functional resemblance of column groups to carbamoyl phosphate. The enzyme binds its two substrates, carbamoyl phosphate and L-aspartate, in an obligatory order, with carbamoyl phosphate leading (Grayson et al., 1979). If column groups are sufficiently similar to carbamoyl phosphate, the enzyme should be adsorbed more tightly in the presence of L-aspartate, owing to the 'locking-on' effect (O’Carra, 1978).

Since no difference could be seen in the elution profiles containing L- and D-aspartate (Fig. 2), it is concluded that (a) the column groups do not resemble carbamoyl phosphate sufficiently to elicit the ordered-binding effect, and (b) column groups do not resemble L-aspartate. Both columns are therefore essentially non-biospecific in their adsorption of the enzyme.

Carbamoyl phosphate has been shown to desorb the enzyme from a variety of mixed-function adsorbents, and evidence has been obtained to show that desorption is biospecific, i.e. involves the catalytically-significant enzyme-substrate complex (Yon, 1980). Part of the evidence depends on comparing the desorbing capabilities of carbamoyl phosphate, P, and 2-glycerophosphate with their dissociation constants determined from steady-state kinetic and inhibition studies. These constants are 0.03 mm, 1.0 mm and 20 mm respectively (Grayson et al., 1979). If desorption is biospecific, these compounds should be effective in the same order as their enzyme-ligand affinities, i.e. carbamoyl phosphate > P, > 2-glycerophosphate. This expectation is confirmed for both adsorbents by the results shown in Fig. 3. However, the weaker ligands, P, and 2-glycerophosphate, were more effective in desorbing the enzyme from Blue Sepharose than from 10-CD-Sepharose; this was also true for carbamoyl phosphate at a much lower concentration (1 μM). Thus the adsorbent that binds the enzyme with lower affinity is also most responsive to biospecific desorption, in agreement with a theoretical prediction (Yon, 1980).

It seems appropriate in the context of the present Colloquium to consider the relevance of this work to 'mainstream' affinity chromatography. Biospecific desorption is widely practised in 'affinity' chromatography, and in the absence of firm evidence to the contrary, purifications are just as likely to be due to mechanisms (2)–(4) above as to mechanism (1). It is unfortunate that, after the initial (and still widely quoted) unambiguous definition of affinity chromatography by Cuatrecasas et al. (1968), the term has come to be used much more loosely; the literature contains many examples of 'affinity chromatography' in which the only certain biospecificity is in desorption, and biospecificity is adsorption is merely assumed. Certainly the time seems overdue for renewed consideration of the meaning we give to this term, and the title of the present Colloquium reflects, I hope, a willingness to come to grips with this problem.

O’Carra, P. (1978) in Chromatography of Synthetic and Biological Polymers (R. Epton, ed.), vol. 2, pp. 131–158, Ellis Horwood Ltd., Chichester

**Triazine-dye affinity chromatography**

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Affinity chromatography provides an elegant and efficient method for the extensive purification of an individual protein from a complex mixture (Lowe & Dean, 1974). In general terms such chromatographic systems employ enzyme substrates, inhibitors or cofactors, or sub-structures of these, as ligands immobilized on agarose beads. Although purification of an individual protein, often to homogeneity, has frequently been achieved on a laboratory scale and the ligand–agarose matrices have proved valuable in enzyme-kinetic and mechanistic studies, scale-up of analytical and laboratory purifications to the development of chromogenic substrates (Ewen, 1973), affinity electrodes (Lowe, 1979), active site probes and affinity labels (Clonis & Lowe, 1980) and other systems are well documented in a recent review (Lowe et al., 1980).

For large-scale affinity chromatography, triazine dyes linked to agarose beads, or indeed other matrices, have enormous advantages over conventional affinity-ligand systems. The dyes themselves are extremely inexpensive; the coupling procedure is both rapid, simple and does not involve the use of toxic chemicals; the chemical stability of the triazine dye–agarose conjugate is excellent and matrices can be stored over many months with little loss in protein-binding capacity, and the matrices are re-usable over many applications (Atkinson & Harvey, 1978). In addition, these matrices are far more stable to biodegradation than conventional affinity matrices, which are susceptible to both ‘fouling’ by unwanted lipid and protein and degradation by proteinases, nucleotidases, phosphatases etc.

Direct formation of an ether link between the triazine ring and the hydroxy groups of agarose can be achieved either by the method described by Baird et al. (1976) or by Atkinson &
Harvey (1978). With the former method, dichlorotriazinyl (MX) dyes produce gels containing markedly higher dye concentrations than the corresponding monochlorotriazinyl (H) dyes. This difference is far less pronounced in the method of Atkinson & Harvey (1978), which is illustrated in Scheme 1. In general, this latter method produces dye concentrations in the region 1.6–2.8 mg of dye (ml of agarose)$^{-1}$ (Sepharose 4B, 6B, 4B-CL or 6B-CL). The individual dyes are not homogeneous, and with some, prior precipitation of the reactive triazine as the potassium salt from aqueous solution gives a more reproducible starting material. Removal of anti-dust agents by ether or acetone has little effect and has therefore been omitted. The effect of a variety of parameters on the coupling of two dyes, Procion Red MX-2B and Procion Red H-3B has been investigated. The coupling of dyes to the matrix is time-dependent. Immobilization of the dichlorotriazinyl (Procion Red MX-2B) dyes is rapid and virtually complete in 2 h. The reaction has a distinct optimum for alkali concentration (0.01 M), and at concentrations above this, the second chlorine atom on the triazine ring is rapidly hydrolysed, giving a sharp decrease in the amount of dye coupled. Any remaining chlorine atoms in the dye structure, after coupling, can be converted to hydroxy groups by incubating the matrix for 2 to 3 days at pH 8.5 at ambient temperature or to amino groups by treating with $\text{NH}_2\text{Cl}$ at pH 8.5 for 4 h at ambient temperature. Immobilization of the monochlorotriazinyl (Procion Red H-3B) dyes is slow at ambient temperature and only reaches a plateau after about 3 days. The reaction rate can be increased by elevated temperature, and with cross-linked agarose at 60°C the reaction rate is 3–4 times that at ambient temperature (20°C). The reaction is relatively insensitive to alkali concentrations in the range 0.05–0.2 M, although there is a slight decrease at
higher concentrations (0.4 M). The amount of dye incorporated is a function of dye concentration up to 2 mg of dye ml⁻¹. Above this the relationship is non-linear, and at concentrations above 4 mg of dye ml⁻¹ little increase in incorporation can be detected. Although a reasonable amount of coupling can be achieved in the absence of NaCl, optimal dye coupling requires a salt concentration above 0.25 M.

The protein-binding capacities of triazine dye-agarose matrices prepared as above are generally in the range 1–6 mg of a specific enzyme from a crude protein mixture (ml of matrix)⁻¹, although capacities for serum albumin are much higher (Atkinson & Dean, 1978; Leatherbarrow & Dean, 1980). Elution of enzyme from these matrices may be effected by a number of methods (Lowe et al., 1980).

On the preparative scale, substrate elution has been employed in the purification of carboxypeptidase G, which hydrolyses the terminal glutamate residue from folacin acid and its analogues, in the purification of carboxypeptidase G, which hydrolyses the terminal glutamate residue from folacin acid and its analogues.

The matrix described above has a much higher capacity for serum albumin than the Procion Red HE-3B matrices described by Baird et al. (1976). A total of 42 triazine dyes have been screened for carboxypeptidase G binding and substrate elution and at least another five dyes give results similar to Red H-8BN.

Substrate elution has also been used to purify glyceraldehyde 3-phosphate dehydrogenase from Bacillus stearothermophilus on a macro scale. After a preliminary screen, 16 matrices were found that the enzyme would bind to at pH 5.5 with elution effected by salt or increasing pH. Three matrices: Procion Blue MX-3G, Procion Blue H-B and Cibacron Blue F3-GA all immobilized on Sepharose 6B bound the enzyme well at pH 7.5 and could be eluted with ATP. After cell breakage, a pH precipitation to remove unwanted protein and gradient DEAE-Sephadex chromatography, 10 mg (10⁶ units) of glyceraldehyde 3-phosphate dehydrogenase, approx. 20% pure, was bound on a 3.5-litre Procion Blue MX-3G-Sepharose 6B column in 25 mM-phosphate, pH 7.5, and eluted as homogeneous protein in 85% yield in 0.1–0.2 column volumes, at a peak fraction concentration of 30 mg ml⁻¹ by a pulse of 5 mM Mg-ATP in the same buffer. As with carboxypeptidase G, relatively high flow rates are needed, but in this case to prevent the binding and ATP-dependent elution of trace quantities of other kinases. The kinetics of ATP-dependent elution of glyceraldehyde 3-phosphate dehydrogenase from Procion Blue MX-3G are interesting in that at low dye concentrations [0.2–0.8 mg ml⁻¹ of Sepharose]⁻¹, binding of the enzyme increases almost linearly, as does the efficiency of ATP elution: although at high dye concentrations [2.6–4.0 mg of dye ml⁻¹ of Sepharose]⁻¹, enzyme binding decreases slowly, whereas the effectiveness of ATP elution decreases rapidly. Although this binding data is predictable, the increase in ATP effectiveness in elution is not. Other systems have also been developed in which glyceraldehyde 3-phosphate dehydrogenase can be bound to Procion matrices in the presence of glycerol (and Mg²⁺ ions) and eluted by their removal.

Both the versatility and advantages of triazine-dye chromatography are illustrated by the preparative-scale purification, employing both cofactor and salt elution, of 3-hydroxybutyrate dehydrogenase (HBDH) and malate dehydrogenase (MDH) from Rhodopseudomonas sphaeroides. Conventional purification of the former is particularly tedious, involving eight steps and an overall 9% yield (Bergmeyer et al., 1967). About 1 kg of cell-free extract was loaded on a 1.8-litre Procion Red H-3B–Sepharose 4B column at pH 7.5 and HBDH eluted with 1 M-KCl in 90% yield and with a 30-fold purification, followed by MDH, eluted with 2 M-NADH in 1 M-KCl. The dialysed pool was then absorbed on 800 ml column of Procion Blue MX-4GD–Sepharose 4B at pH 7.5 and after washing with 1 M-KCl 300 mg of homogeneous HBDH was eluted in 70% overall yield with a pulse of 2 M-NADH in 1 M-KCl. Partially purified MDH, obtained as above, was also applied to the Procion Blue MX-4GD column, and about 1 g of homogeneous enzyme was eluted with a salt gradient. Comparison of the binding and elution of MDH from both R. sphaeroides (RS-MDH) and yeast (Y-MDH) illustrates that the same enzyme from different sources behaves differently on triazine dyes; examination of 28 different triazine matrices revealed that Y-MDH did not bind to five dyes to which RS-MDH did bind; that RS-MDH required NADH for elution on eight dyes from which Y-MDH could be eluted with salt; and that on one dye this situation was reversed.

Even with a relatively non-specific eluant, for example salt, the advantage of the triazine dyes and ‘matching’ the dye to the enzyme can be seen with the purification of pseudocholinesterase from equine serum. After an intensive screen, a 500 ml column of Procion Red HE-7B–Sepharose 4B was used to bind 4200 units of this enzyme, partially purified from serum by DEAE-cellulose chromatography, at pH 6.8 in phosphate. Elution with 1 M-KCl has regularly given recoveries of 4000–6000 units (presumably due to inhibitor removal) with a specific activity increase from 0.08 unit mg⁻¹ to 3.0–5.0 units mg⁻¹, an average 50-fold purification.

Scheme 1. Preparation of triazine-dye matrices
Table 1. Purification of enzymes by triazine-dye affinity chromatography

Methods are detailed in the text. Abbreviations used: PABG, p-aminobenzoylglutamate; HBDH, 3-hydroxybutyrate dehydrogenase; MDH, malate dehydrogenase; MTS, methionyl tRNA synthetase; WTS, tryptophyl-tRNA synthetase; H, homogeneous. Notes: *enzyme at two different stages of purification; ° peak fractions homogeneous; ‡ gradient elution; † removal of an inhibitor gives an artificially high recovery.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Triazine-dye ligand</th>
<th>Eluant</th>
<th>From</th>
<th>To</th>
<th>Yield per step (%)</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboxypeptidase G</td>
<td>Red H-8BN</td>
<td>PABG</td>
<td>2.3*</td>
<td>73</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10°</td>
<td>280</td>
<td>75</td>
<td>60° (H)</td>
</tr>
<tr>
<td>Glycerokinase</td>
<td>Blue MX-3G</td>
<td>ATP</td>
<td>25</td>
<td>120</td>
<td>80</td>
<td>H</td>
</tr>
<tr>
<td></td>
<td>Red H-3B</td>
<td>KCl</td>
<td>0.1</td>
<td>3.1</td>
<td>95</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Blue MX-4GD</td>
<td>NADH/KCl</td>
<td>3.1</td>
<td>19.2</td>
<td>80</td>
<td>H</td>
</tr>
<tr>
<td>MDH</td>
<td>Red H-3B</td>
<td>NADH/KCl</td>
<td>5.4</td>
<td>105</td>
<td>80</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Blue MX-4GD°</td>
<td>KCl</td>
<td>105</td>
<td>292</td>
<td>80</td>
<td>H</td>
</tr>
<tr>
<td>Pseudo-cholinesterase</td>
<td>Red HE-7B</td>
<td>KCl</td>
<td>0.1</td>
<td>4.5</td>
<td>100°</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Green HE-4BD</td>
<td>P°</td>
<td>16</td>
<td>200</td>
<td>70</td>
<td>H</td>
</tr>
<tr>
<td>WTS</td>
<td>Brown MX-5BR</td>
<td>Trp</td>
<td>7.6</td>
<td>1040</td>
<td>88</td>
<td>H</td>
</tr>
</tbody>
</table>

Summaries of all the above purifications are listed in Table 1. One of the more intriguing groups of enzymes, from both the viewpoint of the difficulty of purification and the desirability of developing affinity labels for protein structure probing, is the aminocarboxyl-tRNA synthetases. The binding of 13 of these to 32 immobilized Procion dyes has been studied (Bruton & Atkinson, 1979). The Procion dyes Green HE-4BD, Blue MX-4GD and Red H-8BN proved almost universal adsorbents for these enzymes and certainly the polyanionic nature and sulphonic acid group distribution of the former dye together with its remarkable susceptibility to phosphate rather than chloride elution of these enzymes suggests that this dye may mimic the phosphodiester backbone of nucleic acids. Principally two purification systems to give homogeneous enzyme in high yields were devised (Bruton & Atkinson, 1979); the phosphate-gradient elution of methionyl-tRNA synthetase, from Procion Green HE-4BD giving a 12-fold purification and replacing two conventional columns and, intriguingly, the tryptophan-dependent elution of tryptophanyl-tRNA synthetase from Procion Brown MX-5BR giving a 137-fold purification to homogeneity in one step (Table 1). Since the only other tRNA synthetase significantly eluted from Procion Brown MX-5BR by its free amino acid was the tyrosyl-tRNA synthetase it could be argued that this dye bears some structural resemblance to aromatic amino acids.

Preliminary reports of affinity labelling of enzymes with triazine dyes have been published (Lowe et al., 1980; Clonis & Lowe, 1980). However, since most of the dyes are heterogeneous, affinity-labelling data must be interpreted carefully (Lowe et al., 1980). Recently, techniques have been developed for the separation of individual components of several commercial dye preparations (D. A. P. Small, A. Atkinson & C. R. Lowe, unpublished work) and the inactivation kinetics of a number of enzymes, including dehydrogenases and kinases, have been investigated by employing both homogeneous active and 'inactive' (methoxylated at chlorine atom) triazine dyes.