# REACTIVE DYES IN PROTEIN AND ENZYME TECHNOLOGY

Edited by

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# Preface

The initial observations which led to the first use of reactive dyes in biochemistry predates the elucidation of the genetic code. As with so many critical but unusual and unexpected discoveries, the importance and breadth of applications were hardly even imagined in those early days. In the ten years immediately following the pioneering work, fewer than thirty papers were published on further applications. However, in 1976 alone we have details of thirty-two and the first century was recorded in 1979.

This rapid expansion in the utilisation of reactive dyes followed the combined realisations that the useful properties were not confined to just one or two dye molecules and that, in marked contrast to the earlier theories, the dyes could interact with a broad spectrum of proteins — from kinases to restriction endonucleases and from dehydrogenases to interferon. The research was given additional impetus by the wider availability of dozens of triazine dyes in suitable quantities for biochemical studies in the mid-1970s. All the researchers are greatly indebted to Dr C. V. Stead and his colleagues at the ICI Organics Division for much of this increased availability. We are also grateful for his contribution to this volume, on the basic chemistry of the reactive dyes — a subject which is frequently rather ignored by biochemists and biotechnologists to their cost.

The binding of Cibacron Blue F3G-A to dehydrogenases was first exploited in the development of new techniques for their purification. The entire family of reactive dyes is now used extensively in protein purification. If different species are counted, purification protocols for over a thousand proteins have been published. Two chapters on dye-ligand chromatography, one on the conventional scale and one on larger-scale applications, are very appropriate.

The dye-ligand technology is not limited to conventional matrices. HPLC supports have been modified to incorporate the reactive dyes to provide high-performance dye-ligand chromatography. This can also be operated on a preparative scale, and a further chapter describes these developments.

The binding of the triazine dyes to proteins can be affected by many other molecules and ions. These characteristics have led to dye-ligand

aqueous two-phase systems and metal ion-promoted dye-ligand chromatography. Both these areas are described in this volume.

The observation that many enzymes were specifically eluted from 'dye columns' by substrates or inhibitors suggested that the dyes were mimicking some parts of the structures of those substrates and inhibitors and that they could be used as active-site probes in the elucidation of enzymes' structures and mechanisms. Some particular examples of this novel approach are discussed in Chapter 8.

The Editors thank all the authors for their contributions and hope that these examples will stimulate the readers into further innovative exploitation of these remarkable molecules.

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# 1
# Introduction to the Use of Reactive Dyes in Biotechnology

C. R. Lowe

Dyes are coloured substances which can be applied in solution or dispersion to a substrate such as a textile fibre, paper, leather, hair, fur, plastics, wax, cosmetic base or foodstuff and bestow on the substrate a coloured appearance. In most cases, the substrate to be dyed possesses a natural affinity for the dye and readily absorbs it from solution or aqueous dispersion under suitable conditions of concentration, pH and temperature. As a result of this chemical affinity between substrate and dye, dyed substrates usually show some resistance to washing, although the property of fastness varies considerably. The first commercial synthetic dye, mauveine, a member of the safranine class of azine dyes, was introduced by Perkin in 1856 by the oxidation of aniline containing *o*- and *p*-toluidines, although its chemical constitution was not established until many years later. Working rules relating colour and dyeing properties with chemical composition emerged as more and more dyes were discovered. Thus, in 1868 it was suggested that colour was associated with unsaturation, since all the then known synthetic dyes could be decolorised by reduction (Graebe and Liebermann, 1868). This view was amplified when it was noted that the colour of organic dyes was associated with certain unsaturated functions, termed chromophores, and often comprising nitro, nitroso, azo, ethene and carbonyl groups (Witt, 1876). Later work recognised the importance of substituents on the chromophore, both to deepen its colour and to enhance the affinity of the dye for the natural fibre (Niezki, 1879). By the turn of the century it was suggested that colour might be the result of 'rhythmic vibrations in the ether' caused by oscillations in quinonoid forms of dyes interconnected by a conjugated chain of single and double bonds (Baeyer, 1907; Hewitt and Mitchell, 1907). It is now usual to regard a dye as comprising a substance in which the classical chromophore forms part of a conjugated chain of single and double bonds, often terminating in

a polar function which can exist in two or more adjacent states of covalency. The dye is thus seen as a resonance hybrid of two or more extreme structures with the transition from one to another occasioned by absorption of light and mediated by an electron surge through the conjugated chain, possibly via a series of intermediate structures. Figure 1.1 illustrates the two extreme resonance forms of the triphenylmethane dye Döbner's Violet.

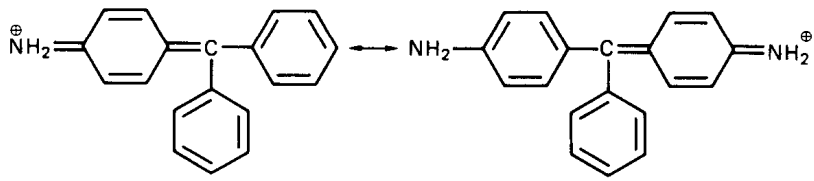
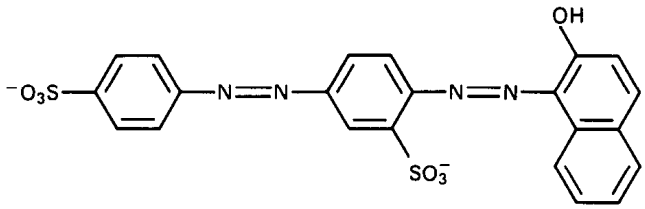
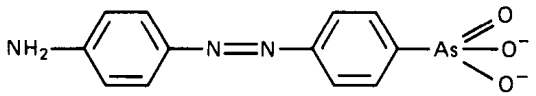


**Figure 1.1** Resonance in the triphenylmethane dye Döbner's Violet

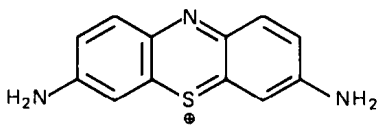
Many of the early dyes were specifically synthesised to stain natural protein substrates such as wool, silk and leather (Allen, 1971). These, mainly acidic azo dyes, interact with the side-chain amino acid residues of the major fibrous protein components, keratin, fibroin and collagen, respectively, via ionic, van der Waals' and hydrophobic forces. In globular proteins, however, it appears that strong binding of aromatic dye molecules occurs predominantly in areas overlapping the binding sites for biospecific ligands such as substrates, coenzymes and prosthetic groups, in preference to other regions of the protein surface (Glazer, 1970). It is likely that only at these sites can the rigid, nearly planar, dye molecules achieve sufficient contacts for tight binding, since elsewhere on the surfaces of globular proteins the complex asymmetric arrangement of side-chain groups precludes the formation of highly specific protein–dye complexes. The special stereochemical arrangement of active sites, their hydrophobicity, ionicity and capacity to hydrogen bond relative to other regions of the protein surface, and, conceivably, the greater flexibility in the polypeptide chains in the vicinity of the site, all contribute to the specific binding of dyes to these sites. Early studies with a number of common proteins and fifty dyes belonging to the azo, acridine, phenothiazine, cyanine, quinone-imine and anthraquinone classes have demonstrated strong stoichiometric dye–protein interactions by spectrophotometry and equilibrium dialysis. For example, equimolar complexes of thionine, Biebrich Scarlet and 4-(4′-aminophenylazo) phenylarsonic acid (Figure 1.2) were demonstrable with the proteases trypsin, chymotrypsin and subtilisin, respectively (Glazer, 1967a,b; 1968a). These protein–dye interactions were shown to be highly specific, with dyes of closely related structure either not being bound or very weakly bound. Furthermore, since



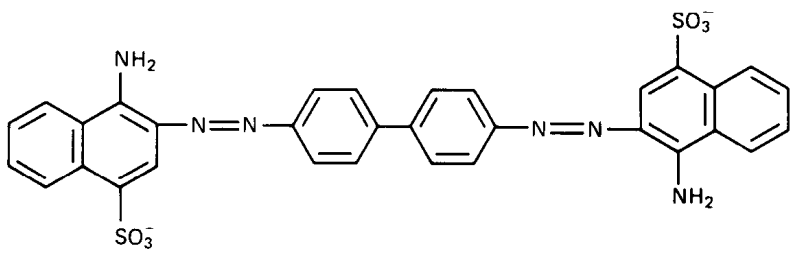

(a)




(b)




(c)




(d)

**Figure 1.2** The structures of (a) Biebrich Scarlet, (b) 4-(4′-aminophenylazo) phenylarsonic acid, (c) thionine and (d) Congo Red

the dyes were not bound by the respective zymogens and were displaced by substrates, competitive inhibitors and specific chemical reagents known to modify active site serine or histidine residues, it was assumed that the strong dye binding sites incorporated at least part of the active site region of the protein. Two striking features of these early investigations, however, were a complete failure to find strong interactions at any other site on the various proteins studied (Glazer, 1968b) and the fact that the structure of the dye bore no obvious relationship to those of the substrates of the proteins (Figure 1.2). Similar conclusions were drawn when 1:1 protein–dye complexes were formed at the active sites of luciferase (DeLuca, 1968) and lysozyme (Rossi *et al.*, 1969), the prosthetic group binding sites of apomyoglobin and apohaemoglobin (Stryer, 1965), the $NAD^+$-binding site of horse liver alcohol dehydrogenase (Brand *et al.*, 1967) and the biotin-binding site of avidin (Green, 1965). Again, these studies support the notion that substrate, prosthetic group or coenzyme binding sites on globular proteins provide a uniquely favourable environment for interaction with a variety of seemingly unrelated organic molecules (Glazer, 1968b, 1970). Dyes bound at enzyme active sites have subsequently been used as direct spectroscopic probes of enzyme structure and function (Perrin and Hart, 1970; Brand and Gohlke, 1972; Edwards and Woody, 1977). The visible absorption bands of dyes are well separated in energy from the region where most proteins absorb, and the perturbation of these bands on interaction with the protein provides a simple experimental monitor for the formation of dye–protein complexes. Such studies with Congo Red (Figure 1.2) and the anthraquinone dye Cibacron Blue F3G-A have shown that these dyes bind tightly to nucleotide-binding enzymes such as dehydrogenases and kinases, although they are not highly specific analogues of nucleotides and coenzymes and apparently do not assume a single unique conformation on different enzymes binding the same coenzyme (Edwards and Woody, 1977, 1979).

The interaction of the anthraquinone dye Cibacron Blue F3G-A with a diverse number of proteins has been the subject of intensive research for well over a decade (Lowe *et al.*, 1981). Interest in this particular dye stems from the fact that a dextran conjugate of Cibacron Blue F3G-A has been used for many years as a void volume marker for gel filtration. It was observed that yeast pyruvate kinase behaved anomalously and cochromatographed with Blue Dextran in the exclusion volume of a Sephadex G-200 gel filtration column, whereas in the absence of Blue Dextran the enzyme behaved as expected and eluted after the void volume (Haeckel *et al.*, 1968). The enzyme could be resolved from the Blue Dextran, and thus purified threefold, by repeating the Sephadex G-200 chromatography in 30 per cent ammonium sulphate. Subsequent studies demonstrated that it was the chromophore of Blue Dextran, Cibacron Blue F3G-A, that was responsible for the binding and not the dextran carrier itself

(Kopperschläger *et al.*, 1968, 1971). Within a few years similar use was made of Blue Dextran coupled with gel filtration to purify yeast phosphofructokinase (Kopperschläger *et al.*, 1971), glutathione reductase (Staal *et al.*, 1969), human erythrocyte pyruvate kinase (Blume *et al.*, 1971; Staal *et al.*, 1971), sweet corn R enzyme (Marshall, 1970) and blood coagulation factors II, VII, IX and X (Swart and Hemker, 1970). By 1973, procedures had been developed for the covalent attachment of Blue Dextran to insoluble supports such as agarose and the use of this material for the purification of various lactate dehydrogenases by dye-ligand chromatography (Ryan and Vestling, 1974). Since then, and to allow more specific elution of the species being isolated, both Blue Dextran and its reactive chromophore, Cibacron Blue F3G-A, have been immobilised to several supports, including Sephadex (Easterday and Easterday, 1974), polyacrylamide (Kopperschläger *et al.*, 1971; Meldolesi *et al.*, 1976) and agarose (Easterday and Easterday, 1974; Ryan and Vestling, 1974). These materials have now been used to purify a plethora of diverse proteins by dye-ligand chromatography, and extensive lists of such applications have now been compiled (Lowe, 1979a; Dean and Watson, 1979; Lowe *et al.*, 1981; Kopperschläger *et al.*, 1982; Lowe, 1984). By covalently attaching the blue dye to an insoluble, hydrophilic, porous support matrix, exposing the crude protein sample to the adsorbent, washing to remove unbound protein and eluting the specifically bound protein, purifications of up to several thousand-fold, in some cases in quantitative yield, can be obtained. Since its introduction in the late 1960s, the technique of affinity chromatography (Lowe and Clonis, 1985) has become established as the pre-eminent tool for the purification of enzymes and other proteins (Lowe and Dean, 1974; Lowe, 1977, 1979a; Lowe and Clonis, 1985). In particular, the development of 'group-specific' media employing an immobilised coenzyme or nucleotide as ligand has greatly extended the versatility of the approach by circumventing the requirement for synthesising new adsorbents for each putative purification (Lowe and Dean, 1971, 1974; Lowe, 1979a; Clonis, 1982). However, the high cost, relatively low protein-binding capacity, lability to both chemical and enzymatic degradation, and marked variation in properties among the various types have seriously undermined the usefulness of these adsorbents. On the other hand, the use of synthetic dyes such as Cibacron Blue F3G-A as ligands for affinity chromatography offers several advantages over immobilised coenzymes and other biological 'group-specific' media. For example, for reasons which are not immediately apparent, the protein-binding capacities of immobilised dye adsorbents exceed those of the natural ligand media by factors of 10–100 (Lowe *et al.*, 1981). The low cost, general availability and ease of coupling to matrix materials represent a major advantage of dyes for large-scale affinity chromatography. Furthermore, synthetic dyes are largely resistant to chemical and enzymatic attack, and the triazine bond, an essential feature of many reactive dyes, is less prone to

leakage than the isouronium linkage introduced during CNBr activation of polysaccharides (Lowe, 1977, 1979a). In addition, the characteristic spectral properties of dyes permit facile monitoring of ligand concentrations and ready identification of column materials. Finally, the general applicability, high capacity and ready release of proteins in good yield, often with significantly enhanced specific activities, favour their application in large-scale chromatography (Lowe *et al.*, 1981; Scawen *et al.*, 1982; Janson, 1984).

Cibacron Blue F3G-A is one example of a considerable range of reactive dyes synthesised in the 1950s for application in the textile and printing industries. These commercial dyes encompass a complete range of shades derived primarily from anthraquinone, azo and phthalocyanine chromophores bonded to suitable reactive functions such as triazinyl and other polyhalogenyl heterocycles, vinyl sulphone, sulphatoethyl sulphone or β-chloroethyl sulphones (Allen, 1971). Anthraquinone dyes produce bright blue and the phthalocyanines bright turquoise shades. Green dyes are characteristic of structures containing mixed anthraquinone–stilbene, anthraquinone–azo or phthalocyanine–azo chromophores, while the remainder of the spectral range are derived mainly from the azo class. Rubine, violet, brown and black dyes are generally metal complexes of *o*,*o*′-dihydroxyazo or *o*-hydroxy-*o*′-carboxyazo chromophores. The structures of a typical anthraquinone dye, Cibacron Blue F3G-A, and a typical azo dye, Procion Red HE-3B, are illustrated in Figure 3.1. Both of these dyes have been immobilised to agarose and other insoluble matrices and extensively exploited for the purification of nucleotide- and coenzyme-dependent enzymes (Dean and Watson, 1979; Clonis and Lowe, 1981; Lowe *et al.*, 1981; Lowe, 1984). The Procion range of reactive dyes was originally developed at ICI in the early 1950s and comprises a number of chromophores linked to either reactive dichlorotriazinyl functions (Procion MX dyes) or less reactive monochlorotriazinyl groups (Procion H range). The Procion and other reactive dyes have a number of characteristics which make their application in biotechnology an attractive proposition: they are readily available at low cost in large quantities and with a variety of chemically distinct chromophores; the dyes display characteristic spectral properties with wide range of $\lambda_{max}$ values covering the entire spectral range and have high molar extinction coefficients, typically in the range 4000–60 000 mol $l^{-1}$ $cm^{-1}$; the triazine group is reactive towards nucleophiles such as the hydroxyls of polysaccharides or metal oxides or the side-chain functional groups of proteins; and the dyes exhibit a remarkable propensity to bind, sometimes biospecifically, to a plethora of proteins and enzymes. These unique properties of the triazine dyes make them ideally suited to a number of preparative and analytical applications in biotechnology, as summarised in Table 1.1

Protein purification by affinity chromatography has been, and will continue to be, the major application of reactive dyes in biochemistry and

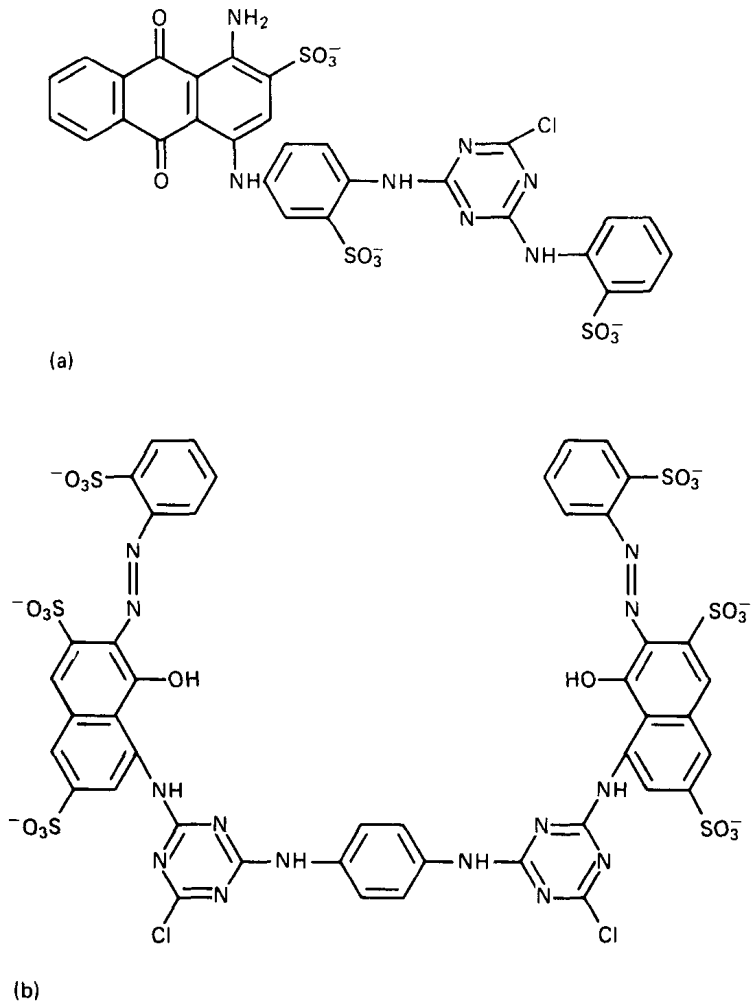



**Figure 1.3** The structures of two typical triazine dyes: (a) Cibacron Blue F3G-A and (b) Procion Red HE-3B

will therefore form the main part of the subject matter of this volume. The literature abounds with examples where immobilised dyes have been utilised to purify individual proteins, sometimes to homogeneity, both on a small scale (Bruton and Atkinson, 1979; Dean and Watson, 1979; Kopperschläger *et al.*, 1982; Lowe, 1984) and on a pilot plant scale (Scawen *et al.*, 1982; Janson, 1984; Lowe, 1984).

Since there a number of reactive dyes that have strongly absorbing chromophores, contain mono- or dichlorotriazinyl functional groups and, at least in part, mimic coenzyme binding, it is not surprising that the free dyes are effective irreversible active site directed affinity labels for a number of

**Table 1.1** Some preparative and analytical applications of triazine dyes

| |
|---|
| Protein purification by affinity chromatography |
| Removal of protein contaminants |
| Resolution of functionally inactive and active enzymes |
| Production of apoenzymes |
| Resolution of isoenzymes and wild type from mutant enzymes |
| Fractionation of multienzyme complexes |
| Coloured substrates for hydrolases |
| Histochemical stains |
| Coloured molecular weight markers for gel filtration and electrophoresis |
| Affinity electrode |
| Radioassay for serum albumin |
| Enzyme immobilisation |
| Active site directed affinity labels |
| Lectin assay |
| High-performance liquid affinity chromatography (HPLAC) |
| Aqueous two-phase separations |
| Affinity electrophoresis |

enzymes (Clonis and Lowe, 1980; Clonis *et al.*, 1981). Thus, a number of reactive dichlorotriazine dyes specifically and irreversibly inactivate pig heart lactate dehydrogenase, yeast glucose-6-phosphate dehydrogenase and yeast hexokinase at sites competitive with $NAD^+$, $NADP^+$ and ATP, respectively (Clonis and Lowe, 1980). These dyes inactivate lactate dehydrogenase in a time-dependent fashion with a hyperbolic dependence of inactivation rate on dye concentration, and protection against inhibition afforded by specific nucleotides and by a mechanism which is irreversible. These observations suggest that triazine dyes are active site directed irreversible inhibitors and that the reactive dichlorotriazine functional group of the dyes is positioned close to a suitable nucleophile in the coenzyme binding site. More recent studies have demonstrated that the dichlorotriazinyl dye Procion Blue MX-R, a structural analogue of Cibacron Blue F3G-A, completely inactivates horse liver alcohol dehydrogenase with the incorporation of 1 mol dye per mol subunit of molecular weight 40 000. Chymotryptic digestion and resolution of the peptides by reverse-phase HPLC yields a single blue peptide, which on sequencing unambiguously identifies the reactive nucleophile as the thiol side-chain of cysteine-174 in the catalytic domain of the enzyme (Small *et al.*, 1982). The apparently essential thiol of cysteine-243 is also the most likely candidate for the alkylation of yeast hexokinase by the copper phthalocyanine dye Procion Green H-4G (Clonis *et al.*, 1981). It is anticipated that future studies involving the use of triazine dyes as active site directed affinity labels will yield much valuable information on how these dyes interact with proteins.

There are a number of other applications (Table 1.1) where the coloured properties of triazine dyes are of paramount importance. For example, the ready solubility, high molar extinction coefficients, minimum aggregation effects and high reactivity make the lower molecular weight mono-azo triazine dyes suitable for histochemical staining. In particular, the fluorescent dye Procion Yellow M4R has been used extensively in neurophysiological investigations (Stretton and Krovitz, 1968; Kennedy *et al.*, 1969; Laties and Liebman, 1970). In addition, reactive dyes have been covalently attached to proteins and used as coloured molecular weight markers for gel filtration or SDS gel electrophoresis (Lowe *et al.*, 1981). Furthermore, a number of chromogenic substrates for the determination of polysaccharidases have been developed. Thus, Remazol Brilliant Blue R–starch has been used as a chromogenic assay for porcine α-amylase (Rinderknecht *et al.*, 1967), while Reactone Red 2 B (Babson *et al.*, 1968) and Cibacron Blue F3G-A have been attached to amylopectine and amylose, respectively, and exploited in the determination of serum α-amylase (Klein *et al.*, 1969, 1970; Hall *et al.*, 1970; Ewen, 1973; Klein and Foreman, 1980). A Procion Blue H-B labelled galactomannan conjugate, Blue Guaran, has been used as a reagent for the assay of lectins from soybean and *Ricinus communis* (Rathaur *et al.*, 1981) and Blue Dextran for the detection of glucose-specific lectins. Furthermore, dyed cellulose derivatives have been used for the continuous spectrophotometric assay of cellulose-solubilising activity (Leisola and Linko, 1976; Ng and Zeikus, 1980).

The affinity of Cibacron Blue F3G-A for human serum albumin has been exploited in two novel analytical procedures for the measurement of serum levels of this protein. The radioaffinity assay is a solid-phase radioimmunoassay which obviates the requirement for antibody and uses Sepharose 4B dyed with Cibacron Blue F3G-A and commercially available $^{125}$I-labelled albumin as tracer (Byfield *et al.*, 1978). The technique is simple, accurate and precise in the low serum albumin range, <30 mg/ml range, and compares favourably with rocket immunoelectrophoresis. Cibacron Blue F3G-A covalently attached to oxidised titanium electrodes has also been used to determine low levels of human serum albumin by a novel potentiometric procedure (Lowe, 1979b). The 'affinity electrode' response is linear up to 15 μg albumin/ml and can be used continuously over a period of many months without apparent loss in response.

The versatility of reactive dyes for the purification of macromolecules by affinity chromatography is now well documented and is beginning to percolate through to pilot plant operations. In recent years the principle of pseudo- or quasi-affinity chromatography on immobilised dyes has been greatly extended to include other purification techniques such as high-performance liquid affinity chromatography and aqueous two-phase separation systems, as well as a variety of analytical techniques such as affinity

electrophoresis, electrodes and immunoassay techniques and assay procedures for hydrolytic enzymes and lectins (Table 1.1). It is confidently predicted that the applications of reactive dyes in biotechnology will continue to flourish.

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