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# Design and applications of biomimetic anthraquinone dyes

# III. Anthraquinone-immobilised C.I. Reactive Blue 2 analogues and their interaction with horse liver alcohol dehydrogenase and other adenine nucleotide-binding proteins

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SUMMARY

C.I. Reactive Blue 2 analogues were bonded onto an agarose support matrix by a novel method which entailed immobilisation by the anthraquinone ring 1-amino group as opposed to the usual triazine ring coupling methods. Dyes with spacer arms attached to the anthraquinone ring 1-amino group were synthesised by reacting methoxytriazine analogues of C.I. Reactive Blue 2 with chloroacetyl chloride and ethylenediamine. Unlike the blue parent dyes, all C.I. Reactive Blue 2 analogues with derivatised anthraquinone ring 1-amino groups were of a characteristic red colour. This change of chromaticity was entirely expected since the anthraquinone ring 1-amino group is an important component of the C.I. Reactive Blue 2 chromophore. Chromatographic studies indicated that, in comparison to adsorbents comprising triazine ring-immobilised dyes, adsorbents formed from C.I. Reactive Blue 2 analogues immobilised by the anthraquinone ring were better suited to the isolation of horse liver alcohol dehydrogenase and other adenine nucleotide-requiring enzymes. Similarities between C.I. Reactive Blue 2 analogues immobilised by the anthraquinone ring and $N^6$-(6-aminohexyl)adenine nucleotide derivatives could be identified which may account for these observations. These studies confirm that highly effective affinity ligands based on synthetic textile dyes can be designed in a rational manner.

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## INTRODUCTION

Immobilised reactive textile dyes have been used to purify a vast number of proteins by affinity chromatography[1–4]. Despite the success of reactive dyes as affinity ligands, relatively little is known about the interaction of these compounds with their complementary binding sites on proteins. One would surmise that a greater understanding of the interactions between synthetic dyes and proteins could subsequently lead to the identification of dye ligands with improved binding selectivities.

In a previous publication[5], the effect of modifications to the terminal aminobenzenesulphonate ring of C.I. Reactive Blue 2 on the interaction of the dye with horse liver alcohol dehydrogenase was reported. Analogues of C.I. Reactive Blue 2 with various neutral, anionic and cationic groups located on the terminal phenyl ring displayed large variations in affinity for the enzyme. However, the effects of these modifications on the binding of alcohol dehydrogenase were masked when the C.I. Reactive Blue 2 analogues were immobilised by the triazine ring moiety to an agarose support matrix. This observation suggested that the method of dye immobilisation did not promote optimal dye–protein interaction, despite the use of a spacer arm coupling regime.

From X-ray crystallographic studies on the binding of C.I. Reactive Blue 2 by horse liver alcohol dehydrogenase[6], it is apparent that the triazine ring of the dye is bound within a deep cleft situated at the junction of the coenzyme and catalytic domains of the enzyme. This region of the protein is normally associated with binding the pyrophosphate bridge of $NAD^+$ or NADH. Thus, for steric reasons, immobilisation of C.I. Reactive Blue 2 via the triazine ring is not conducive to optimal interaction with horse liver alcohol dehydrogenase. The anthraquinone ring 1-amino group represents a more suitable point of dye attachment, since this group, like the exocyclic adenine $N^6$-amino group of bound $NAD^+$, is exposed to the exterior solvent[6,7]. Thus, the synthesis of affinity adsorbents composed of C.I. Reactive Blue 2 analogues immobilised by a spacer arm attached to the 1-amino group of the anthraquinone ring is reported here. These adsorbents displayed improved properties for the purification of horse liver alcohol dehydrogenase and were found to be effective in binding other adenine nucleotide-requiring enzymes.

## EXPERIMENTAL

### *Materials*

*p*-Dimethylaminobenzaldehyde, 1,1′-carbonyldiimidazole (CDI), cyanogen bromide (CNBr) and glucose 6-phosphate were purchased from Sigma (Poole, U.K.). N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) and ethylenediaminetetraacetic acid (EDTA) were supplied by BDH (Poole, U.K.), whilst chloroacetyl chloride and ethylenediamine were obtained from Fisons (Loughborough, U.K.).

The following biochemicals were purchased from Boehringer (Lewes, U.K.): nicotinamide-adenine dinucleotide ($NAD^+$); reduced nicotinamide-adenine dinucleotide (NADH); nicotinamide-adenine dinucleotide phosphate ($NADP^+$); phosphoenolpyruvate (PEP); adenosine 5′-diphosphate (ADP); adenosine 5′-triphosphate (ATP); horse liver alcohol dehydrogenase (alcohol:$NAD^+$ oxidoreductase; EC

1.1.1.1; 2.7 U $mg^{-1}$); yeast glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase; EC 1.1.1.49; 140 U $mg^{-1}$); rabbit muscle lactate dehydrogenase (L-lactate:$NAD^+$ oxidoreductase; EC 1.1.1.27; 550 U $mg^{-1}$); yeast hexokinase (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1; 140 U $mg^{-1}$) and rabbit muscle pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase; EC 2.7.1.40; 200 U $mg^{-1}$).

The chromatographic materials Sepharose 4B and Sephadex LH-20 were obtained from Pharmacia (Milton Keynes, U.K.). Intermediates used in the synthesis of C.I. Reactive Blue 2 analogues were donated by ICI Organics Division. All other reagents and solvents were of analytical grade and purchased from the usual sources.

*Synthesis of C.I. Reactive Blue 2 analogues with anthraquinone-linked spacer arms*

Methoxylated C.I. Reactive Blue 2 analogues II–IV (Fig. 1) were synthesised, as described previously[8], by reacting 1-amino-4-(4'-amino-3'-sulphoanilino)anthraquinone-2-sulphonic acid with 2,4-dichloro-6-methoxy-*s*-triazine, followed by reaction of the product with the relevant substituted phenylamine derivative.

Chloroacetylated C.I. Reactive Blue 2 analogues V–VIII (Fig. 1) were synthesised by stirring a solution of the methoxylated dyes I–III (25 mmol) in dimethylformamide (DMF; 150 ml) with chloroacetyl chloride (250 mmol) for 30 min at 20–25°C. The reaction end-point was established by analytical thin-layer chromatography (TLC), whereupon saturated NaCl solution (800 ml) was added and the red chloroacetyl product filtered, washed with 10–15% (w/v) NaCl solution (300 ml), re-suspended in acetone (1 l), filtered and dried under vacuum at 50°C.

Dyes with $\beta$-aminoethylaminoacetylamino spacer arms (VIII–X; Fig. 1) were formed by reacting chloroacetyl dyes V–VII (15 mmol in 75 ml DMF) with ethylenediamine (150 mmol) for 25 min at 20–25°C. The reaction end-point was determined by analytical TLC, whereupon the mixture was diluted with saturated NaCl solution (800 ml) and the product suspension adjusted to pH 7.0 with concentrated HCl. The red precipitate was filtered, washed with 10% (w/v) NaCl solution (500 ml), acetone (100 ml) and dried under vacuum at 50°C.

*Analysis of C.I. Reactive Blue 2 analogues*

Analytical TLC was performed using silica foils (Schleicher & Schuell, Dassel, F.R.G.) and a propan-2-ol–ammonia-water (7:2:1, v/v/v) solvent. The presence of dyes which contained a primary amine was confirmed by treating dried TLC foils with Ehrlich's reagent, a solution of *p*-dimethylaminobenzaldehyde (1 g) in methanol–concentrated HCl (9:1, v/v) (100 ml).

Visible absorption spectra of purified C.I. Reactive Blue 2 analogues were recorded (800–400 nm) from 0.1 m*M* aqueous solutions in 10 mm path-length cells. The dyes were purified to near homogeneity by lipophilic chromatography on Sephadex LH-20 as described previously[9]. Infrared spectra were recorded from paraffin oil mulls of purified dye.

*Immobilisation of C.I. Reactive Blue 2 analogues to beaded agarose*

Purified dyes VIII–X were immobilised to CDI-activated Sepharose 4B according to established procedures[5]. Immobilised dye concentrations were determined by acid hydrolysis and spectrophotometric estimation of the solubilised dye[5]. For the

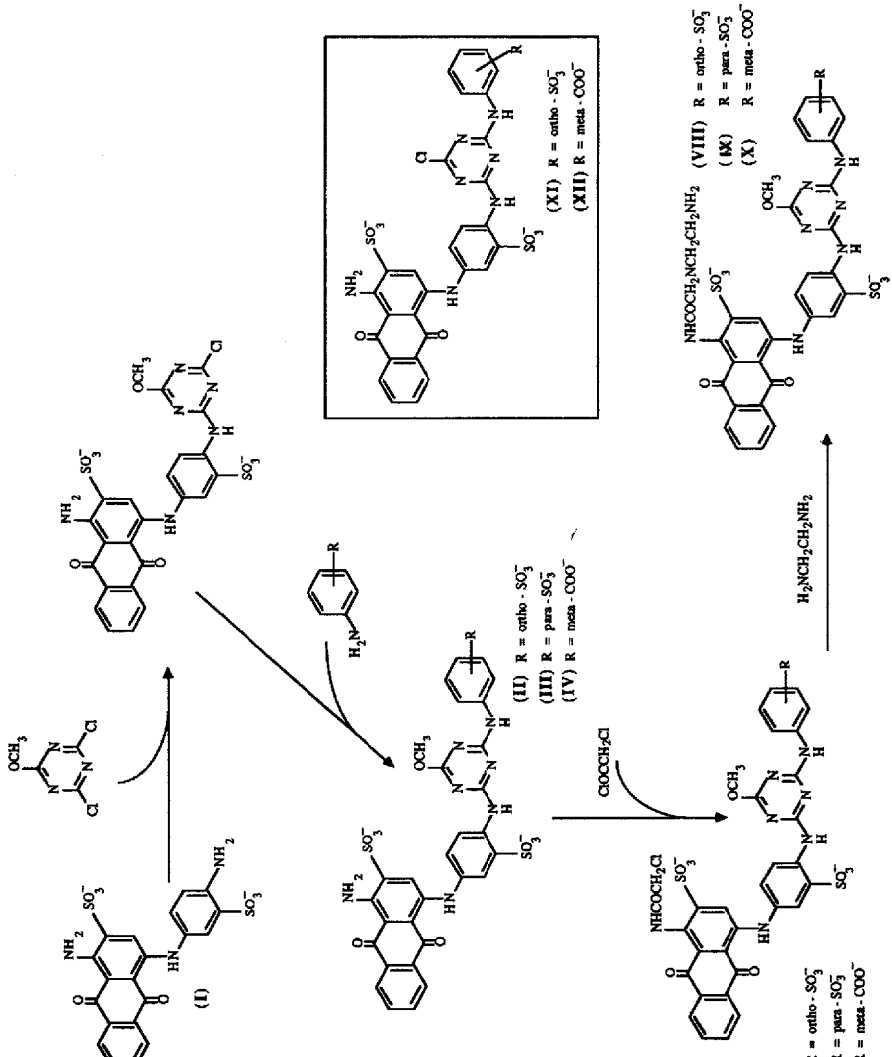


Fig. 1. Synthesis of C.I. Reactive Blue 2 analogues with anthraquinone-linked spacer arms.

purposes of immobilised dye determination, molar extinction coefficients of purified dyes VIII–X were determined following the incubation of aqueous dye solution (0.2 m*M*; 100 μl) with 5 *M* HCl (200 μl) for 5 min at 60°C. The solution was neutralised by adding NaOH (10 *M*; 100 μl) and potassium phosphate buffer (1 *M*; 600 μl), whereupon the absorbance of the solution was measured at 516 nm against a no-dye blank.

Trial immobilisations to CNBr-activated agarose were also performed. Activated Sepharose 4B (2 g moist gel; synthesised according to Lowe[10]) was added to a solution of dye (25 mg) in sodium carbonate buffer, pH 9.5 (0.4 *M*; 5 ml) and tumbled for 16 h at 4°C. Dyed gels were washed sequentially with water (100 ml), 1 *M* NaCl solution (100 ml), water (100 ml), 50% (v/v) dimethylsulphoxide (DMSO) solution (20 ml), water (100 ml) and stored in 0.02% (w/v) sodium azide solution (4°C) until required.

*Preparation of crude horse liver extract*

A cell-free horse liver extract was prepared by a method based on a previous report[11]. All procedures were performed at 4°C.

Fresh horse liver (1 kg) was washed with distilled water and ground in a meat grinder. The liver pulp was stirred (16 h; 4°C) in 10 m*M* HEPES–NaOH buffer, pH 7.0 (500 ml) and debris removed by screening through a nylon mesh. The retained liver pulp was re-slurried in 500 ml buffer and screened a second time. The crude extracts were combined, centrifuged (4 h; 23 000 *g*; average distance from the centre of rotation: 8.9 cm), the lipid layer was removed and the supernatant decanted to yield 900 ml cell-free extract. The extract was dialysed (3 days) against 64 volumes potassium phosphate buffer, pH 7.0 (20 m*M*) containing reduced glutathione (1 m*M*) before use.

*Affinity chromatography of horse liver alcohol dehydrogenase on immobilised C.I. Reactive Blue 2 analogues*

The interaction of purified horse liver alcohol dehydrogenase with dyes VIII–X immobilised to CDI-activated agarose was established by analytical affinity chromatography as described previously[5]. The enzyme was purified from a crude horse liver extract as follows.

Glass columns (10 × 0.5 cm I.D.) were slurry packed with adsorbent (1.0 g moist gel) and the gel bed was equilibrated with HEPES–NaOH buffer, pH 7.5 (20 m*M*) containing reduced glutathione (1 m*M*). Horse liver extract was dialysed (16 h; 4°C) against 150 volumes equilibration buffer, and 2 ml (130 mg protein) were chromatographed at a flow-rate of 30 ml $h^{-1}$ $cm^{-2}$. The column was flushed with equilibration buffer (10 ml) and bound enzyme eluted with equilibration buffer containing 1 *M* KCl. Fractions (1 ml) were collected throughout the procedure and 20-μl samples removed for assay of enzyme activity and protein concentration.

*Interaction of adenine-nucleotide-binding proteins with immobilised C.I. Reactive Blue 2 analogues*

The interaction of various dehydrogenases and kinases with immobilised C.I. Reactive Blue 2 analogues was investigated by affinity chromatography at 4°C. Columns of adsorbent were equilibrated with HEPES–NaOH buffer, pH 7.5 (20 m*M*) containing $MgCl_2$ (5 m*M*), EDTA (0.4 m*M*) and 2-mercaptoethanol (2 μ*M*). Suspensions of pure enzyme were dialysed (16 h; 4°C) against 2000 volumes equilibration

buffer, and 5–10 U were applied to the columns at a flow-rate of 30 ml $h^{-1}$ $cm^{-2}$. Buffer flow was interrupted for 10 min following which the adsorbent was washed with equilibration buffer (6 ml) and bound enzyme displaced by a linear 0–1.0 *M* KCl elution gradient. Fractions (1 ml) were collected, from which 20-$\mu$l samples were removed for assay of enzyme activity.

*Enzyme and protein assays*

Glucose-6-phosphate dehydrogenase was assayed by following the reduction of $NADP^+$ at 340 nm (25°C). In a total volume of 1 ml were: triethanolamine–NaOH buffer, pH 7.6 (86 $\mu$mol); $MgCl_2$ (6.6 $\mu$mol): glucose 6-phosphate (1.2 $\mu$mol); and $NADP^+$ (360 nmol). 1 U of activity is defined as the amount of enzyme required to reduce 1 $\mu$mol $NADP^+$ per min at 25°C. Alcohol dehydrogenase was assayed as described previously[5].

Hexokinase activity was followed with a glucose-6-phosphate dehydrogenase-linked assay. In a total volume of 3 ml were: triethanolamine–NaOH buffer, pH 7.6 (250 $\mu$mol); $MgCl_2$ (20 $\mu$mol); glucose (660 $\mu$mol); ATP (8.1 $\mu$mol); $NADP^+$ (2.2 $\mu$mol); and glucose-6-phosphate dehydrogenase (50 $\mu$g; 2.5 U). 1 U of hexokinase activity is defined as the amount of enzyme required to effect the reduction of 1 $\mu$mol $NADP^+$ per min at 25°C.

Pyruvate kinase activity was determined with a lactate dehydrogenase-linked assay. In a total volume of 3 ml were: triethanolamine–KOH buffer, pH 7.6 (0.26 mmol); $MgSO_4$ (7.5 $\mu$mol); KCl (30 $\mu$mol); PEP (1.6 $\mu$mol); ADP (14 $\mu$mol); NADH (0.6 $\mu$mol); and lactate dehydrogenase (50 $\mu$g; 15 U). 1 U of pyruvate kinase activity is defined as the amount of enzyme required to promote the oxidation of 1 $\mu$mol NADH per min at 25°C.

Alcohol dehydrogenase concentration was determined by absorbance at 280 nm ($\varepsilon_{280}$ = 0.455 ml $mg^{-1}$ $cm^{-1}$)[12]. Protein concentrations of crude mixtures were determined by the method of Warburg and Christian[13].

## RESULTS

Triazine dyes are commonly immobilised to chromatographic support materials by direct reaction between the chlorotriazine group of the dye and nucleophilic groups (frequently hydroxyl ions) on the surface of the matrix. To prevent a similar reaction occurring with C.I. Reactive Blue 2 analogues possessing anthraquinone-linked spacer arms, unreactive methoxylated triazine dyes were synthesised (Fig. 1). This was achieved by reacting the blue chromophore of C.I. Reactive Blue 2 (I; Fig. 1) with 2,4-dichloro-6-methoxy-*s*-triazine, a procedure which also eliminated any reaction between ethylenediamine and the triazine ring in the final stage of synthesis.

Three methoxylated C.I. Reactive Blue 2 analogues were produced which possessed *o*-sulphonate, *p*-sulphonate and *m*-carboxylate groups on the terminal aminobenzene ring of the dye (Fig. 1). Reaction of dyes II–IV with chloroacetyl chloride was accompanied by a colour change from royal blue to burgundy red. The reaction occurred readily in DMF but did not occur in water or aqueous DMF mixtures. Reaction of chloroacetyl dyes V–VIII with ethylenediamine also proceeded smoothly in DMF, although appreciable product hydrolysis was observed if the reaction was allowed to proceed for longer than 30 min. This was readily apparent since as a result

of amide bond hydrolysis in the alkaline reaction mixture, the precursory blue methoxy dye was regenerated, thus causing the reaction mixture to darken.

Spectrophotometric analysis of purified dyes VIII–X (Table I; Fig. 2) revealed maximal light absorption in the region of 520 nm. This represented a hypsochromic shift of approximately 100 nm relative to the $\lambda_{max}$ of the *o*-sulphonate isomer of C.I. Reactive Blue 2 (XI; Fig. 1). The shift in absorption maximum from 617 to 520 nm was also accompanied by a reduction in extinction (Table I). These spectral changes were entirely expected since the anthraquinone ring 1-amino group is a principal electron-donating group of the C.I. Reactive Blue 2 chromogen[14,15]. Consequently, any alteration of the electronic distribution of the 1-amino group will be manifested as a change in the amount of energy required for electronic transition. Therefore the wavelength and intensity of light absorption will be altered following reactions involving the anthraquinone ring 1-amino group. Chloroacetyl dyes had a $\lambda_{max}$ marginally lower (approximately 10 nm) than dyes with $\beta$-aminoethylaminoacetylamino spacer arms (Table I). This discrepancy may reflect differences in electronegativity between chlorine and substituted nitrogen atoms.

Blue 1,4-diaminoanthraquinone dyes are readily protonated by mineral acids to form quaternary amine salts[15,16]. A large hypsochromic shift is associated with this process. The visible absorption spectrum of the *o*-sulphonate terminal ring isomer of C.I. Reactive Blue 2 (XI) recorded in 4.5 *M* HCl resembled the absorption spectra of C.I. Reactive Blue 2 analogues with modified anthraquinoid 1-amino groups (Fig. 2). The absorption peak of the protonated dye was shifted 91 nm relative to the $\lambda_{max}$ of the dye in distilled water (526 *versus* 617 nm). However the $\lambda_{max}$ of V in 4.5 *M* HCl was very similar to that recorded in distilled water (502 *versus* 508 nm; Fig. 2). This observation suggested that the 1-amino group of chloroacetylated dyes was not susceptible to protonation, presumably since this group had been derivatised. Additional evidence of the formation of a 1-chloroacetylamino group was obtained by infrared spectroscopy. Dye V clearly showed the presence of an absorption peak at 1660 $cm^{-1}$ which is indicative of an amide carbonyl group. This peak was absent in the spectrum of the blue precursor dye (II).

Successful coupling of chloroacetyl dyes to ethylenediamine was confirmed using Ehrlich's reagent. Dye VIII rapidly turned an orange colour on exposure to Ehrlich's reagent, confirming the presence of a primary amine. No reaction was observed with compound V, the chloroacetyl precursor of compound VIII.

TABLE I

SPECTRAL DATA DETERMINED FOR C.I. REACTIVE BLUE 2 ANALOGUES

| *C.I. Reactive Blue 2 analogue*[a] | $\lambda_{max}$[b] *(nm)* | $\varepsilon_{\lambda}$[b] *(l mol$^{-1}$ cm$^{-1}$)* |
|---|---|---|
| V | 508 | 5800 |
| VII | 516 | 5500 |
| IX | 520 | 5400 |
| X | 520 | 4900 |
| XI | 617 | 12600 |

[a] For structures, see Fig. 1.
[b] Data obtained from 100 $\mu M$ aqueous solutions.

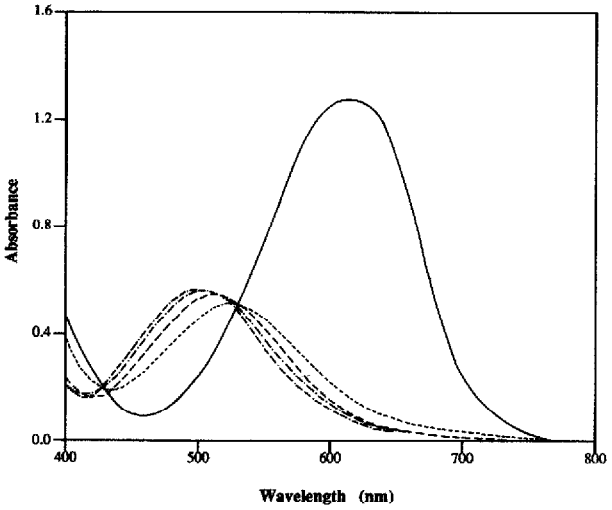


Fig. 2. Absorption spectra of C.I. Reactive Blue 2 analogues. Spectra were recorded from 0.1 m*M* aqueous dye solutions in 10 mm path length cells (25°C) against a distilled water–4.5 *M* HCl blank:– = XI in distilled water; . . . . . = XI in 4.5 *M* HCl; – . – . – = V in distilled water; – . – . – . – = V in 4.5 *M* HCl; - - - - = VIII in distilled water.

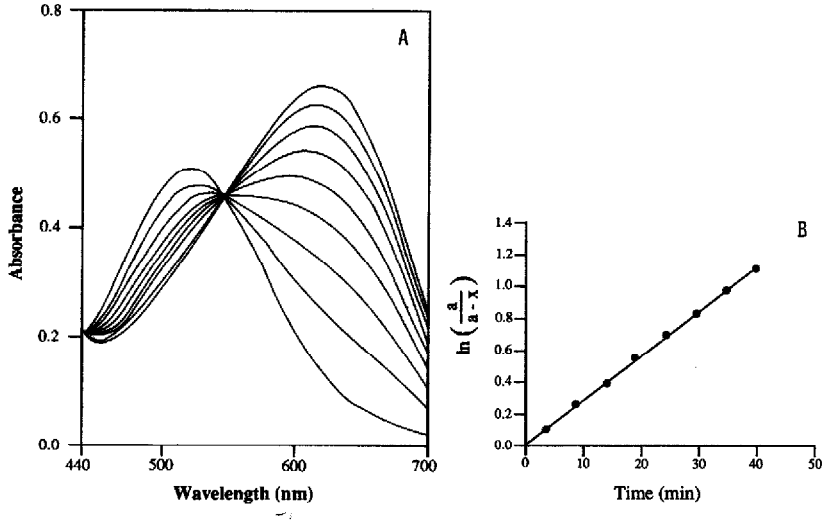


Fig. 3. Base-catalysed hydrolysis of C.I. Reactive Blue 2 analogue V. (A) Visible absorption spectra of a solution containing, in 1 ml total volume, 100 nmol of compound V and 100 $\mu$mol of NaOH, were recorded at 5-min intervals against a distilled water blank; path length 10 mm. (B) Plot of ln [initial dye concentration ($a$)/dye concentration at time $t$ ($a - x$)] against time; values were determined from the absorbance at 516 nm ($\varepsilon_{516}$ = 2400 l mol$^{-1}$ cm$^{-1}$). The slope of the line gives an estimate of the pseudo-first-order rate constant ($k_1$).

C.I. Reactive Blue 2 analogues with anthraquinone-linked spacer arms were rapidly hydrolysed in strongly alkaline solutions. A time-dependent reduction in absorbance at 518 nm and a concomitant increase in absorbance at 618 nm was observed when compound VIII was treated with 0.1 *M* NaOH (Fig. 3). An isosbestic point at 545 nm was clearly visible, indicating direct conversion of compound V to the blue compound absorbing at 618 nm. Absorbance at 618 nm reached a steady maximum after 4 h. Analysis of the hydrolysate by TLC revealed the blue species had an identical $R_F$ value to precursor compound II. Thus, the initial 1-aminoanthraquinone dye is regenerated as a result of alkaline hydrolysis. The reaction was found to observe typical pseudo-first-order kinetics with a first-order rate constant of $4.64 \cdot 10^{-4}$ $s^{-1}$ (Fig. 3).

Dyes with anthraquinone ring-linked spacer arms (VIII–X) were immobilised to CDI-activated Sepharose 4B to yield red gels. Dye hydrolysis in the mildly alkaline coupling medium (pH 10.0) was not observed spectrophotometrically over a period of 14 h at 15°C. Immobilised dye concentrations were determined as described in the Experimental section in an effort to account for potential acid-catalysed dye hydrolysis during gel dissolution with 5 *M* HCl. However, under these conditions dye hydrolysis was limited. Determined $\varepsilon_{515}$ values were 4600, 4600 and 4400 l $mol^{-1}$, $cm^{-1}$ for compounds VIII, IX and X, respectively. A trial immobilisation of compound V to CDI-activated Sepharose 4B was also performed. As expected, a very weakly substituted gel was produced (immobilised dye concentration 0.1 $\mu$mol $g^{-1}$ of moist gel), the low level of coupling being attributable to reaction between the chloroacetyl group of the dye and unsubstituted hydroxyl groups on the matrix.

A number of published reports describe the direct immobilisation of C.I. Reactive Blue 2 to activated matrices, supposedly by reactions involving the anthraquinone ring 1-amino group[17–19]. However, in view of our findings, we were surprised to find no mention of a colour change upon immobilisation by this method. Thus, a number of C.I. Reactive Blue 2 analogues were reacted with CNBr-activated Sepharose 4B, essentially as described by Jankowski *et al.*[17]. Immobilised dye concentrations were determined by a previously reported method[5]. Purified II did not immobilise to CNBr-activated Sepharose 4B, whilst purified XI coupled to give a very weakly substituted gel (Table II). However, significant immobilisation of the blue chromophoric base compound I and commercial-grade C.I. Reactive Blue 2 (supplied by ICI Organics Division) was observed (Table II). In all instances blue gels were produced as opposed to the characteristically red gels obtained when dyes VIII–X were immobilised. These findings suggested that C.I. Reactive Blue 2 does not couple directly to

TABLE II

IMMOBILISATION OF C.I. REACTIVE BLUE 2 ANALOGUES TO CNBr-ACTIVATED AGAROSE

| *C.I. Reactive Blue 2 analogue* | *Immobilised dye concentration ($\mu$mol $g^{-1}$ of moist gel)* |
|---|---|
| Pure I | 0.3 |
| Pure II | 0.0 |
| Pure XI | 0.1 |
| Crude C.I. Reactive Blue 2 | 0.6 |

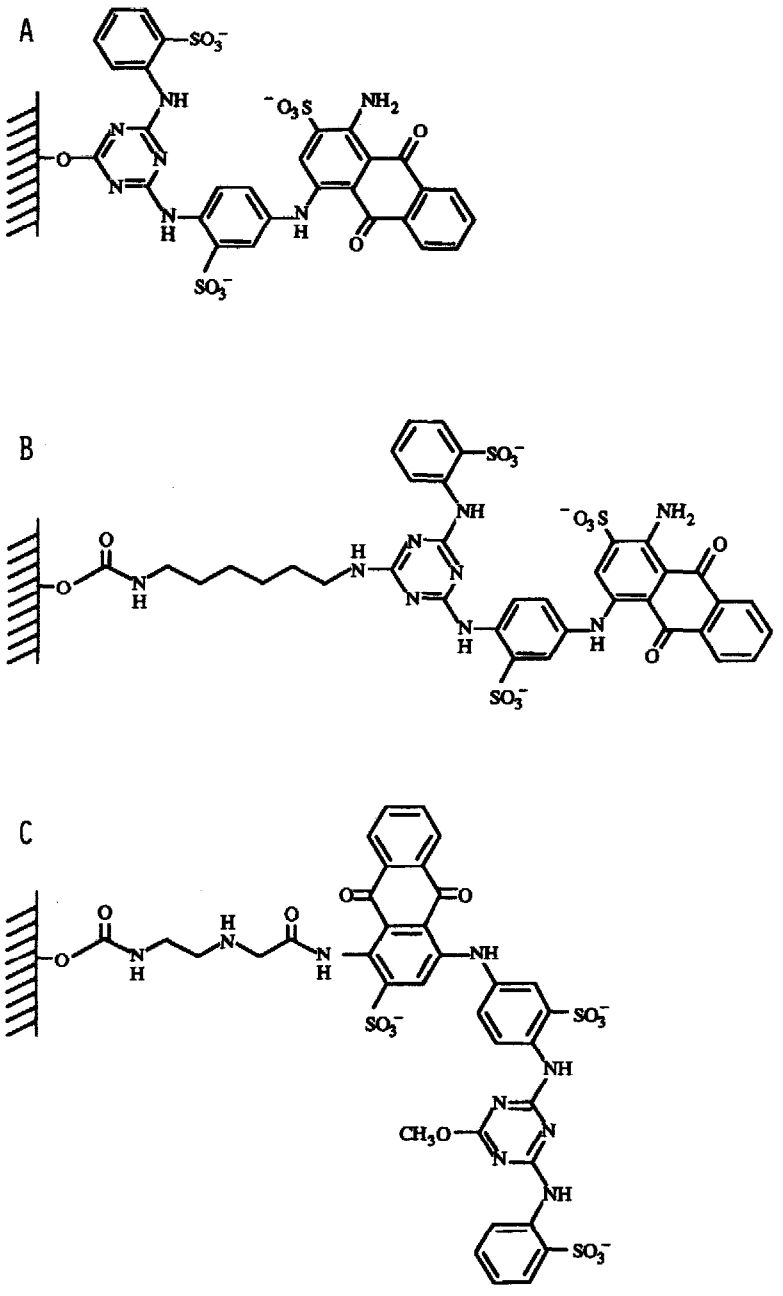


Fig. 4. Structures of immobilised C.I. Reactive Blue 2 analogues. (A) Compound XI coupled directly to agarose; (B) compound XI coupled to CDI-activated agarose by a triazine ring-coupled 6-aminohexyl spacer arm; (C) compound VIII immobilised to CDI-activated agarose.

activated matrices by the anthraquinone ring 1-amino group. The low level of substitution observed for compound XI is probably due to reaction of the chlorotriazine ring with free hydroxyl groups on the matrix. Therefore, the higher level of substitution observed for crude C.I. Reactive Blue 2 is most likely to be attributable to immobilisation of chromophoric contaminants known to be present in commercial dye preparations[9].

Affinity chromatography of pure horse liver alcohol dehydrogenase was performed using C.I. Reactive Blue 2 analogues VIII–X coupled to CDI-activated Sepharose 4B (Fig. 4). All three dyes bound the enzyme, compound X exhibiting the highest affinity (Table III). The concentration of NADH required to elute adsorbed alcohol dehydrogenase correlated directly with the apparent dissociation constant ($K_d$) of the dye in free solution (determined by difference spectroscopy[5] using methoxylated dyes II–IV). The concentration of NADH required for elution from immobilised X ($K_d$ 0.27 $\mu M$) was almost double that required for elution from immobilised IX ($K_d$ 5.7 $\mu M$). When compound XI is immobilised to beaded agarose by a 1,6-diaminohexane spacer arm attached to the triazine ring of the dye, an unduly high concentration of NADH is required to displace bound horse liver alcohol dehydrogenase[5]. Steric factors associated with terminal phenyl ring structure and the point of spacer arm attachment were thought to be responsible. Elution of the enzyme from immobilised VIII (which possesses an equivalent terminal ring structure to compound XI) was achieved in a manner entirely consistent with the observed $K_d$ of the dye in free solution. This suggested that immobilisation of C.I. Reactive Blue 2 analogues by the anthraquinone ring 1-amino group was advantageous when using analytical affinity chromatography to investigate the effects of terminal phenyl ring modifications on enzyme binding.

Horse liver alcohol dehydrogenase was effectively purified from a crude liver extract by affinity chromatography on anthraquinone ring-immobilised C.I. Reactive Blue 2 analogues. A non-selective desorption technique was adopted (1 *M* KCl) so that an indication of adsorbent specificity could be gained. On the basis of previous work[1,2], one would predict that considerably higher degrees of purification would be achieved by use of a selective eluent such as NADH. Immobilised C.I. Reactive Blue 2 analogue VIII gave the highest degree of enzyme purification with a 10.3-fold increase in specific activity (Table IV; Fig. 5). Dye IX gave the lowest degree of purification, a

TABLE III

AFFINITY CHROMATOGRAPHY OF HORSE LIVER ALCOHOL DEHYDROGENASE ON IMMOBILISED C.I. REACTIVE BLUE 2 ANALOGUES

| *Immobilised C.I. Reactive Blue 2 analogue* | $K_d$ *(μM)*[b] | *Immobilised dye concentration (μmol g⁻¹ of moist weight gel)* | *NADH concentration required for elution (μM)*[a] | *Activity recovered (%)* |
|---|---|---|---|---|
| VIII | 0.66 ± 0.05 | 2.1 | 108 | 87 |
| IX | 5.7 ± 0.5 | 2.2 | 68 | 91 |
| X | 0.27 ± 0.07 | 2.3 | 113 | 69 |

[a] pH 7.5, 4°C.

[b] Determined for the methoxylated precursor dyes II–IV.

TABLE IV

PURIFICATION OF HORSE LIVER ALCOHOL DEHYDROGENASE FROM A CRUDE LIVER EXTRACT BY AFFINITY CHROMATOGRAPHY ON IMMOBILISED C.I. REACTIVE BLUE 2 ANALOGUES

| *C.I. Reactive Blue 2 analogue* | *Immobilised dye concentration (μmol $g^{-1}$ of moist gel)* | *Specific activity of applied sample (U $mg^{-1}$)* | *Specific activity of eluted enzyme (U $mg^{-1}$)* | *Purification (fold)* | *Yield (%)* |
|---|---|---|---|---|---|
| VIII | 2.1 | 0.018 | 0.185 | 10.3 | 86 |
| IX | 2.2 | 0.019 | 0.058 | 3.1 | 36 |
| X | 2.3 | 0.015 | 0.085 | 5.6 | 74 |
| XI[a] | 2.1 | 0.015 | 0.065 | 4.3 | 87 |
| XI[b] | 2.2 | 0.014 | 0.103 | 7.3 | 84 |

[a] Direct coupling through chlorotriazine group.
[b] 6-Aminohexyl coupling.

possible reflection of the relatively low affinity of this analogue for horse liver alcohol dehydrogenase. Similar purification experiments were also performed with adsorbents composed of XI coupled directly to Sepharose 4B (synthesised according to Lowe *et al.*[20]) and 6-aminohexyl-XI coupled to CDI-activated Sepharose 4B (synthesised as described previously[5]). The method of dye immobilisation (Fig. 4) was found to have a profound effect on adsorbent specificity (Table IV). The commonly

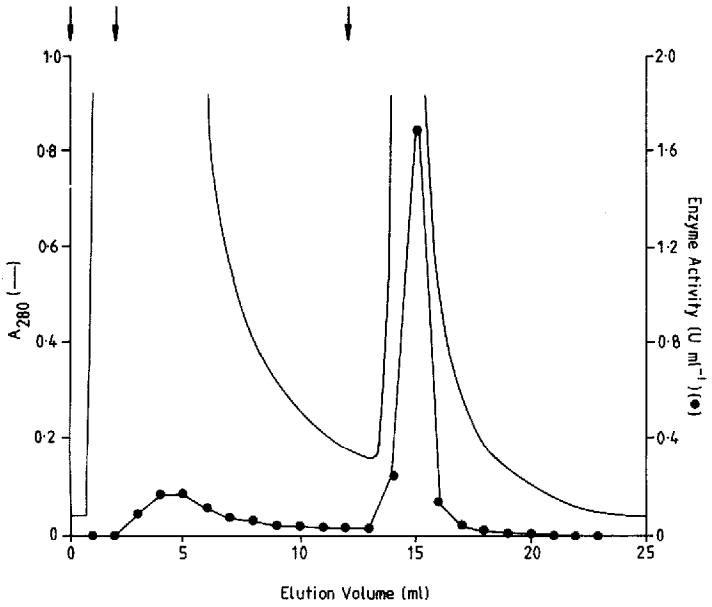


Fig. 5. Purification of alcohol dehydrogenase from horse liver extract by affinity chromatography on agarose-immobilised C.I. Reactive Blue 2 analogue VIII. Dialysed horse extract (2 ml; 130 mg protein) was applied at a linear flow-rate of 30 ml $min^{-1}$ $cm^{-2}$ to a 10 cm × 0.5 cm I.D. glass column packed with 1.0 g of adsorbent equilibrated in HEPES–NaOH buffer, pH 7.5 (20 m*M*) containing glutathione (1 m*M*). The column was flushed with equilibration buffer (10 ml) and bound protein eluted with buffer containing 1 *M* KCl. Fractions (1 ml) were collected and assayed for enzyme activity.

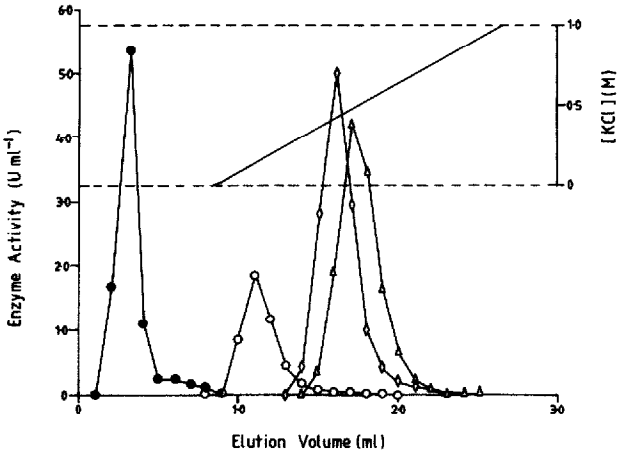


Fig. 6. Affinity chromatography of dehydrogenases and kinases on immobilised C.I. Reactive Blue 2 analogue X. Glass columns (10 cm × 0.5 cm I.D.) containing Sepharose 4B-immobilised X (1.0 g moist gel; total column volume, 2.2 ml) was equilibrated with HEPES–NaOH buffer, pH 7.5 (20 m*M*) containing 2-mercaptoethanol (2 $\mu M$), EDTA (0.4 m*M*) and $MgCl_2$ (5 m*M*). Dialysed enzyme [yeast hexokinase, 10 U (●); rabbit muscle pyruvate kinase, 10 U (△); horse liver alcohol dehydrogenase, 5 U (○); or yeast glucose-6-phosphate dehydrogenase, 10 U (◇)] was applied to the columns at a flow-rate of 30 ml $h^{-1}$ $cm^{-2}$ and incubated with the adsorbent for 10 min. The columns were flushed with equilibration buffer (6 ml) and adsorbed enzyme was eluted with a KCl gradient (0–1.0 *M*; 20 ml). Fractions (1 ml) were collected and samples (20 $\mu$l) assayed for enzyme activity.

adopted direct immobilisation technique resulted in a low degree of enzyme purification (4.3-fold). Spacer arm-coupled dyes gave notably higher degrees of purification, linkage to the anthraquinone ring proving the most effective method of immobilisation.

Investigations with immobilised nucleotides show that the point of spacer arm attachment can dramatically affect the affinity of the immobilised ligand for nucleotide-binding proteins[21–23]. Consequently, affinity chromatography was used to investigate the interaction of selected dehydrogenases and kinases with compounds X and 6-aminohexyl-XII (synthesised as reported previously[5]), immobilised to CDI-

TABLE V

AFFINITY CHROMATOGRAPHY OF DEHYDROGENASES AND KINASES ON C.I. REACTIVE BLUE 2 ANALOGUES IMMOBILISED TO SEPHAROSE 4B BY SPACER ARM COUPLING TO THE ANTHRAQUINONE RING (X) OR THE TRIAZINE RING (6-AMINOHEXYL-XII)

| *Enzyme* | *Concentration of KCl required for elution (M)* | |
|---|---|---|
| | *Immobilised X* | *Immobilised XII* |
| Horse liver alcohol dehydrogenase | 0.15 | 0.12 |
| Yeast glucose-6-phosphate dehydrogenase | 0.44 | 0.47 |
| Rabbit muscle pyruvate kinase | 0.45 | 0.30 |
| Yeast hexokinase | —[a] | —[a] |

[a] Enzyme eluted in void volume.

activated Sepharose 4B (Fig. 6). Rabbit muscle pyruvate kinase bound more tightly to compound X than to 6-aminohexyl-XII (as adjudged by the concentration of KCl required for elution), whilst horse liver alcohol dehydrogenase and yeast glucose-6-phosphate dehydrogenase were bound by both adsorbents with approximately equal affinity (Table V). Yeast hexokinase was unretarded by both adsorbents as might have been deduced from previous dye-binding studies[24]. Thus C.I. Reactive Blue 2 analogues immobilised by the anthraquinone ring 1-amino group retain their ability to interact with nucleotide-binding proteins. In he case of rabbit muscle pyruvate kinase, binding is favoured by this method of immobilisation.

## DISCUSSION

The interaction of affinity ligands with the ligand binding sites of proteins is frequently enhanced by the provision of a spacer arm between the ligand and the support matrix[10]. In addition, the orientation of the immobilised ligand can also influence its affinity for proteins. Studies with immobilised nucleotides such as adenosine 5'-monophosphate (AMP) or $NAD^+$ have shown that spacer arm linkage and ligand orientation can have a profound effect on protein adsorption[21–23]. Rather surprisingly, such considerations are seldom applied to reactive dye ligands, despite the fact that these commonly used compounds have been applied to the purification of a large number of nucleotide-binding proteins[1–4]. The predominant immobilisation technique adopted for reactive dyes, regardless of application, parallels the direct reaction of chlorotriazine dyes with cellulose textiles[4].

From previous investigations on the interaction of C.I. Reactive Blue 2 analogues with horse liver alcohol dehydrogenase, we concluded that immobilisation by the triazine ring was responsible for interferences with dye binding[5]. A more suitable point of attachment appeared to be the anthraquinone ring 1-amino group, since this component is exposed to the exterior solvent when dye is bound to the enzyme[5,6]. We find that immobilisation of C.I. Reactive Blue 2 analogues by a spacer arm linked to the anthraquinone ring 1-amino group, as opposed to the chlorotriazine ring, not only increases the affinity of the immobilised analogues for horse liver alcohol dehydrogenase, but also improves their specificity.

Previously, an immobilised C.I. Reactive Blue 2 analogue with an *m*-orientated carboxyl group on the terminal phenyl ring of the dye ($K_d$ 0.06 $\mu M$) bound horse liver alcohol dehydrogenase with an affinity such that 90 $\mu M$ NADH was required for elution[5]. In this work, analogue X (which also possessed an *m*-orientated terminal ring carboxyl group; $K_d$ 0.27 $\mu M$) immobilised by the anthraquinone ring, as opposed to the triazine ring, bound the enzyme more tightly so that 113 $\mu M$ NADH was required for desorption (Table III). Since both experiments were performed under identical conditions, the results suggest that immobilisation by the anthraquinone ring 1-amino group promotes the interaction of C.I. Reactive Blue 2 analogues with horse liver alcohol dehydrogenase. Presumably steric hindrance in the vicinity of the triazine ring is reduced on immobilising the dye by the anthraquinone ring.

The increase in immobilised dye specificity was particularly marked for compound VIII. Following alcohol dehydrogenase purification from a crude liver extract, a 2.4-fold increase in enzyme specific activity was achieved over the level of purification obtained with essentially the same dye (XI) immobilised by direct coupling

(Table IV). Enzyme recovery upon elution was virtually identical for both adsorbents (86–87%). Spacer arm coupling, by the anthraquinone ring or the triazine ring, resulted in a higher degree of enzyme purification as compared to the performance of directly coupled dyes (Table IV). Confirmation was provided by additional chromatographic data (not presented) determined for other immobilised C.I. Reactive Blue 2 analogues. This finding suggests that immobilisation of reactive dyes by a spacer arm linkage may enhance selective dye–protein interactions at the expense of non-selectgive binding, thereby promoting an overall increase in adsorbent selectivity. Similar conclusions may be reached from binding studies performed with blue dextran Sepharose. Human fibroblast and leucocyte interferons have greater affinity for Cibacron Blue F3G-A immobilised by a dextran linker, as opposed to dye coupled directly to beaded agarose[17]. Conversely, rat brain hexokinase and pigeon liver $NAD^+$ kinase display a reduced affinity for blue dextran Sepharose[24,25]. Thus dye specificity may be modulated by the use of spacer arm linkage regimes.

C.I. Reactive Blue 2 analogues immobilised by the anthraquinone ring 1-amino group were capable of interacting with a number of dehydrogenases and kinases (Fig. 6). Interestingly, the anthraquinonoid 1-amino group of C.I. Reactive Blue 2 bound to horse liver alcohol dehydrogenase is located in a similar position to the adenyl $N^6$-amino group of bound $NAD^+$[6] (ref. 6). Both $NAD^+$ and AMP immobilised by a hexyl spacer arm attached to the adenyl $N^6$-amino group often provide very effective adsorbents for the purification and resolution of dehydrogenases and kinases. Therefore, C.I. Reactive Blue 2 immobilised by the anthraquinone ring may bind a variety of adenine nucleotide-requiring proteins, possibly with an affinity and specificity greater than the conventionally immobilised dye.

Reaction of the C.I. Reactive Blue 2 anthraquinone ring 1-amino group with electrophiles is hindered due to the close proximity of the quinone oxygen and 2-sulphonate groups. In addition, the $2P_z$ electrons of the 1-amino group conjugate with the anthraquinone ring $\pi$ electron cloud, causing a reduction in basicity of the 1-amino group. For these reasons, the 1-amino group is not particularly reactive towards electrophiles, accounting for its lack of reactivity with cyanuric chloride in aqueous solution[9]. However, reactivity of the 1-amino group probably increases in DMF due to suppression of anthraquinone-2-sulphonate group ionisation. This factor, coupled with the absence of water, may allow reaction of the anthraquinone ring 1-amino group with chloroacetyl chloride to yield an amide bond.

Conjugation of the 1-amino group $2P_z$ electrons with the electron cloud of the anthraquinone ring gives rise to a charge-transfer absorption peak in the visible region of the spectrum, brought about by $2P_z$–$\pi^*$ transition[14]. Addition of a second electron donor group in the 4-position causes a bathochromic shift and increase in extinction[14,16]. Thus, 1-amino anthraquinone absorbs at 475 nm with a molar extinction coefficient of 6300 l $mol^{-1}$ $cm^{-1}$, whilst 1,4-diaminoanthraquinone absorbs at 550–590 nm with an extinction coefficient of 15 850 l $mol^{-1}$ $cm^{-1}$ (values determined in methanol)[15,16]. If the electron density of the amino groups are reduced in any way (by acetylation for example), a hypsochromic shift and reduction in extinction are observed[14,26]. Reactions involving the anthraquinone ring 1-amino group will therefore result in marked spectral changes.

The spectral perturbations observed upon chloroacetylation of C.I. Reactive Blue 2 analogues (Fig. 2) are entirely consistent with the known behaviour of ami-

noanthraquinone chromophores[14,16]. However, our findings are at odds with a number of reports in the biochemical literature which infer that C.I. Reactive Blue 2 may be coupled to activated matrices via the anthraquinone ring 1-amino group without significant changes of chromaticity[17–19,25]. We find that purified C.I. Reactive Blue 2 couples very poorly to CNBr-activated Sepharose, the coupling observed probably being attributable to reaction of the chlorotriazine moiety with matrix hydroxyl groups. Significant blue coloration was observed if impure dye was immobilised, suggesting that chromophoric contaminants (probably compounds I and 2,4-dichloro-*s*-triazin-6-yl-I) were immobilised on previous occasions and not C.I. Reactive Blue 2. A possible consequence of this may be the reportedly low affinity of C.I. Reactive Blue 2 towards protein when supposedly immobilised by the anthraquinone ring 1-amino group[17–19,25]. These findings have also been reported in several review articles[1,2,27]. In fact, C.I. Reactive Blue 2 analogues genuinely immobilised by the 1-amino group are very effective affinity ligands. It is worthwhile noting that analogue X bound yeast glucose-6-phosphate dehydrogenase avidly, whereas an adsorbent formed by reacting Cibacron Blue F3G-A with succinyl-polyacrylic hydrazide agarose did not bind the enzyme[18]. Thus, great care must be exercised when interpreting chromatographic results obtained on using affinity adsorbents constructed from heterogeneous commercial dye preparations.

As with most small affinity ligands, the protein binding selectivity of reactive dyes is influenced by the immobilisation technique adopted and the point of attachment to the matrix. Such considerations should not be overlooked when designing chromophoric ligands for the chromatographic purification of proteins. Optimally immobilised biomimetic dyes, with structures designed to interact with selected proteins, should therefore have considerably improved specificities to conventional textile dyes.

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REFERENCES

1 C. R. Lowe, in A. Wiseman (Editor), *Topics in Enzyme and Fermentation Biotechnology*, Vol. 9, Ellis Horwood, Chichester, 1984, pp. 78–161.
2 G. Kopperschläger, H.-J. Böhme and E. Hoffman, in A. Feichter (Editor), *Advances in Biochemical Engineering*, Vol. 25, Springer, Berlin, Heidelberg, 1982, pp. 101–138.
3 C. R. Lowe and J. C. Pearson, *Methods Enzymol.*, 104 (1984) 97–113.
4 Y. D. Clonis, A. A. Atkinson, C. J. Bruton and C. R. Lowe (Editors), *Reactive Dyes in Protein and Enzyme Technology*, Macmillan, Basingstoke, 1987.
5 S. J. Burton, C. V. Stead and C. R. Lowe, *J. Chromatogr.*, 455 (1988) 201–216.
6 J.-F. Biellman, J.-P. Samama, C.-I. Branden and H. Eklund, *Eur. J. Biochem.*, 102 (1979) 107–110.
7 H. Eklund, J.-P. Samama and T. A. Jones, *Biochemistry*, 23 (1984) 5982–5996.
8 J. C. Pearson, S. J. Burton and C. R. Lowe, *Anal. Biochem.*, 158 (1986) 382–389.
9 S. J. Burton, S. B. McLoughlin, C. V. Stead and C. R. Lowe, *J. Chromatogr.*, 435 (1988) 127–137.
10 C. R. Lowe, in T. S. Work and E. Work (Editors), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 7, North-Holland, Amsterdam, 1979, pp. 267–522.
11 L. Andersson, H. Jornvall, A. Akeson and K. Mosbach, *Biochim. Biophys. Acta*, 364 (1974) 1–8.
12 D. J. Cannon and R. H. McKay, *Biochem. Biophys. Res. Commun.*, 35 (1969) 403–409.

13 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1941) 384–421.
14 V. Y. Fain, in R. W. A. Oliver (Editor), *The Electronic Spectra of Anthraquinones*, 1970.
15 P. F. Gordon and P. Gregory, *Organic Chemistry in Colour*, Springer, Berlin, Heidelberg, 1983.
16 R. H. Peters and H. H. Summer, *J. Chem. Soc.*, (1953) 2101–2110.
17 W. I. Jankowski, W. Muenchhausen, E. Sulkowski and W. A. Carter, *Biochemistry*, 15 (1976) 5182–5187.
18 R. S. Beissner and F. B. Rudolph, *J. Chromatogr.*, 161 (1978) 127–135.
19 R. J. Leatherbarrow and P. D. G. Dean, *Biochem. J.*, 189 (1980) 27–34.
20 C. R. Lowe, M. Hans, N. Spibey and T. W. Drabble, *Anal. Biochem.*, 104 (1980) 23–28.
21 M. J. Harvey, C. R. Lowe, D. B. Craven and P. D. G. Dean, *Eur. J. Biochem.*, 41 (1974) 335–340.
22 C. R. Lowe, *Pure Appl. Chem.*, 51 (1979) 1429–1441.
23 C. R. Lowe, I. P. Trayer and H. R. Trayer, *Methods Enzymol.*, 66 (1980) 192–208.
24 J. E. Wilson, *Biochem. Biophys. Res. Commun.*, 72 (1976) 816–823.
25 D. K. Apps and C. D. Gleed, *Biochem. J.*, 159 (1976) 441–443.
26 Z. Yoshida and F. Takabayashi, *Tetrahedron*, 24 (1968) 913–943.
27 F. Quadri, *Trends Biotechnol.*, 3 (1985) 7–12.