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CHREV. 122

PROTEIN PURIFICATION USING IMMOBILISED TRIAZINE DYES

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1. INTRODUCTION

Dextran conjugates of Cibacron blue F3GA* (Fig. 1) have been used for many years to measure the void volume in gel filtration[1]. It was discovered that dye conjugates when co-chromatographed with proteins selectively interacted with various enzymes. Furthermore, it was shown in 1968 that the dye chromophore rather than the dextran is responsible for the interaction with enzymes[2,3].

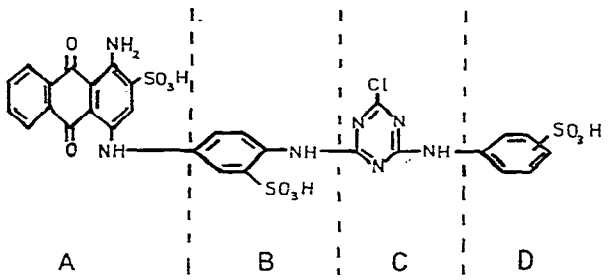

Fig. 1. Structure of Cibacron blue F3GA. The dye is a mixture of two forms: one with the $-SO_3H$ group in moiety D meta to the –NH– bridge, the other with this group para to the –NH– bridge. In Blue Dextran the chlorine atom in ring C is replaced by O-Dextran 2000.

Since this discovery, triazine dye "affinity" adsorbents have been shown to bind a wide variety of different proteins[4–118] (Table 1). In addition to Cibacron blue, one other dye has attracted interest recently, namely Procion red HE 3B, the structure for which is given in Fig. 2[6,10,94,117,118].

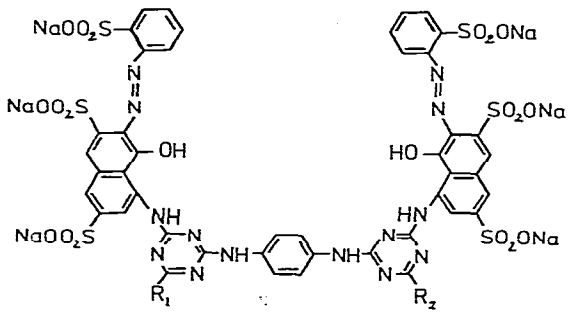

Fig. 2. Structure of Procion red HE 3B.

This article sets out to review (a) the chemistry of dye immobilisation; (b) the applications of immobilised triazine dyes; (c) ways of modifying the binding of enzymes to triazine dye columns and (d) the mechanism of action of immobilised dye-protein interactions.

* Also known as Procion blue H-B, colour index 61211 (ref. 33).

TABLE 1

INTERACTION OF PROTEINS WITH IMMOBILISED TRIAZINE DYES

Eluent: N = nucleotide; S = salt; Su = substrate; SuA = substrate analogue; P = product; D = Cibacron blue F3GA; N-Su = nucleotide-substrate adduct; A = aprotic solvent; E = electrophoretic desorption; X = not stated; O = other. Adsorbent: BDA = Blue Dextran–Agarose; CBA = Cibacron Blue F3GA–Agarose; PRA = Procion red HE 3B–Agarose; BD-X, CB-X, PR-X = Blue Dextran, Cibacron blue F3GA or Procion red HE 3B, respectively, coupled to other support; BD + X = free Blue Dextran chromatographed on gel filtration medium.

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Dehydrogenases and reductases				
Alcohol dehydrogenase	Horse liver	N	BDA, BX-X	4, 5
	Human	N	CBA	164
	Yeast	S	CBA, PRA	6, 151
	Rainbow Trout	N	CBA	7
	Ox brain	S	PRA	119
	Cotton seeds	N	BDA	8
	Rape seed	E	BD-X	9
Aldehyde reductase	Rat liver	S	CBA, PRA	10
	Sacch. cerevisiae	S	CBA	11
	Sacch. cerevisiae	S	BDA	12
	Ox brain	S	PRA	119
Dihydrofolate reductase	*L. casei*	S	CBA, PRA	6, 151
		E	CB-X, PR-X	
	Ox brain	N	PRA	118
Dihydropteridine reductase	Ox brain	N	PRA	118
Glucose-6-phosphate dehydrogenase	Yeast	S	BDA, BD-X	4
	Yeast	E	BD-X	9
	Yeast, beef liver	S	BDA	13
	Yeast	S	CBA, PRA	6, 151, see 167
	Heterolactic bacteria	S, N	BDA	14
	B. stearothermophilus	S	PRA	121
	A. niger	—	BDA	171
	L. buchneri	S, E	BDA	15
Glutamate dehydrogenase	Beef liver	S, E	CBA, PRA, CB-X, PR-X	6, 151
	Neurospora crassa	S	CBA, PRA	
	Yeast	S	CBA, PRA	
	A. niger	—	CBA	171
Glutathione reductase	Yeast	S	CBA, PRA	6, 151
	Human erythrocytes	X	BX + X	16
Glyceraldehyde-3-phosphate dehydrogenase	Rabbit muscle	N	BDA, BD-X	4
	Rabbit muscle	S	BDA	17
	A. niger	—	BDA	171
Glycerol-3-phosphate dehydrogenase	*E. coli*	X	BDA	18
3-Hydroxyl-3-methyl glutaryl CoA reductase	Chicken liver	S	BDA	41
	Rat liver	S	BDA	42, 160
3(17)β-Hydroxy steroid dehydrogenase	Rat erythrocytes	N	CBA	47
Isocitrate dehydrogenase	Human heart	N + A	BDA	19
	A. niger	—	BDA	171
	B. stearothermophilus	O	BDA	20
	Pig heart	S	CBA, PRA	6, 151

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TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
9-Ketoprostaglandin dehydrogenase	Human placenta	S	CBA	21
Lactate dehydrogenase	Rabbit muscle	N	BDA, BD-X	4, 5, 22, 29, see also 167
	Beef heart	E, N	BD-X	9, 129
	Beef heart			
	Rabbit muscle	N	BDA	13
	Pig heart	S	CBA, PRA	6, 151
	Beef muscle	E	CB-X, PR-X	
	Rat heart	N	CBA, PRA	10
	Rat liver			
	Rat hepatoma	N	BDA	23, 24
	Soya bean	X	BDA	25
	Isoenzymes M + H	N	BDA	168
	A. niger	—	BDA	171
	B. Thermus aquaticus	N	BDA	27
	L. casei	N–Su	CBA	26
	Heterolactic bacteria	S	BDA	17
	Mouse, dog, rat, amphibia, fish tissues	N, S, P, Su, SuA	BDA	28
	Mustelus canis, whiting, skate tissues	N, S	BDX	29–31
Malate dehydrogenase	Pig heart	N	BDA	5, 13, see 167
	Rat heart	N	CBA, PRA	10
	Mustelus canis	N	BD-X	29
	Sacch. cerevisiae	N, S	BD-X	144
	Yeast	S	CBA, PRA	6, 151
		E	CB-X, PR-X	
	Yeast	X	BD-X	32
	A. niger	—	BDA	171
Methylene tetrahydrofolate reductase	Bovine liver	S	CBA, PRA	10
	Ox brain	S	PRA	118
NADH-cytochrome b_5 reductase	Rabbit liver	A	BDA	33
Nitrate reductase	*Chlorella fusca*	N	BDA	34
	N. crassa	N	BDA	35
	N. crassa	N	BDA	39
	Rhodotorula glutinis	N	BDA	36
	Spinacea oleracea	S	BDA	31, 37
	Chloracea	N	BDA	38
2-Oxoaldehyde reductase	Rat liver	S	BDA	40
15-Hydroxyprostaglandin dehydrogenase	Human placenta	S	BDA	43
	Human placenta	N	BDA	44
Pyruvate dehydrogenase complex	*Azotobacter vinelandii*	S	BDA	45, 46
6-Phosphogluconate dehydrogenase	Yeast	N	BDA	4, 5, 13
	Yeast	S	CBA, PRA	6, 149, 151
	B. stearothermophilus	S	PRA	121
	A. pseudoplatanus	S	PRA	121
	A. niger	—	BDA	171
	Heterolactic bacteria	S	BDA	14

TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Shikimate dehydrogenase	*Physcomitrella patens*	S	CBA	48
	N. crassa	N	CBA	49
Kinases				
Adenylate kinase	Rabbit muscle	N	BDA	5
	Pig heart	S	BDA	50
cAMP-dependent protein kinase	Beef brain	N, S	BDA	51
cGMP-dependent protein kinase	Calf lung	S	BDA	52
Creatine kinase	Rabbit muscle	E, N	BD-X	136, 168
	Snake venom	X	BD-X	53
Cytidylate kinase	Human erythrocytes	N, S	BDA, CBA	136, 54
Deoxynucleoside kinases	*L. acidophilus*	X	CBA	55, 56
	Various mammalia	N	CBA	57
Hexokinase	Rat brain	X	BDA	13
	Yeast	X	CBA	4
	A. niger	—	CBA	171
Myokinase	Rabbit muscle	E	BD-X	9
NAD kinase	Pigeon liver	D	BDA, CBA	58
Phosphofructokinase	*E. coli*	N	BDA	3, 5, 59, 61
	L. plantarum	N	CB-X	62
	L. acidophilus			
	Thermus X-1	N	BDA	60
	Yeast	N	BD-X, BD + X	63–65
		Su	CB-X	66, see 167
		X	CB	163
	A. niger	X	BDA	171
	Sacch. carlsbergensis	S	CBA	67
	Pig kidney	S	CB-X	68, 63
	Human erythrocytes muscle, yeast		CB-X	69, 156
Phosphoglycerate kinase	*B. vulgaris*	—	CBA	158
	Yeast	N	BDA	70
	Yeast	N, Su	BDA	22, 33
		S	BDA	129
	Horse erythrocytes	N, Su	BDA	71
	A. niger	—	BDA	171
Pyruvate kinase	*Phaseolus aureus*	X	BDA	72
	Yeast	S	BD + X	2, 3
		S	CB-X	73
		N	BDA	70
	Rabbit muscle	Su	BDA	22
	Rabbit muscle	E	BD-X	9
	Human kidney	N	CBA	74
	Human erythrocytes	S, Su	BD + X, BDA	70, 75, 76
	Human liver	Su	BDA	77
		S	BDA	168
	A. niger	—	BDA	171
Other proteins				
Adenylate cyclase	Beef brain	N	BDA	33, 78

(Continued on p. 306)

TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Adenylate kinase	Rabbit muscle	S	BDA	5
Nucleoside diphosphokinase	Human erythrocytes	N, S	CBA	54
Adenyl succinate synthetase	*Az. vinelandii*	N	BDA	79
Albumin	Chicken egg	S	CBA, PRA	6
	Chicken egg	E	BD-X	9
	Human serum	O, E	CBA, CB-X	6, 80
	Human serum	S	BDA, CBA	82, 83
	Human serum	S	CBA	85
	Human serum	O	CBA	84, 148
	Bovine serum	E	BD-X	9
Aldolase	Rabbit muscle	S	BDA	22
	Rabbit muscle	E	BD-X	9
	A. niger	—	BDA	171
Aminoacyl tRNA synthetase	*Sacch. cerevisiae*	S	BDA	86
	E. coli	N, S	BDA	87, 88
Arom multienzyme complex	*N. crassa*	S	CBA	89
Arylsulphatase	Rat brain, liver			
	Human urine	O	CBA	90
	Sheep brain			
Blood clotting factors	Human	S	BD + X	91
	Human	S	BDA	92
C-3 Factor of complement	Human	X	BDA	148
cAMP phosphodiesterase	X	S	BDA	33
Ceruloplasmin	Human blood	X	BDA	148
Cyclic nucleotide phosphorylase	Beef heart	N	CB-X	103
Carboxypeptidase G	*Pseudomonas* ATCC 25301		PRA	94
Choline acetyl transferase	Human brain, placenta	S	BDA	95
Chymotrypsin / Chymotrypsinogen	X	N	BDA, BD-X	4, 168
Citrate synthease	X	S	BDA	33
	A. niger	—	BDA	171
Cytochrome *c*	Horse heart	N, S	BDA, BD-X	4, 5
3-Dehydroquinate hydrolase	*Physcomitrella patens*	S	CBA	48
DNA-polymerase I	*E. coli*	N	BDA	5
	Hela cells	S	BDA, BD-X	5, 96
Enolase	Rabbit muscle	Su, P, S	BDA	22
α-Fetoprotein	Human	S	CBA	85
Flavocytochrome *c*	Yeast	S, A, N	BDA	93
Fructose diphosphatase		N	BDA	5, 22, 168
Follicle stimulating hormone	Human	X	BD + X	97, 98
Glyoxylase I	Sheep liver	X	BD	157
Haptoglobulin	Human blood	X	BDA	148

TABLE 1 *(continued)*

Protein	*Source*	*Eluent*	*Adsorbent*	*Reference*
Interferon	Human fibroblasts	A	BDA	138, 81
	Human leukocytes	S	BDA	81
	Mouse	O	BDA	162
	Mammalian	S	BDA	169
Macroglobulin	Human blood	X	CBA	148
Malic enzyme	*A. niger*	S, N	CBA	171
Methylmalonyl CoA isomerase	X	S	BDA	33
NAD^+-glycohydrolase	Calf spleen	N, Su, S	BDA	100, 128
Orotate decarboxylase	Human		BDA	102
Orotate phosphoribosyl transferase	Yeast	S, Su, P	DBA	101
Phosphoglucomutase } Phosphoglyceromutase }	Rabbit muscle	Su	BDA	5, 22
Phosphorylase a	Rabbit muscle	N	BDA	22
Phosphodiesterase	Beef heart	N, S, S + N	CB-X	103
	Snake venom	S	BDA, CBA	53
Poly(A)DPR polymerase	Calf thymus	S	BDA	104
Polynucleotide phosphorylase	*E. coli*	N	BDA	105
Polynucleotide kinase	T4 phage	S + N	BDA	106
Pregnancy protein SP_1	Human	O	CBA	148
Propyl hydroxylase	Neonatal rat dermis	S	CBA	107
Pyruvate carboxylase	*A. niger*	S, N	CBA	171
R. enzyme	Sweet corn	S	BD + X	108
Restriction endonucleases	Various bacteria	S	CBA	109, 110
Retinol-binding protein	Human blood	X	CBA	148
RNA-polymerase II	Wheat germ	X	CBA	111
RNA-polymerase, β-subunits	*B. subtilis*	S	BDA	112
Ribonuclease type I-A	Beef pancreas	N, S	BDA	4, 5
Sex hormone binding globulin	Human		CBA	113
Succinyl CoA transferase	Pig heart	X	BD + X	114, 115
Succinyl CoA transferase	Sheep kidney	S	CBA	116
Thiosulphate sulphur transferase	Bovine liver	Su	CBA	170
Transferrin	Human	O	CBA	148
Trypsin	X	N	CBA	168

2. IMMOBILISATION OF DYES —THE SUPPORT MATRIX

As with many affinity chromatographic ligands, triazine dyes have been immobilised to a wide variety of support matrices in the search for an ideal system. Some of the supports that have been examined in this way include agarose, dextrans (cross-linked or uncross-linked in either case), polyacrylamide, agarose–polyacrylamide copolymers, cellulose and glass[30,80,94].

Whilst Cibacron blue has mainly been used when attached to a solid support, several applications have been described where a high-molecular-weight (but soluble) Blue dextran–protein complex was separated from low-molecular-weight material by

TABLE 2

PROTEINS WHICH HAVE BEEN OBSERVED NOT TO INTERACT WITH IMMOBILISED CIBACRON BLUE F3GA

Proteins shown to interact by other workers (see Table 1)

Adenylate cyclase[10], albumin (chicken egg)[4,81], alcohol dehydrogenase[73], aldolase[63], 4-aminoacyl-t-RNA synthetase[86], cytochrome *c* (horse heart)[9], dihydrofolate reductase (bacterial)[5], glyceraldehyde-3-phosphate dehydrogenase[63], phosphofructokinase[64], pyruvate kinase[63].

No references showing otherwise

Acetate kinase[15], rabbit, chicken, bovine serum albumins[80,92], apoflavodoxin[5,11], aspartate transcarbamylase[22], cytidine kinase[136], guanylate kinase[114], hemoglobin[4], heterolactic bacterial and yeast hexokinases[15,22], *N. crassa* NAD^+-glycohydrolase[100], potato lactate dehydrogenase[117], micrococcal nuclease[5,13], phosphate acetyl transferase[15], yeast phosphoglucoisomerase[22], yeast phosphoglucomutase[22], subtilisin[5,11], superoxide dismutase[83], thermolysin[33], thyroglobulin[4], triosephosphate isomerase[22], uridine kinase[54].

gel filtration[2,3,63,73,91,92]. Blue dextran has also been used as an "entrapped" ligand in polyacrylamide[63,66].

Rather few reports actually compare different support matrices[80,82]. When viewed simply from the standpoint of capacity of the column (either moles of protein bound per column volume or per mole of ligand) it has been shown that agarose is superior to cellulose[80], polyacrylamide[69] and Ultrogel[30]. Sephadexes have been claimed either to be at least as good as agarose[94] or inferior[33]. Blue dextran coupled to porous glass or silica has been successfully operated at higher capacities when compared with AMP-glass or agarose[30].

Table 3 shows a comparison of adsorbent capacities for 6-phosphogluconate dehydrogenase from *Acer pseudoplatanus* L. on a range of different matrices containing immobilised Procion red HE 3B (ref. 120). In this study only three supports were of high working capacity. These were: uncross-linked Sepharose, cross-linked Matrex gel (see Table 3) and Sephadex G-200. In contrast to previous work, there did not appear to be any correlation between the concentration of KCl coincident

TABLE 3

CAPACITIES OF IMMOBILISED PROCION RED HE 3B FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE (6PGDH) FROM *ACER PSEUDOPLATANUS*[120]

Support matrix	*Ligand concentration (mg/g wet weight)*	*Capacity for 6PGDH (Units/g wet weight)*
Matrex gel (Amicon)	3.6	62
4% Agarose (IBF)	0.5	2
6% Agarose (IBF)	0.3	5
Sepharose 6B (Pharmacia)	2.6	44
Sepharose C16B (Pharmacia)	3.7	16
Sephadex G-200 (Pharmacia)	1.0	50
Sephacryl S-200 (Pharmacia)	2.4	15
Ultrogel AcA 54	0.8	0.2
Agarose (marine colloids)	2.8	14
Spheron (Hydron)	3.9	1
Cellulose (Whatman)	2.6	0.1

(used by some workers as a measure of binding "affinity") with the peak of eluted enzyme and the matrix capacity (see ref. 6).

In a separate study[121] with thermophilic 6-phosphogluconate dehydrogenase from *B. stearothermeophilus*, Sephadex-immobilised Procion red HE 3B is able to bind the enzyme only when the Sephadex has a larger pore size than G-50. But above the latter (G-50), capacities were similar within the Sephadex series and were proportional to the ligand concentration. In addition, ligand effectiveness was superior to many forms of agarose. In one case (Matrex gel red) higher capacities were obtained probably because of the greater ligand concentration obtainable with this gel.

High ligand concentrations alone do not necessarily determine the potential capacity of a support for a protein. For example, cellulose is more highly substituted with dye than agarose and dye concentrations in agarose are higher than in Ultrogel (an agarose–polyacrylamide copolymer) but only the agarose based dye is effective[80]. In instances where the dye couples less readily to a matrix, polyethyleneimine (or polylysine) can improve substitution levels[6].

The combination of affinity chromatography with gel filtration (affinity gel filtration[12]) remains an attractive proposition. Much of the early work was hampered by the fact that cyanogen bromide adversely affects cross-linked dextran gels. However, several efficient triazine dye columns have been prepared using G-100[94] and G-200[120] (Table 3), Sephadex or Sephacryl[80] (see also Table 4). The successful use of cross-linked dextrans as matrices for triazine dyes is probably due to the mild nature of the reaction between the triazine moiety and the polysaccharide. Wood and co-workers[125,141] have advocated the general use of triazines to immobilize ligands. One prediction which is now apparent from the above is that affinity gel filtration could be performed on dextran-based gels with any of the usual affinity ligands provided that the cyanogen bromide activation step is replaced by a triazine-based reaction either by preassembling the ligand plus triazine (or less satisfactorily by prior attachment of the triazine followed by reaction with the ligand).

In summary, it is likely that triazines will represent a considerable improvement in the methods available for the attachment of ligands to support and that preassembled defined molecular species will be more accessible by this route. Another

TABLE 4

SOME SUPPORT MATRICES

Support	*Trade name*	*Company*
Crosslinked 6% Agarose	Matrex gel red	Amicon
Agarose	Matrex gel blue	
Agarose	Affigel blue	Biorad
Polyacrylamide-Agarose copolymer	Ultrogel	LKB
2,4,6% Agarose	Sepharose	Pharmacia
2,4,6% Agarose	CL Sepharose	
Cross-linked Agarose	CL Sepharose	
Cross-linked 6% Agarose	Blue Sepharose	
Cross-linked dextran	Sephadex	
Cross-linked dextran (polyacrylamide bridges)	Sephacryl	Whatman
Cellulose	Ultrogel	Whatman

prediction is that more precise affinity gels can be constructed where non-uniform ligand distribution is hopefully eliminated (see below).

3. TRIAZINE DYES AND SPACERS

Two questions seem relevant regarding spacers: (a) Does the addition of a hydrophobic spacer arm to a "detergent" or "hydrophobic" ligand have any significant role? (b) Are dextrans or other soluble polysaccharides true spacers when coupled to triazine dyes prior to their immobilisation?

Table I shows that about 60% of the cited examples use dextran conjugates of Cibacron blue. The conjugates have been immobilised either (a) via the dextran moiety using periodate oxidation[31]; (b) via cyanogen bromide activation of the dextran[30]; (c) via the anthraquinone amino group using cyanogen bromide-activated adsorbents (this method presumably not only cross-links the agarose but also links the conjugate to the agarose by the same sort of bridges)[23] or (d) via carboxyl groups previously attached to the matrix[58] (Fig. 3). In the examples (a) and (b) the dextran can be viewed as a spacer whilst in (c) and (d) the dye may act in part as a bridge between two much larger polysaccharide species (dextran and agarose). In the latter case the dextran is more likely to operate in a restricting sense making the dye less available to some proteins on the basis of size or accessibility of dye binding sites. Alternatively, since

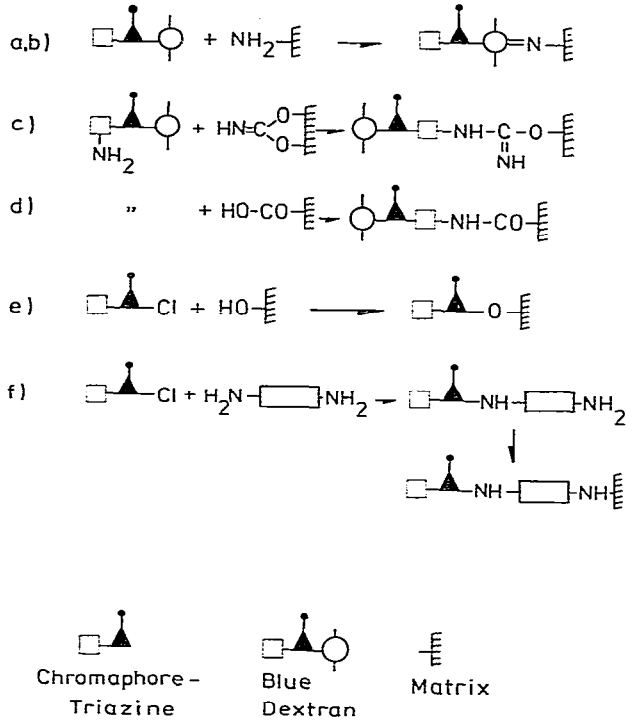

Fig. 3. Structure of some Cibacron blue conjugates. The structure of Blue Dextran indicates multiple sites of attachment of the dye to the dextran polymer.

presumably several dye molecules are bound to each dextran chain and fewer dye molecules react with the activated agarose, the dextran in these cases could also act as a spacer for a proportion of dye molecules.

On the other hand, triazine dyes can be bound directly via the triazine ring to polysaccharide supports ((e) in Fig. 3)[47,69,73,83,123] or via polyethyleneimine ((f) in Fig. 3)[6,124] by prior coupling of the dye to the spacer and the assembled conjugate is then coupled to a cyanogen bromide- (or triazine-) activated matrix.

There have been a few studies comparing the properties of triazine dyes immobilised in different ways. These studies show that directly coupled dye is less selective than the dye coupled via polyethyleneimine, dextran or hexyl spacers[6,13,58,83].

Whether the dye is immobilised via a spacer arm or polymer, or which type of support matrix is used is probably best determined separately for each application. The above discussion can only be used as a guideline (see 152).

However, it is desirable that adsorbents should be compared at similar immobilised ligand concentrations[30]. In parallel studies with immobilised nucleotides[127] and hydrophobic ligands it has been shown that variation of the ligand concentration considerably alters the properties of the column.

Lang *et al.*[125] have made an observation of note concerning hydrophobic spacer arms for immobilization of oxamate. If the uncertainties of cyanogen bromide chemistry are replaced by the triazine linkage then the involvement of the hexamethylene spacer, with or without the oxamate becomes clearer. Oxamate can only recognise lactate dehydrogenase when both a hydrophobic arm and NAD(H) are present —replacement of hexyl spacer by two three carbon chains joined by an amino group abolishes binding— in O'Carra's words[142]: "It is clearly necessary to exercise great care in distinguishing bio-affinity from non-specific adsorption".

4. DETERMINATION OF DYE CONCENTRATIONS

Determinations of triazine dye concentrations by spectrophotometric means are complicated by several factors (a) deviations from Lambert-Beer's law due to association of dye molecules at higher concentrations[126]; (b) many commercial dye preparations are multi-component; (c) a concentration-dependent red shift is observed with Cibacron blue[143] and is likely to exist for dyes of similar structure.

Immobilised dye concentrations have been measured by spectrophotometric measurements of (a) the gel[80] (b) the dye released by digesting the matrix with 3 *M* hydrochloric acid or 50% acetic acid[80,118,159], or (c) by the difference between added and unbound dye at the time the gel is made. For Cibacron blue, extinction coefficients of 12.3 to $13.6 \cdot 10^3$ $l \cdot mol^{-1} \cdot cm^{-1}$ at 610 nm (pH 7) have been measured[58,118,126,129,137] and for Procion red the extinction coefficient is reported as $1 \cdot 10^4$ to $3 \cdot 10^4$ $l \cdot mol^{-1} \cdot cm^{-1}$ at 522 nm (pH 7) at 18° (Ref. 121).

Very few studies have incorporated prior purification of the commercial dye preparations. Nevertheless, contaminants may be removed by solvent precipitation[58], cellulose chromatography[128] or by isoelectrofocusing (using the latter technique many distinguishable bands are observed) (see also ref. 167).

5. MECHANISM

The mechanism of the binding of triazine dyes to enzymes is not simple. The above considerations hardly touch on some of the complications that can arise in such mechanistic studies. Many of the analyses of such interactions have been based upon biospecific elution (low substrate or nucleotide concentrations elute the retarded protein whereas equivalent or higher (50 m*M*) salt concentrations have no effect). This has been taken to indicate a "biospecific" interaction (*i.e.* via a nucleotide binding domain[5]). However, Yon[139] has shown that several enzymes, including dehydrogenases, may be eluted from "detergent" adsorbents under these conditions. Whilst biospecific elution is a useful tool both in affinity and other types of adsorption chromatography[139], information relating to the adsorption phase (and its mechanism) is not obtained by studying desorption[127]. One concludes that the elution of dehydrogenases by nucleotides from immobilised Cibacron blue does not imply any biospecificity in the mechanism of adsorption.

It is nevertheless possible that Cibacron blue, and other polyaromatic sulphonates in general, can mimic certain aspects of the binding of nucleotides to the nucleotide binding sites of proteins[5,33,78,129,130] but more elaborate claims for the use of the dye to test for the dinucleotide fold[33,131,132] must be viewed with caution. Indeed, Stellwagen has recently carried out a careful analysis of the interaction between Cibacron blue and a variety of enzymes and concludes "we find that Cibracon blue F3GA has suffered the fate of many biological inhibitors namely to appear less specific as its use becomes more widespread"[143]. This is in agreement with other analyses[63,69,126].

6. METHODS FOR MODIFICATION OF PROTEIN BINDING TO TRIAZINE DYE COLUMNS

6.1. Decreasing protein binding

In order to operate any chromatographic separation successfully, attention should be paid to the correct selection of a number of variables. These include (apart from the choice of support matrix, spacer and ligand which have been discussed above) column dimensions[145], flow-rate[6], sample size in relation to column capacity[145], sample buffer, pH and ionic strength[146] and temperature[147].

In our hands, the operation of triazine dye columns does not differ very widely from similar separations carried out using immobilised nucleotides. However, several points can be made to underline some important differences.

6.1.1. pH. The correct choice of adsorption pH is essential. Proteins tend to bind to triazine dyes more tightly at lower pH values, presumably because of a contribution from ionic interactions favoured by increasing positive charges on interacting protein molecules. However, this change in binding is not necessarily determined by the p*I* of each protein. Thus the p*I*s of emergent proteins were not related to the elution of plasma proteins from Cibacron blue–agarose[148]. Conversely, the desorption pH can be critical: gradients to higher pH values are successful in separating contaminating yeast proteins from 6-phosphogluconate dehydrogenase on Procion red HE 3B-agarose[149].

6.1.2. Ligand concentration. Careful adjustment of the triazine dye ligand concentration can result in useful alterations in the purification factors achieved with these columns.

This adjustment can be made (a) uniformly *i.e.* each bead has the same ligand concentration or (b) with unsubstituted beads[50].

The application of the methods described in (a) and (b) should be considered when a protein binds more tightly than is desirable. Instances where glucose-6-phosphate dehydrogenase has bound apparently irreversibly have been resolved by altering the adsorption pH to higher values, by lowering the ligand concentration or changing the support matrix from agarose (6%) to crosslinked dextran (G-200 Sephadex)[120]. Electrophoretic desorption may also resolve this problem[6].

6.1.3. Choice of eluent. KCl or nucleotides have been widely used to selectively elute enzymes from immobilised nucleotides. Indeed one of the advantages of the latter would seem to be the wide range of different elution procedures available[140]. In our hands, nucleotides such as NADP are not particularly successful as eluents in the purification of various dehydrogenases from sheep liver, yeast, *B.stearothermophilus*, *A.pseudoplatanus* L. Either KCl or pH elution seem to produce sharper peaks of higher specific activity[120,121,149,155]. Although these observations have been made both with Cibacron blue F3GA and Procion red HE 3B columns it is probably best to investigate each application separately.

6.1.4. Temperature. Few studies have examined the effects of adsorption and desorption temperature in affinity processes[147,151]. The salt-dependent binding of a thermophilic enzyme (6-phosphogluconate dehydrogenase from *B.stearothermophilus*) has been compared with that of the same enzyme from a mesophile (*S.cereviseae*)[121]. The salt concentration required to elute both enzymes from either Cibacron blue F3GA or Procion red HE 3B columns increased with increasing temperature up to 45°. Whilst this effect was observed for thermophilic enzymes on AMP–agarose[151] the opposite was found for mesophilic enzymes[147].

6.2. Increasing protein binding

Often all that is needed is an increase in ligand concentration: this may require a change of support matrix (4–6% agarose) or the use of a different type of agarose (Ref. 150 and Table 4).

Lowering the pH of adsorption has also been found to succeed in increasing protein binding[148]. Raising the temperature favours an increase in binding in our experience[121].

Finally, increasing the applied enzyme concentration has been shown to give greater retardation[152].

7. SCOPE OF APPLICATIONS AND CHOICE OF DYE

At present the majority of applications of triazine dyes have involved Cibacron blue F3GA because of its early availability. The wide variety of applications is reflected in the number of papers referred to in Table 1. These include the resolution (a) of isoenzymes[28,59,61,90,144], (b) of subunits of protein aggregates[51,52], (c) of wild type from mutant enzymes[6,18,70] and (d) of enzymes from nucleotide enzyme complexes[17]. Many straightforward protein purifications have been described

and include even multienzyme complexes[45,46,89]. Purification factors for triazine dye steps in isolation procedures are often in excess of ten-fold and may be in excess of fifty-fold[5,14,15,22,31,41,56,60,78,87,111,119,144].

Preliminary comparisons of Cibacron blue and Procion red would suggest that the former is better suited to the purification of NAD^+-dependent dehydrogenases whilst the latter is more selective for $NADP^+$-linked enzymes[6,10,121,155]. This generalisation is likely to be a guideline worth following although many proteins other than dehydrogenases are retarded by immobilised Cibacron blue F3GA and Procion red HE 3B (Table 1 Kinases and Other proteins). We have found it beneficial to run columns of both types[121] during the purification of any one dehydrogenase.

There are many other triazine dyes besides the two discussed above. Screening programmes are already under way in several laboratories to attempt to find useful applications for these dyes.

In addition to the triazine dyes, two recent dyes have been described for base-specific fractionation of nucleic acids[153,154]. These are: A-T specific malachite green and G-C specific phenyl neutral red. A comparison of these dyes with the triazine dyes already mentioned suggests that useful triazine dyes for the future might contain positively charged substituents in place of negative sulphonate groups (see ref. 167).

8. DISADVANTAGES

Many triazine dye preparations are multicomponent and although fillers and minor impurities are removable this aspect has received scant attention.

9. ADVANTAGES

9.1. Stability

Triazine linkages are less prone to ligand leakage than cyanogen bromide-activated polysaccharides. Thus unlike the latter, triazine dye columns can be operated in glycine, Tris or ethanolamine buffers (which can displace ligands from cyanogen bromide-activated supports by nucleophilic attack).

9.2. Uniformity

At commonly used cyanogen bromide concentrations it has been shown that ligands are probably unsymmetrically distributed in agarose beads[155]. The same has not been proven for triazine-linked systems and if prior assembly of the triazine-ligand is carried out, cross-linking should not occur and the problem of asymmetric coupling therefore unlikely to be encountered. Furthermore one can avoid the hazards of cyanogen bromide activation.

9.3. Ease of preparation

The widespread availability of many triazine dyes and their ease and speed of coupling is a major advantage when comparing these ligands with defined nucleotide affinity adsorbents. The low cost of these dyes is also a major consideration when designing large scale systems[6,30].

9.4. Capacity

The binding capacities for proteins of triazine dye columns is far higher than the values obtained for immobilised nucleotides[4,5,6,30,31].

9.5. Special properties

Apart from the facile identification of dye columns from their colour, the red shift which is observed when proteins adsorb to triazine dye columns, is a potentially useful indicator of a successful choice of adsorption conditions and in addition can be used to examine the interaction between protein and immobilized dye (using wavelengths that are well away from absorbances associated with the protein, linkage and matrix).

9.6. Choice of eluent

Triazine dye columns are more effectively eluted with salt or pH changes which are cheaper than with substrates and hence could be important for larger scale separations.

9.7. Ease of storage

Less care is needed to prevent ligand leakage. No evidence of bacterial or other contamination has been observed when dye-agarose columns are stored in the cold room for two years[4,8,31].

9.8. Selectivity and usefulness of triazine chemistry

The rates of reaction of the three chlorine atoms in *s*-trichlorotriazine (cyanuric chloride) are markedly different[125,167]. This enables one to produce precisely coupled ligand assemblies.

10. OPTIMIZED COUPLING PROCEDURES

10.1. Monochlorotriazine dyes (e.g. Cibacron blue, Procion red HE 3B and Procion H dyes in general)

Agarose (either Matrex gel or Sepharose 6B; 20 g moist weight) is suspended in water (70 ml) and a solution of the dye (200 mg) in water (20 ml) is added. The mixture is placed in a rotary mixer (Coulter) for 5 min after which 20%(w/v) sodium chloride (10 ml) is added*. Mixing is continued for 30 min at room temperature. A solution of 5 *M* NaOH or 1 *M* Na_2CO_2 (0.5 ml) is added**. (Any other base may be used to elevate the pH at this stage providing it does not contain nucleophiles such as amino groups[167].) After the addition of base the mixture is incubated for three days, filtered and extensively washed with water, 1 *M* sodium chloride, 4–8 *M* urea and water.

10.2. Dichlorotriazines (Procion MX series)

The above procedure is exactly repeated except that the final incubation is for 1 h at room temperature. The washing procedure is the same.

* This salt step is essential to "salt" the dye into the matrix; as a result faster reaction times result and less hydrolysis of the triazine moiety occurs[167].

** NaOH tends to lead to higher ligand concentrations[167].

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12. SUMMARY

This review attempts to identify proteins which selectively interact with immobilised triazine dyes such as Cibacron blue F3GA and Procion red HE 3B. Different support matrices are compared by examining the capacities of these dyes for proteins. Various approaches to the immobilisation of triazine dyes are considered together with the use of spacers.

Some theories of the mechanism of protein retardation by immobilised dyes are discussed. A number of methods are suggested for the measurement of dye concentrations and for the modification of the binding of proteins to dye columns. The variety of elution methods is compared with a view to optimizing purifications. The scope of applications is reviewed as well as the choice of dye. Some advantages of triazine dyes over other affinity ligands are given.

It is concluded that although no satisfactory mechanism for the binding of triazine dyes to proteins has yet been proposed, these dyes possess considerable potential for protein purification, particularly when applied on the large scale.

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