbed proteins were immediately washed off the column with buffer (10–12 ml) and elution of lactate dehydrogenase activity was effected with a linear gradient of NADH (0–20 μ*M*; total volume 20 ml). Fractions (1.5 ml) were collected at a flow-rate of 23 ml/h and assayed for lactate dehydrogenase activity (O—O), bovine serum albumin (●—●) and eluent (— · — ·). a, Native or unmodified gel; b, gel treated with sodium dithionite; c, gel treated with sodium dithionite followed by HNO_2 followed by NaOH; d, gel treated with sodium (or lithium) borohydride.

A similar general picture was obtained on chromatography of yeast glucose-6-phosphate dehydrogenase on native and chemically modified immobilized Procion dyes. For example, treatment of native Procion Yellow H-5G with sodium borohydride resulted in gels with significantly lower binding strengths for yeast G6PDH as judged from the concentrations of $NADP^+$ required to elute peak activity of the enzyme (Table III). The inert protein, bovine serum albumin, was (quantitatively) recovered in the void volume which, along with the low concentrations of $NADP^+$ required to elute the enzyme, suggests that chemically treated Procion Yellow H-5G may prove useful in the purification of G6PDH.

Yeast hexokinase was also tested for its ability to bind to immobilized native and chemically modified Procion dyes. At first, this enzyme was not adsorbed onto any of the four different Sepharose-bound triazine dyes tested. However, in the pres-

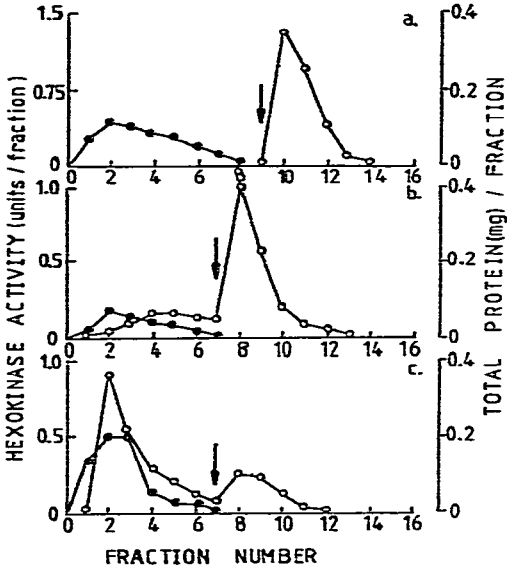

Fig. 3. Elution of yeast hexokinase from Sepharose-bound native and chemically modified Procion Green H-4G. A sample (100 μl) containing dialysed enzyme (4.2 units) and bovine serum albumin (0.66 mg) was applied to each column (3.2 × 0.43 cm) of immobilized dye (0.5 g moist gel; 2.6 μmol dye per g moist gel) equilibrated in 30 m*M* Tris–HCl buffer, pH 7.5, containing 10 m*M* $MgCl_2$. Non-adsorbed proteins were immediately washed off the column with buffer (10–12 ml) and elution of the hexokinase activity effected by omitting the Mg^{2+} from the irrigating buffer (↓). Fractions (1.4 ml) were collected at a flow-rate of 23 ml/h and assayed for hexokinase activity (○) and bovine serum albumin (●). a, Native gel; b, gel treated with sodium dithionite; c, gel treated with sodium (or lithium) borohydride.

ence of 10 m*M* $MgCl_2$ in the irrigating buffer, quantitative binding of yeast hexokinase only onto Procion Green H-4G was achieved, whereas the enzyme passed unretarded through all the other dye-adsorbents, native or chemically modified. These observations are in agreement with previous reports[3,26]. The enzyme bound native Procion Green H-4G–Sepharose could be desorbed in good yield (70%) when Mg^{2+} was removed from the irrigating buffer (Fig. 3a). Chemical treatment of the native immobilized dye has a profound effect on its ability to bind yeast hexokinase. Treatment with sodium dithionite weakens the dye–enzyme interaction (Fig. 3b) and the enzyme is recovered (*ca.* 20%) in the buffer washings. This is probably due to removal of a terminal sulphonated polycyclic moiety. Furthermore, treatment of the native gel with borohydride produces a gel in which the enzyme is largely unretarded and appears in the early fractions (Fig. 3c) with *ca.* 70% recovery of enzyme activity. In each case the enzyme retained on the Procion Green H-4G adsorbent was recovered by removal of Mg^{2+} from the irrigating buffer. Alternatively, yeast hexokinase quantitatively adsorbed to native immobilized dye could be recovered by applying a pulse of 20 m*M* ATP (100% recovery of enzyme activity) or 20 m*M* D-glucose (60% recovery) both in the presence of 10 m*M* $MgCl_2$. The unique behaviour of yeast hexokinase towards Procion Green H-4G has been discussed earlier[26].

It appears, therefore, that removal of sulphonated (poly) cyclic parts from the

dye structure results, in general, in weakened dye–macromolecule interactions. This may prove useful in dye chromatography because:

(i) Non-specific proteins such as bovine serum albumin appear in the buffer washings in higher recoveries than from unmodified dye-gels

(ii) The binding of enzymes on the modified adsorbents is weaker, allowing higher recovery at lower eluent concentrations

(iii) Points (i) and (ii) should lead to enzyme preparations with higher yields and specific activities than the unmodified dye-gels

Finally, chemical treatment of immobilized Procion dyes is economically feasible because of the cheap reagents utilized and the ease with which the modified gels are prepared in a short time.

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