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USE OF TRIAZINE DYES IN THE AFFINITY CHROMATOGRAPHIC PURIFICATION OF ALKALINE PHOSPHATASE FROM CALF INTESTINE

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SUMMARY

Dye-ligand chromatography was examined as a method for the purification of alkaline phosphatase (EC.3.1.3.1). Forty six dye–Matrex Gels were assessed for their ability to bind alkaline phosphatase. Most dye adsorbents bound significant quantities of the enzyme. Three dye columns were examined in more detail for their selectivity using gradients of potassium chloride to desorb enzyme protein. Purification of alkaline phosphatase using Cibacron blue 3GA–Sepharose 6B chromatography was enhanced by using affinity elution. The best purifications (290-fold) were obtained using pulsed elution with the substrate α-naphthyl phosphate although the inhibitor, inorganic phosphate, was also useful (128-fold purification).

INTRODUCTION

The purification of alkaline phosphatases from a variety of sources has presented many problems[1]. These include removal of contaminating enzymes[1], particularly phosphodiesterases[2,3] and overall yields of the enzyme after purification[4]. The enzyme from calf intestinal mucosa has been of particular interest[5] because of its high specific activity[1] and potential in enzyme-coupled immunoassays. Methods for the purification of this enzyme have been described which incorporate both conventional[6] and affinity[1] procedures. The former methods produce low yields of enzyme whilst the latter require organic synthetic work to prepare the affinity columns. Immobilised fibre-reactive dyes[7] are proving useful additions to the chromatographic systems available for enzyme purification. Recent work on the applications of these dyes suggests that they may be useful for the purification of many enzymes, not necessarily those with nucleotide binding sites[8,9].

In the light of this and in view of the difficulty of isolation of pure alkaline phosphatase by previously described methods, we decided to examine the use of immobilised triazine dyes for the enzyme's purification. Furthermore, the latter media are more readily available than those described for the affinity chromatography of alkaline phosphatases[2,4]. When crude alkaline phosphatase was chromatographed on immobilised dye columns it was bound to most of the immobilised dyes tested. In this paper we report on the chromatographic properties of selected dye columns and the effects of various elution procedures.

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EXPERIMENTAL

Materials

4-Nitrophenyl disodium orthophosphate was obtained from BDH (Poole, Great Britain); β-naphthyl phosphate acid, α-naphthyl phosphate (disodium salt), bovine serum albumin and Fast Blue BB salt were purchased from Sigma, St. Louis, MO, U.S.A.; crude extracts of calf intestine containing alkaline phosphatase were obtained from Biozyme (Blaenavon, Great Britain); Procion dyes were obtained from I.C.I. Organics Division (Blackley, Great Britain); Sepharose 6B was purchased from Pharmacia (London, Great Britain); Matrex Gel, Model 202 ultrafiltration cell and PM10 membranes were supplied by Amicon (Lexington, MA, U.S.A.).

Methods

Cibacron blue and Procion dye derivatives of Sepharose 6B and Matrex Gel were prepared by the method of Heyns and De Moor[10].

Alkaline phosphatase was assayed at 25°C in semi-micro cuvettes in a total volume of 1.0 ml. The reaction mixture consisted of 3 m*M* 4-nitrophenyl disodium orthophosphate, 1 m*M* $MgCl_2$, 0.1 m*M* $ZnCl_2$, in 0.1 *M* glycine–NaOH, pH 10.3. The reaction was initiated by the addition of enzyme solution and was followed by *p*-nitrophenol liberation at 405 nm ($\varepsilon = 18.5 \cdot 10^3$ l mol^{-1} cm^{-1}). Ligand concentrations were determined using "wet gel" weight. Samples of all dyed agaroses, Sepharose 6B and Matrex Gel were hydrolysed by boiling for 10 min in 50% (v/v) acetic acid.

Cibacron blue concentrations were determined at 610 nm ($\varepsilon = 13 \cdot 10^3$ l mol^{-1} cm^{-1} at pH 7.0). Protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as standard.

Analytical polyacrylamide–disc gel electrophoreses were run in 7.5% gels in Tris–glycine, pH 8.5 (ref. 12).

Salt concentrations were measured using a Radiometer CDM3 conductivity meter and were determined by comparison with standard salt solutions. Gels were stained for alkaline phosphatase activity by incubation at roomtemperature in the dark in a 0.1 *M* glycine–NaOH buffer, pH 10.3 containing β-naphthyl phosphate acid (2 mg/ml) and Fast Blue BB salt (1 mg/ml). When the bands were satisfactorily developed, the staining solution was replaced with 7% (v/v) acetic acid.

RESULTS AND DISCUSSION

The result of screening of 46 immobilised dye adsorbents (1 ml columns) is summarized in Table I. The table shows that all but two of the columns were able to bind the enzyme from crude calf intestinal extracts albeit to different degrees. It is also clear from the protein appearing in both void volumes (data not shown) and potassium chloride eluates that (at the ligand concentrations used) different dyes show widely differing capacities for alkaline phosphatase.

The screening of these dye columns was conducted at relatively high pH since it has been observed[13] that only more tightly bound proteins are adsorbed at these pH values. This approach would therefore tend to select those columns which bound the enzyme most tightly.

Further examination of Procion yellow HE-3G–Sepharose 6B was made using

TABLE I

CAPACITIES FOR DYE–MATREX GEL ADSORBENTS FOR CALF INTESTINE ALKALINE PHOSPHATASE

Dye–Matrex Gel columns (6 × 0.5 cm, 1 ml) were equilibrated in 50 m*M* Tricine–NaOH buffer pH 8.0. A sample of crude calf intestine extract containing alkaline phosphates (20 U) was applied to each of the dye columns of the same buffer (in 0.2 ml). The columns were washed with the same buffer (20 ml), and alkaline phosphatase which was eluted by addition of 1 *M* KCl (in equilibration buffer) was assayed (see Experimental). Ligand concentrations (*L*) expressed as mg dye per ml gel, were determined by the procedure described (see Experimental). Control gel contained undyed Matrex Gel.

		L (mg/ml)	*Enzyme (units) eluted with KCl*
Procion blue	P3BN	1.9	3.3
	MX2G	4.3	16.0
	MXG	0.8	9
	HGR	3.8	1.2
	SP3R	1.8	1.3
	MX 7RX	3.9	0
	H5R	1.9	9
	MXR	4.7	5.5
	MX3G	4.8	7
Cibacron blue	3GA	3.5	11
Procion red	MX5B	3.8	2
	MX8B	4.4	
	H3B	5.6	9
	HE7B	4.8	11
	MX2B	5.0	1
	MXG	2.1	1.2
	P 3BN	3.5	1.4
Procion yellow	HA	2.9	0
	HE3G	2.3	16
	H5G	1.6	6
	MX6G	3.6	2
	MXR	3.7	2
	H3R	2.7	4
	M4G	2.6	7.5
	MX 3R	5.2	1.6
	MXGR	4.7	4.5
	MX4G	2	5
	HE4R	4	5
Procion green	HE4BD	3.9	7
Procion brown	H2G	4.2	5.5
	HGR	2.2	8.4
	H5BR	6.3	3.5
	H5R	3.9	5.6
	MX5BR	6.4	6.6
	H4RD	2.7	1.5
Procion scarlet	MXG	5.3	2
	HRN	3.8	6
Procion navy	MXRB	4.9	3.5
	H4R	3.8	3.6

(Continued on p. 524)

TABLE I *(continued)*

		L (mg/ml)	*Enzyme (units) eluted with KCl*
Procion turquoise	H5G	0.9	2.2
	H7G	5.8	1.8
	MXG	1.1	4.5
Procion olive	H7G	6.0	10
	MX3G	5.1	10
Procion orange	H2R	3.7	5.6
	HER	4.8	9.2
Control		0	0

a larger column (25 × 1 cm, 20 ml; Fig. 1). Crude alkaline phosphatase (100 U) was applied to the column, which was washed with equilibration buffer (70 ml) and then with a gradient of 0 to 1 *M* KCl (100 ml). Fractions containing enzyme activity (62 units = 62% yield) were pooled and showed an increase in specific activity of 8.5-fold. The peak of emergent activity contained 0.2 *M* KCl.

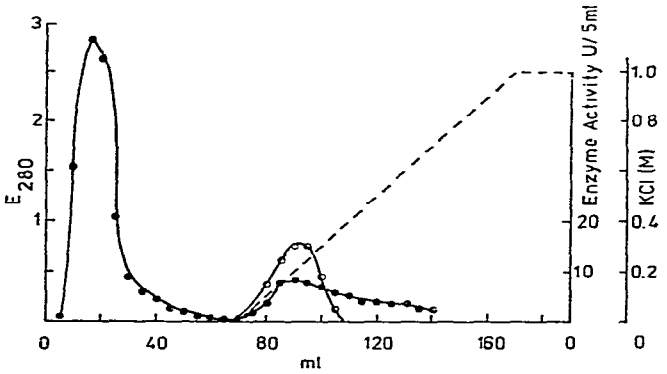

Fig. 1. Chromatographic profile of alkaline phosphatase from calf intestine extracts using Sepharose-6B–immobilised Procion yellow HE3G. A column of Procion yellow HE3G–Sepharose 6B (25 × 1 cm, 20 ml) was equilibrated in 50 m*M* Tricine–NaOH buffer pH 8.0. A sample of crude extract from calf intestine containing alkaline phosphatase (100 U) in equilibration buffer (2 ml) was applied to the column. The column was washed with the same buffer (70 ml). The column was then eluted with a linear gradient of KCl, 0–1 *M* (100 ml) in the same buffer. Fractions (5 ml) were collected and assayed for alkaline phosphatase activity and protein (see Experimental). Fractions containing enzyme activity were pooled to permit the determination of the overall purification factor. ●, E_{280}; ○, alkaline phosphatase activity; – – – –, KCl concentration. Flow-rate, 5 ml/h.

A similar experiment was conducted with Sepharose 6B–immobilised Procion scarlet MXG (100 ml, Fig. 2). Crude extract containing 100 units of enzyme activity were applied. The column was washed with equilibration buffer (500 ml) and then with a linear gradient of 0 to 1 *M* KCl (500 ml). Alkaline phosphatase (92 units = 92% yield) was recovered in a narrow peak (which centred on 0.1 *M* KCl). The increase in specific activity was 43-fold.

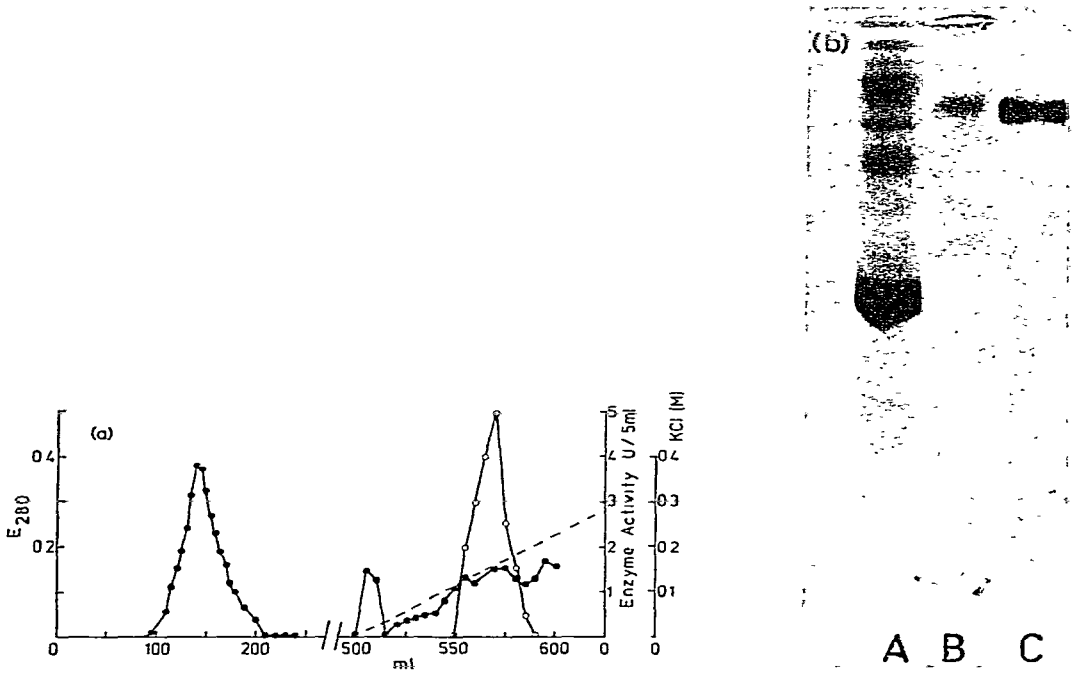

Fig. 2a. Chromatographic profile of alkaline phosphatase from calf-intestine extracts using Sepharose 6B–immobilised Procion scarlet MXG. A column of Procion scarlet MXG–Sepharose 6B (16 × 2.8 cm, 100 ml) was equilibrated in 50 m*M* Tricine–NaOH buffer pH 8.0. A sample of crude extract from calf intestine (3 ml) containing alkaline phosphatase (100 U) was applied to the column. The column was washed with equilibration buffer (500 ml) and a linear KCl gradient of 0–1 *M* (500 ml) in the equilibration buffer was applied. Fractions (5 ml) were collected and assayed for alkaline phosphatase activity and protein (see Experimental). Fractions containing enzyme activity were pooled. ●, E_{280}; ○, alkaline phosphatase; – – – –, KCl concentration. Flow-rate, 20 ml/h.
Fig. 2b. Polyacrylamide–gel electrophoresis of purified alkaline phosphatase from calf intestine prepared by elution of bound enzyme from immobilised Procion scarlet MXG using KCl gradients. Gel (A), electrophoresis of crude calf intestine extract stained with 1% (w/v) amido black in 7% (v/v) acetic acid. Gel (B), electrophoresis of protein eluted with KCl. Gel (C), was as (B) but stained for alkaline phosphatase activity.

A column of immobilised Cibacron blue 3GA–Sepharose 6B (25 × 1.1 cm, 25 ml, Fig. 3) was used to purify the intestinal crude extract containing alkaline phosphatase (150 units). The column was washed with equilibration buffer (80 ml) followed by a linear gradient of 0 to 1 *M* KCl (100 ml).

The enzyme which emerged was pooled (98 units = 66% yield) had an overall specific activity of 24.5 U/mg, an increase of 17-fold over the initial extract.

It is evident from the above experiments that pulses of salt may be used to desorb the calf intestine alkaline phosphatase from triazine dye columns. Furthermore, larger increases in specific activity of 10- to 40-fold may be obtained using salt gradients rather than pulses of this eluent.

We conclude that dye ligand chromatography can provide useful purifications of alkaline phosphatase. Polyacrylamide gel electrophoresis of these purified preparations (Fig. 2b) shows substantial reduction in the number and intensity of visible

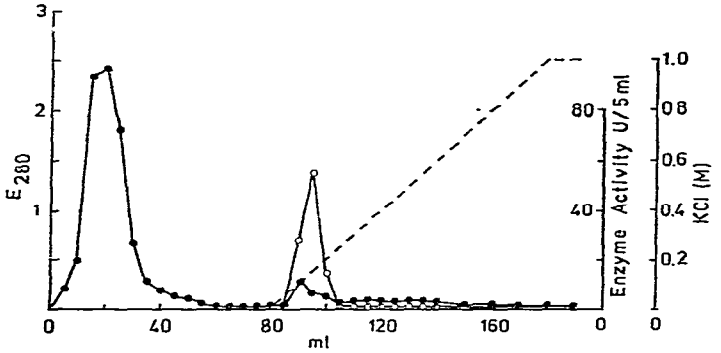

Fig. 3. Chromatographic profile of alkaline phosphatase from calf intestine extracts using Sepharose 6B-immobilised Cibacron blue 3GA. A column of Sepharose 6B–Cibacron blue 3GA (25 × 1.1 cm) 25 ml) was equilibrated in 50 m*M* Tricine–NaOH buffer pH 8.0. A sample of crude extract from calf intestine (2 ml) containing alkaline phosphatase (150 U) was applied to the column. The column was washed with the equilibration buffer (80 ml) and a linear gradient 0–1 *M* (100 ml) KCl in the equilibration buffer was applied. Fractions (5 ml) were collected and assayed for alkaline phosphatase activity and protein (see Experimental). Fractions containing enzyme activity were pooled. ●, E_{280}; ○, alkaline phosphatase activity; – – – –, KCl concentration. Flow-rate, 5 ml/h.

protein bands but clearly there are several contaminating proteins that do not coincide with alkaline phosphatase activity. Therefore, we examined the use of affinity elution to eliminate these contaminating proteins.

When used as an eluent, inorganic phosphate, a competitive inhibitor of alkaline phosphatase[14], increased the specific activity of the enzyme eluted from immobilised Cibacron blue 3GA to 180 U/mg representing approximately 130-fold purification (Fig. 4). In this case a gradient of inorganic phosphate was unnecessary

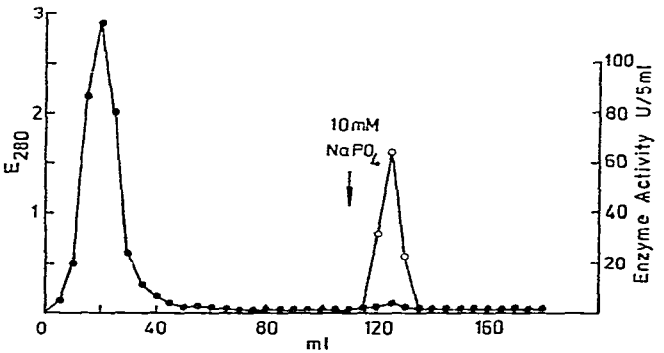

Fig. 4. Affinity elution of alkaline phosphatase from Cibacron blue 3GA–Sepharose 6B using phosphate. A column of Cibacron blue 3GA–Sepharose 6B (25 × 1.1 cm, 25 ml) was equilibrated in 50 m*M* Tricine–NaOH buffer, pH 8.0. A sample of crude extract from calf intestine (2 ml) containing alkaline phosphatase (135 U) was applied to the column. The column was washed with equilibration buffer (110 ml). Alkaline phosphatase was eluted using 10 m*M* Na_2HPO_4 (100 ml) in the equilibration buffer. Fractions (5 ml) were collected and assayed for alkaline phosphatase activity and protein (see Experimental). Fractions containing enzyme were pooled. Flow-rate, 5 ml/h. ●, E_{280}; ○, alkaline phosphatase activity.

and pulsed elution gave good yields (88%) and useful purifications. Furthermore, the yield of alkaline phosphatase could be increased by altering the phosphate concentration (Fig. 5). 10 m*M* phosphate is optimal since it gave the best yield at the lowest concentration of eluate.

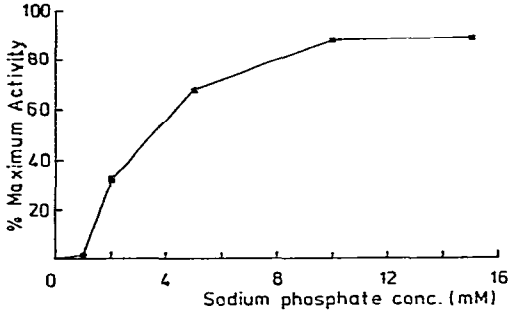

Fig. 5. The effect of the concentration of the affinity eluent phosphate on the recovery of alkaline phosphatase from Cibacron blue 3GA–Sepharose 6B. The experiments were conducted exactly as described in Fig. 4, except that the concentration of phosphate was varied from 1–15 m*M*. The recovery is expressed as a percentage of the total enzyme applied to the column (135 U).

A comparison with the work of Brenna *et al.*[1] and Landt *et al.*[2] who describe affinity chromatographic methods for this enzyme shows that similar purifications could be obtained using phosphate as eluent. Brenna *et al.*[1] obtained a 52-fold purification (to homogeneity) using phosphate gradients, whilst Landt *et al.*[2] used a single step increase in phosphate concentration to produce a 10-fold purification. These examples and the experiments described in Fig. 4 illustrate the value of affinity desorption irrespective of the mechanism of the adsorption phase of the chromatographic process.

Figs. 6 and 7 show that affinity elution with 10 m*M* α-naphthyl phosphate yields higher purification factors than inorganic phosphate (295-fold compared with the crude extract, 89% yield). Seargeant and Stinson[4] obtained 400-fold purification of human liver alkaline phosphatase using β-naphthyl phosphate elution from a phosphonic acid gel. These results indicate that naphthyl phosphates (α and β) produce higher purification factors than phosphate alone when used with either dye ligand or affinity adsorbents. Seargeant and Stinson[4] found that most of the protein applied to their phosphonic acid adsorbent was bound non-specifically which necessitated the use of affinity elution with α-naphthyl phosphate.

Brenna *et al.*[1] have synthesised two types of adsorbent (Fig. 8D) (1) with an arsonic acid group; (2) with a sulphonic acid as a control. The capacity of the sulphonated adsorbent for alkaline phosphatase was only 10% when compared with the arsonate analogue. Table I shows many of the dye-agarose derivatives examined have capacities comparable to those of the arsonate gel described by Brenna *et al.*[1]. This work suggests that careful screening of immobilised dyes can produce effective adsorbents which when coupled with affinity elution will provide useful purification steps in the purification of alkaline phosphatase and other enzymes.

The similarity between the dye-ligand structures and those used by Brenna *et al.*[1] and Landt *et al.*[2] and Seargeant and Stinson[4] is shown in Fig. 8, together with

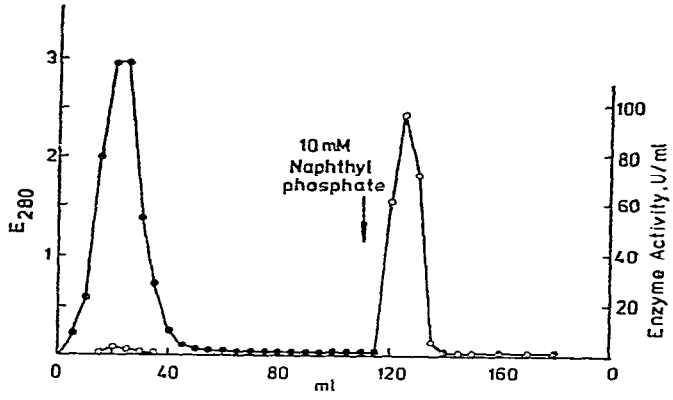

Fig. 6. Affinity elution of alkaline phosphatase from Cibacron blue 3GA–Sepharose 6B by α-naphthyl phosphate. A column of Cibacron blue 3GA–Sepharose 6B (25 × 1.1 cm, 25 ml) was equilibrated in 50 m*M* Tricine–NaOH buffer pH 8.0. A sample of crude extract from calf intestine (2 ml) containing alkaline phosphatase (260 units) was applied to the column. The column was washed with equilibration buffer (110 ml). Alkaline phosphatase was eluted by a pulse of 10 m*M* α-naphthyl phosphate (65 ml) in the same buffer. Fractions (5 ml) were collected. Fractions containing enzyme activity were pooled, concentrated by ultrafiltration (see Experimental), dialysed and then reassayed for enzyme activity and protein (see Experimental). ●, E_{280}; ○, alkaline phosphatase activity. Flow-rate, 5 ml/h. Measurement of E_{280} was discontinued after the addition of α-naphthyl phosphate because of the optical density of the latter.

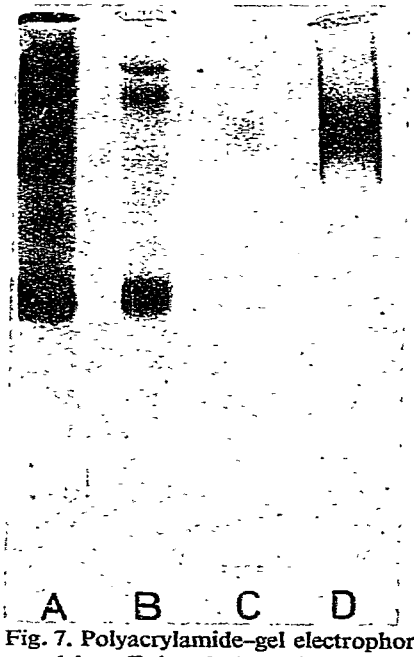

Fig. 7. Polyacrylamide–gel electrophoresis of purified alkaline phosphatase from calf intestine prepared by affinity elution with α-naphthyl phosphate from Cibacron blue 3GA–agarose. Gel (A), electrophoresis of crude calf intestine extract stained with 1% (w/v) amido black (in 7%, v/v, acetic acid); Gel (B), electrophoresis of protein appearing in the void volume of the Cibacron blue 3GA–agarose column (Fig. 6) stained as in gel (A). Gel (C) and (D) electrophoresis of protein eluted with 10 m*M* α-naphthyl phosphate from the Cibacron blue 3GA–agarose column (Fig. 5), gel (C) was stained with amido black as in (A) whereas gel (D) was stained for alkaline phosphatase activity, see Experimental.

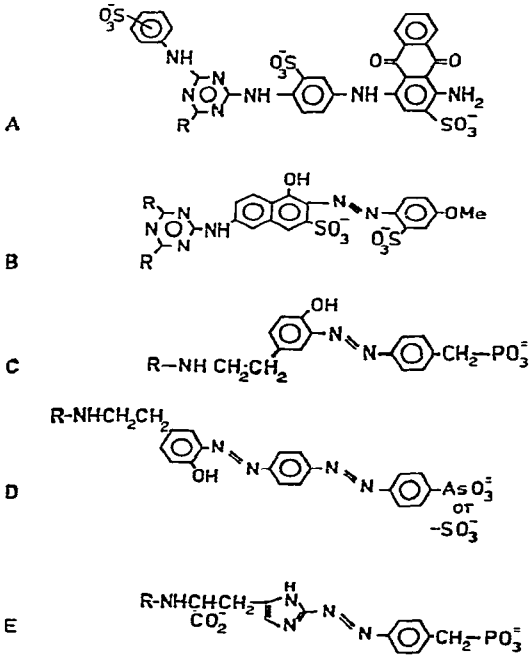

Fig. 8. Structures of two of the dyes investigated in this work and three affinity ligands reported in the literature for the chromatography of alkaline phosphatase. A = Cibacron blue 3GA; B = Procion scarlet MXG; C = phosphonate derivative described by Seargeant and Stinson[4]; D = arsonate and sulphonate derivatives described by Brenna *et al.*[1]; E = phosphonate derivative described by Landt *et al.*[2]; R = the support matrix.

the published structure of two of the dyes used in this study. In view of the close similarity in disposition of hydrophobic, hydrophilic and charged species it is perhaps not surprising that alkaline phosphatases are observed to behave in a similar fashion on both types of adsorbent.

The specific activity of the alkaline phosphatase obtained after the use of affinity elution with α-naphthyl phosphate from immobilized Cibacron blue 3GA was lower than that observed by Brenna *et al.*[1]. However, we have found only one band on polyacrylamide–gel electrophoresis which stained for enzymic activity while these authors reported two bands. We were unable to obtain the latter two bands even with crude extracts. It is possible that in the extraction of the enzyme from calf intestine protein, one of the proteins described by Brenna *et al.*[1] is lost.

Unlike the triazine dyes, many affinity adsorbents are complex chemical structures requiring difficult and expensive synthetic routes for their preparation. The poor stability (both chemical and biological) of numerous affinity adsorbents (such as immobilised nucleotides) often leads to deterioration of the ligand after only a few uses. On the other hand, immobilised triazine dyes are stable to bacterial attack (the free dyes are bacteriostatic) and their capacities are often very high per ml of gel bed volume. Furthermore, the coupling of preactivated triazine dyes to supports is easily carried out and is not hazardous when compared with cyanogen bromide activation (for a review, see Dean and Watson[15]). Thus, the triazine dye adsorbents appear to be satisfactory as alternatives to previously described affinity media.

CONCLUSION

We have examined a range of triazine dyes as ligands for the chromatographic purification of alkaline phosphatase. Salt elution can be used to screen a large number of such dyes. Affinity elution with naphthyl phosphate was then used to examine a few selected dyes. The latter procedure has yielded highly purified alkaline phosphatase from calf intestine extracts.

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