DYE-LIGAND AFFINITY CHROMATOGRAPHY: THE INTERACTION OF CIBACRON BLUE F3GA® * WITH PROTEINS AND ENZYMES

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I. INTRODUCTION

Cibacron Blue F3GA (CB) has ascended in status from a lowly textile dye to a glorified "biospecific" ligand in less than a decade. Along the way, the dye has also acquired the distinction of being a "universal pseudoaffinity ligand". In addition, the dye has been described to possess a discriminating ability to bind to selected proteins containing a specific structure called the "dinucleotide fold". While it turned out to be true for a large number of enzymes, such a specificity was later found to be neither universal among proteins with the "dinucleotide fold" nor confined to those proteins which bind nucleotides. The background and the genesis of such a concept will be discussed in a separate section, but it must be mentioned here that CB has a structural analogy to the nucleotides. During the 1970s, CB gained a lot of attention and, in the immobilized form, it has been used to purify and separate a wide variety of proteins and enzymes, from albumin to zymogens. In this sense, CB is a dye in search of its own identity. The versatility of CB arises from a judicious combination of aromatic (nonpolar) and sulfonate (ionic) groups in the same molecule. In fact, there is something unique about this combination conferring special characteristics on several dyes binding strongly at the coenzyme-substrate sites of several enzymes in a non-specific mode. Several cases of strong binding of dyes to globular proteins at their active sites in preference to other regions of the protein surfaces have been described by Glazer.[1] The dyes are mainly hydrophobic, interspersed with some charged groups. CB thus represents a specialized case of a molecule which combines structural and stereochemical features together so as to be able to offer a pseudospecificity to a large number of proteins. Within this framework, it is still possible to utilize the specificity of the dye in a restricted sense by an appropriate alteration of operating conditions.

Several reviews[2-5] have appeared in the last few years covering the affinity chromatographic details of separation and purification of several proteins and enzymes on immobilized CB columns using various support matrices. The reviews by Dean and Watson[4] and Burgett and Greenley[5] enumerate a large number of enzymes separated on immobilized CB columns emphasizing the specific details on the conditions of loading and elution. Stellwagen[3] has pioneered the concept of dinucleotide-fold specificity of CB and has provided an excellent background leading to that concept. In our current effort, we will not dwell on the details of purification of all the enzymes nor will we attempt to update the list. The primary thrust of this review is to critically evaluate the interactions leading to the binding of the dye to

* Cibacron Blue F3GA® is a registered trademark.

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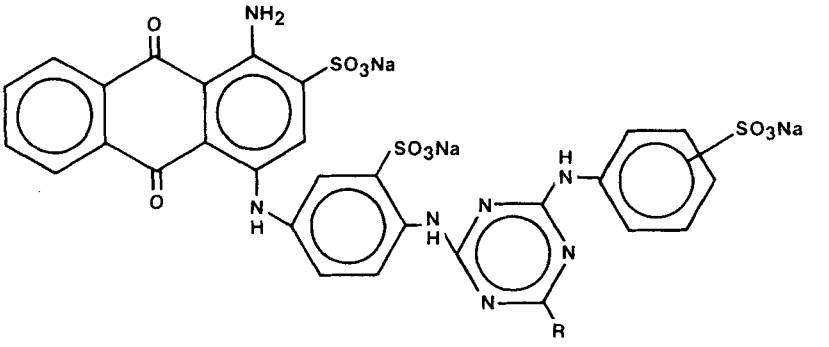
proteins in solutions, the binding of proteins to immobilized CB columns, the conditions of binding and elution, and the mutual interactions between the dye and other molecules on protein surfaces. In the last few years, a number of studies have been made on the dye itself in aqueous solutions, the binding of the dye with proteins which were not expected to bind the dye, and the kinetics of CB inhibition of several enzymes. All of these studies have shed significant light on the nature of the interactions involving CB and the proteins. It is thus our primary aim to try to analyze the available information in molecular terms. Such an understanding should lead to any potential new applications or refinement of existing applications, in addition to clarifying some floating misconceptions. Our approach is to delineate the simpler interactions emanating from studies on the interactions of CB with small molecules and apply them in the analysis of the interactions of the dye with proteins. A unified analysis will be attempted so as to incorporate the column binding studies as well as kinetic and equilibrium studies in solution. However, for the sake of convenience, the discussion will be split into several groups of proteins and enzymes, each group having a common function. The immobilization procedures, the types of matrices involved, the efficiency of the columns, the necessary precautions to be observed, and novel applications will also be covered.

II. EVOLUTION OF CB AS AN AFFINITY CHROMATOGRAPHY LIGAND

A. History

The development of CB into an affinity chromatographic ligand is a classic case of serendipity. Blue Dextran 2000 (BD) is commonly used as a visual indicator of the quality of packed gel beds and has been used as a void volume marker in gel permeation studies for several years. Blue Dextran is a high molecular weight (averaging 2×10^6 daltons) anhydroglucose polymer to which the sulfonated polyaromatic blue dye, CB, is covalently attached. The structure of the dye (and the modified forms) is shown in Figure 1. The dye itself is a mixture of meta- and para- forms which respect to the terminal (benzene) sulfonate group. In Blue Dextran Sepharose (BDS) the Sepharose is attached to BD through the amino group of the anthraquinone ring. BD does not interact with most gel permeation media,[2] although a strange case of an aged sample of BD adhering to the bottom of a column of Sephadex G-200 was reported.[6] In this case, the sweet-corn R-enzyme when applied to the Sephadex column did not elute at the fraction where it should, but was found retained by the column which was then eluted by 0.5 *M* NaCl, with 100% recovery. It turned out that the Blue Dextran trapped in Sephadex was responsible for the binding of the enzyme and caused the artefact. It is now clear, in retrospect, that the particular sample of "aged" Blue Dextran got immobilized on the Sephadex column either by forming Blue Dextran Sephadex or the blue chromophore was separated from the dextran linkage and formed Blue Sephadex on the column. This and similar other[7,8] "accidental" binding of enzymes to BD at low ionic strength led to the development of BD as a means of purifying several enzymes of interest. Initially, the use of BD in protein purification involved gel filtration of the BD-enzyme complex at the void volume of the column and rechromatographing the BD-enzyme complex in presence of high concentrations of salt, a condition disruptive for the complex. The enzyme separates from BD under these conditions and is recovered at its regular elution volume. Subsequently, BD was immobilized in polyacrylamide, and in CNBr-activated agarose. The Blue Dextran-Sepharose prepared by Ryan and Vestling[8] became very popular and started the field of Blue Dextran-Sepharose chromatography for enzyme purification. The proteins that bound to BD were found to be interacting with the blue chromophore, since control experiments with unmodified Dextran indicated no affinity for the enzymes.



R = -Cl = Cibacron Blue
R = -O-Dextran = Blue Dextran
R = -O-Sepharose = Blue Sepharose

FIGURE 1. The structure of Cibacron Blue F3GA. The dye is a mixture of para and meta forms with respect to the sulfonate group in the terminal phenyl ring. In Blue Dextran Sepharose, Blue Dextran is linked via the 1-amino group on the anthraquinone ring, to the Sepharose.

This led to a further advancement in the field, i.e., the direct coupling of the free dye to matrices like Sephadex and Sepharose.[7,9]

B. Types of Cibacron Blue Affinity Media and Their Relative Utility

Most of the immobilization procedures involving CB or BD follow the general procedure of Cuatrecasas.[10] Cross-linked dextran (Sephadex) and beaded agarose derivatives (Sepharose) are ideally suited for immobilization of the dye on the basis of flow properties, mechanical and chemical stabilities of the gel after the coupling, and the ability of the gel to form a loose porous network. Consequently, Sepharose, Sephadex, and polyacrylamide matrices (among others) have been used widely to immobilize either the free dye or Blue Dextran.[7-11] The preparation of BDS was described by Travis and Dannell[11] and Ryan and Vestling.[8] In broad outline the procedure adopted is as follows:[11] 100 mℓ of settled Sepharose 4B was activated with 16 g of CNBr at pH 11 and 10°C. When the reaction subsided, the Sepharose was washed with 0.1 *M* $NaHCO_3$ at pH 9.5. One gram of BD in 100 mℓ of the same buffer was added and let stand at 4°C for 24 hr. The BDS conjugate that resulted was washed with the buffer, urea, and water, followed by 0.5 *M* NaCl solution at pH 8. This preparation was found to bind 10 mg of albumin per milliliter of packed gel.[11] Finer details in the handling are given by Ryan and Vestling.[8] BDS can be used repeatedly if stored refrigerated with NaN_3 to prevent biodegradation. Blue Sepharose was prepared by Easterday and Easterday[9] by coupling the free dye with Sepharose 6B cross-linked with epichlorhydrin. Blue Sephadex was first prepared by Bohme et al.[7] by a straightforward reaction between the dye and Sephadex at 60°C. The advantage of using the dye and a nonactivated gel (such as Sephadex or Sepharose) as opposed to activated gel (such as CNBr-activated Sepharose) is that the unreacted dye can be washed out, while unreacted activated gel can impart additional binding sites.[2] It is important to try more than one affinity medium and a different coupling procedure since the protein of interest, although known to bind the free dye, may not bind to a particular immobilized form for reasons of steric and chemical compatibility.

Blue Sepharose has been shown by and large to bind several proteins without any obvious chemical or steric constraints.

C. Coupling Stability and Linkage

All the immobilization procedures referred to in the previous paragraph involve thorough washing of the final column material to remove excess free dye, since "irreversible" binding of proteins may occur more rapidly to the free dye than immobilized dye. Sometimes it is necessary to use a detergent, e.g., Tween-80, to wash off any free dye tenaciously bound to the matrix.[2] The stability of hydrated affinity gels varies with storage conditions. With Blue Sepharose, the dye-matrix linkage is less stable at pH values below 4, but at pH values between 6 and 10, at which a majority of protein binding experiments are conducted, Blue Sepharose is known to be very stable[2] for several years.

There are two types of chemical linkages possible with Cibacron Blue. In the first type, the chlorotriazine ring is coupled with hydroxyl groups in the matrix of choice — whether it is dextran, Sephadex, Sepharose, cellulose, or Sephacryl. In this process HCl is released and an ether linkage is formed. If dextran is used, soluble Blue Dextran is the product. With the other matrices mentioned, gels are obtained. Blue Agarose (Pierce Chemical) and Affi-Gel Blue (Bio-Rad Laboratories) belong in this class. In the second type, the amino group in the anthraquinone ring is coupled with CNBr-activated Sepharose (or agarose). However, unless the chlorotriazine ring is deactivated as in the case of Blue Dextran, this type of gel will be reactive and bind proteins irreversibly. If Affi-Gel 10 or 15 (Bio-Rad Lab) is used to couple the dye, the coupling will occur through the amino group of the dye since the support matrix is activated N-hydroxysuccinimide ester of succinyl aminopropyl agarose. In such a case, many proteins may be vulnerable for irreversible binding to the columns by virtue of the reactivity of the "naked" chlorine in the triazine ring. Triazine linkages are also less prone to ligand leakage than coupling effected by CNBr-activated polysaccharides.[2] In addition, the ligand distribution is more symmetrical in the triazine linkage. Accordingly, the triazine linkage immobilization has become the most widely used procedure.

D. Choice of Matrix, Degree of Dye Substitution, and Binding Capacity

The ideal matrix will be rigid, highly porous, permit high ligand substitution, and not otherwise interact with the sample. Using excessive amounts of CB, Easterday and Easterday[9] found that the degree of dye substitution in Sephadex gels was higher than in Sepharose since dextran contains more free hydroxyl groups per unit weight of the gel than Sepharose.

Chambers[12] described a simple procedure for the determination of the amount of covalently bound CB present in preparations of Blue Sephadex and Blue Dextran Sepharose. This method involves hydrolysis of the Sephadex and Sepharose dye derivatives in 6 *N* hydrochloric acid at 40°C for 60 min and measuring the optical absorbance of the supernatant solution at 515 nm.

The effect of the matrix on the binding of pyruvate kinase (PK) and creatine kinase (CK) was studied.[9] PK binds poorly to Blue Sephadex G-50, but strongly to Blue Sephadex G-200, Blue Sepharose 2B, and Blue Sepharose 6B. However, CK bound strongly to all four of the gels. On the basis of extensive studies, Haff and Easterday[2] concluded that Sepharose CL-6B was the best matrix for immobilization of CB. Angal and Dean[13] studied the binding and elution of human serum albumin to derivatives of Sepharose, Sephacryl, Cellulose, and Ultrogel using CB as the ligand. They found that the higher the agarose content of the matrix, the more chromophore was immobilized. However, the ability of each immobilized gel to retain albumin was not strictly dependent on the ligand content alone. Cellulose, despite having higher ligand concentration, retained albumin poorly. All the derivatized

Sepharoses and Sephacryl have high affinity constants for albumin. The affinities of derivatized cellulose and ultrogels are lower by a factor of 10.

The protein binding capacity of the immobilized blue dye ligand depends, among other conditions, on the degree of substitution of the dye in the matrix, the uniformity of such distribution, steric constraints of the chromophore in the gel, and the type of linkage of the chromophore with the matrix. For example, Blue Sephadex G-200 binds approximately 40 times more phosphofructokinase than Blue Dextran polyacrylamide.[7] Elution of the enzyme from the latter gel could be accomplished at low concentrations of ATP, while a high concentration of ammonium sulfate is needed to elute the enzyme from Blue Sephadex G-200 gel. This phenomenon is possibly due to the difference in the type of linkage of the chromophore in the two gels. In Blue Sephadex the linkage is through the triazine ring. The amino group also contributes to the binding of the enzyme and thus requires a high ionic strength for elution. In Blue Dextran polyacrylamide the amino group is also linked up and consequently the binding of the enzyme is weaker. As an extreme case, Blue Dextran Sepharose does not show any affinity to phosphofructokinase. This may reflect the unavailability of the amino group for the binding, in addition to any steric constraints imposed by the dextran and Sepharose components of the matrix. The influence of the polysaccharide carrier in the strength of binding is manifested by the inability of ATP or high ionic strength to elute phosphofructokinase bound to Blue Cellulose whereas it could be eluted with 10 m*M* ATP from Blue Sepharose CL-6B.[14] It is thus imperative to try different affinity support matrices for optimal effects in a particular application.

E. Mechanism of Protein Binding and Elution from the Column

The binding of proteins to immobilized blue gel columns has been variously characterized as "nucleotide-specific", "ionic", and "hydrophobic". This is reminiscent of the fabled description of an elephant by six blind men, each one describing the elephant as a pillar or a wall by virtue of limited touch perception. In a sense, the description of CB as capable of each or all of the interactions mentioned above is probably accurate because that is what the dye appears to engage in. Depending on the environment that obtains, be it hydrophobic or electrostatic, the dye seems to, in a large number of cases, optimize the available interactions. This is not to deny the importance of specificity. CB is not a biologically specific molecule like NAD or ATP. However, restricted biological functionality as applied to binding only can be assumed by the synthetic dye if structural similarity to certain biological molecules is imminent. The details of such interactions will be analyzed in a later section, but suffice it here to say that CB has a broad specificity, be it for nucleotide-binding proteins or simply a stereoselective electrostatic binding.

Most dehydrogenases and kinases bind to the immobilized Blue gel columsn via their nucleotide-binding sites and correspondingly elute from the column by low concentrations of the appropriate nucleotides.[3] This has been taken to mean "biospecific" binding and elution. However, it must be realized that information concerning the mechanism of adsorption cannot be obtained by studying desorption. So it has to be concluded that the elution of dehydrogenases from immobilized Cibacron Blue columns by nucleotides does not necessarily imply any biospecificity in the adsorption process.

Since the Blue chromophore contains both amino and sulfonate groups, the so called "nonspecific" ion-exchange effects would also be expected. Easterday and Easterday[9] have shown that a wide variety of proteins ranging from albumin and aldolase to hemoglobin and cytochrome C exhibit nonspecific binding to Blue Sepharose at low ionic strengths. The key to nonspecific binding is low ionic strength. Nonspecific binding can be either hydrophobic or ionic or both. Usually hydrophobic binding is disrupted by high concentrations of chaotropic salts and so is ionic binding. However, hydrophobic interactions can be

enhanced in presence of high concentrations of either ammonium or sodium sulfate or KH_2PO_4. Such salts also tend to inhibit protein denaturation.* When the nonspecific binding is ionic in nature, elution of the protein from the blue gel columns can be achieved with high salt concentrations or by changing the pH. It has been observed that most proteins bind to the Blue chromophore columns below their isoelectric points[15] and elute at a pH higher than the pI. Although this is a guide, factors such as the location of the charged groups could make variations from this norm. For example, if the active site of an enzyme has a cluster of basic amino acid side chains, the enzyme may remain bound to the column even at a pH well beyond the pI. Thus, it is possible that the binding of most proteins to immobilized blue columns covers the entire range from nonspecific ion-exchange interaction to nucleotide-specific binding. Elution can be achieved by specific nucleotides, change of pH, ionic strength, or even organic solvents if the interaction is sufficiently hydrophobic.

III. INTERACTION OF CB WITH ITS ENVIRONMENT

A. Spectral Properties

The visible absorption spectrum[16] of Cibacron Blue in dilute solution in water consists of a broad band with a maximum at 610 to 615 nm, and a molar extinction coefficient[17] of 13,600 cm^{-1} (as determined by dry weight measurements). At concentrations above 5 μM, the dye exhibits a hypochromic deviation from Beer's law, which implies self-aggregation.[16] Blue Dextran and Blue Sepharose display absorption spectra similar to those of the free dye, although the long-wavelength maxima (which are affected least by light scattering) are red shifted by 5 to 10 nm. While the position of λ_{max} does not change upon self-aggregation (unpublished results), it does change in different solvents.[18] For example, the λ_{max} is 619 nm in methanol, 626 nm in ethanol, and 637 nm in pyridine. In 6 *M* HCl, however, the dye solution was purple[12] and the spectrum had a λ_{max} at 515 nm with an extinction coefficient of 5610 M^{-1} cm^{-1}. In this instance, the sulfonate groups of the dye remain completely protonated.

* It is important to recognize that salt effects on macromolecule interactions could manifest in an "electrostatic" or "hydrophobic" mode depending on the position of the anion and/or cation of the salt in the Hofmeister series. Typically the anion and the cation series are described as: $SO_4^{--} < CH_3OO^- < Cl^- < Br^- < NO_3^- < ClO_4^- < I^- < SCN^-$ and $NH_4^+ < K^+$, Na^+, $Cs^+ < Li^+ < Mg^{++} < Ca^{++}$ in the *increasing* order of destabilizing the "native" conformation of macromolecules.[14a] This order could also be taken as equivalent relative effectiveness of disruption of hydrophobic interactions. Ions such as ClO_4^-, I^-, and SCN^- disrupt the aqueous solvent structure extensively and thereby remove the *raison d'etre* for hydrophobic interactions. Thus, these ions are potent destabilizers of the ordered structure. On the other hand, sulfate and phosphate ions presumably fit into the existing water structure through extensive hydrogen bonding, with the result they reinforce the hydrophobic interactions in the macromolecule. Na_2SO_4 and $(NH_4)_2SO_4$ thus act as potent stabilizers of the native conformations and agents for precipitating a protein from solution. It is known[33] that Na_2SO_4 at a high concentration (1.9 *M*) makes isocitrate dehydrogenase from human heart bind tightly to the Blue-Dextran Sepharose column. However, this effect is not due to the precipitating action of Na_2SO_4 since in 2.3 *M* Na_2SO_4, 89% of isocitrate dehydrogenase remains soluble. Na_2SO_4 presumably promotes the hydrophobic interaction of the enzyme with the dye chromophore of the column by modifying the solvent structure in a way that is conducive to enhanced hydrophobic interactions. As for the cations, NH_4^+ ions act to reinforce the water structure while Ca^{+2}, and Mg^{+2} act as destabilizers of ordered conformation by binding to the macromolecules. The stabilizing action of Na_2SO_4, and CH_3COO Na arise from their participation in the extended solvent structure and consequent increase in the surface tension of water while Ca^{+2}, and Mg^{+2}, although increasing the surface tension of water, destabilize proteins by binding to them.[14b] Similar effects would apply to the elution of proteins from Blue Dextran Sepharose columns; the elution could result from either simple "electrostatic" ion exchange by salts like NaCl and KCl which occupy the middle positions in the Hofmeister series or "hydrophobic" disruption through the solvent by salts like $NaClO_4$, NaI, and KSCN, which are at the right extreme of the series. The ions at the left extreme of the series such as sulfate, phosphate, and acetate would not be able to elute the protein by virtue of their stabilizing action on the solvent structure, since this stabilization has reciprocal effects in enhancing hydrophobic interactions between the dye and the protein.

It was known[17] that the difference spectrum of the dye in 50% ethylene glycol vs. that in water had positive absorption with maximum at ~650 nm. However, no extensive studies on the spectral properties of the dye as a function of the composition of the solvent were undertaken until recently.[19] Subramanian[19] studied the difference spectra of the dye in binary aqueous solutions containing different amounts of dioxan, t-butanol, NaCl, urea, K phosphate, and other additives. The absorption spectra of the dye in these binary aqueous solutions show varying magnitudes of hyper- and hypochromic effects with or without shifts in λ_{max}. For example, the λ_{max} shifts to 630 nm (from 610 nm in water) in 50% (v/v) aqueous dioxan, aqueous t-butyl alcohol, and in neat tetramethylurea. This red shift is indicative of a nonpolar environment for the dye. Hyperchromicity was evident in aqueous solvent mixtures containing dioxan, tetramethylurea, and t-butyl alcohol indicating disaggregation of partially aggregated dye. In contrast, in aqueous solutions of 3 *M* NaCl or 1 *M* HCl, a large hypochromic effect was observed in the 610-nm region along with a shoulder in the 670- to 680-nm region. This would indicate that highly ionic media encourage self-aggregation through electrostatic shielding of the negative charges on the dye. In Figure 2, the effects of addition of small amounts of water to the solution of the dye in dioxan are shown. In 96.77% dioxan-3.23% H_2O (v/v) vs. water (or 0.1 *M* K-phosphate buffer), the difference spectrum of CB (curve 1 in Figure 2A and 2B) shows a positive peak at 690 nm, a shoulder around 630 nm, and a negative trough at 590 nm. Further small additions of water shift the position of the positive peak in the difference spectrum continuously to a point (curve 6 has a solvent composition 93.75% dioxan-6.25% H_2O, v/v) after which only intensity changes were noticed without shift in wavelength. In Figure 3 are shown the difference spectra of the dye in 2.8 *M* NaCl, 7 *M* urea, 50% aqueous dioxan (v/v), and 91% t-butyl alcohol-9% water (v/v) all referenced against water. The features are very different from one medium to another. The difference spectrum in 7 *M* urea is rather inconspicuous since in 7 *M* urea solution the structural changes in the solvent compared to water are minimal. The difference spectrum in 2.8 *M* NaCl vs. water has a positive peak at 690 nm and double minima at 630 and 585 nm. The spectra in 50% (v/v) aqueous dioxan and aqueous t-butyl alcohol (91% t-butyl alcohol, v/v) are very similar to each other with positive peaks at 655 nm and shoulders at 610 nm.

The construction of a space-filling model of CB indicates that the dye is a fairly rigid molecule with very little conformational freedom and that the rings are not coplanar to each other. The visible transitions of Cibacron Blue are localized on the anthraquinone ring and to a smaller extent on the contiguous phenyl ring.[20] Since there is very little delocalization of electrons between the aromatic rings in the dye molecule, only those interactions with the aminoanthraquinone ring, the neighboring phenyl ring, and the two attached sulfonate groups would be expected to show changes in absorption spectra. The sulfonic acid groups have low pK values of about 1.0 with the result that they are ionized at neutral and higher pH values. (The commercially available dye is the sodium salt.)

Any hyperchromic effect in the dye spectrum normally indicates a disruption of aggregation between dye molecules. Aggregation results from an unfavorable interaction with water, as an offsetting hydrophobic interaction. This aggregation typically takes the form of stacking which produces hypochromism in the spectral transition. A concentration difference spectrum[19] of the dye has a shape similar to the difference spectrum obtained with 3 *M* NaCl. The two situations need to be clarified. In the case of increasing dye concentration, the aromatic rings of the dye molecule cannot be solvated by water without a decrease in entropy. Hence the dye molecules stack against each other producing a hypochromic effect. However, the stacking will be repelled by the negative charges on the sulfonate groups. In order to counter this, the cations form a tight ion pair with the sulfonate and this interaction produces a red shift in the spectrum as evidenced by the positive peak at 690 nm and negative trough at 580 nm. With 3 *M* NaCl, the sequence is reversed with the result being the same. The high concentration of sodium ions effectively shields the sulfonate groups (producing the ionic

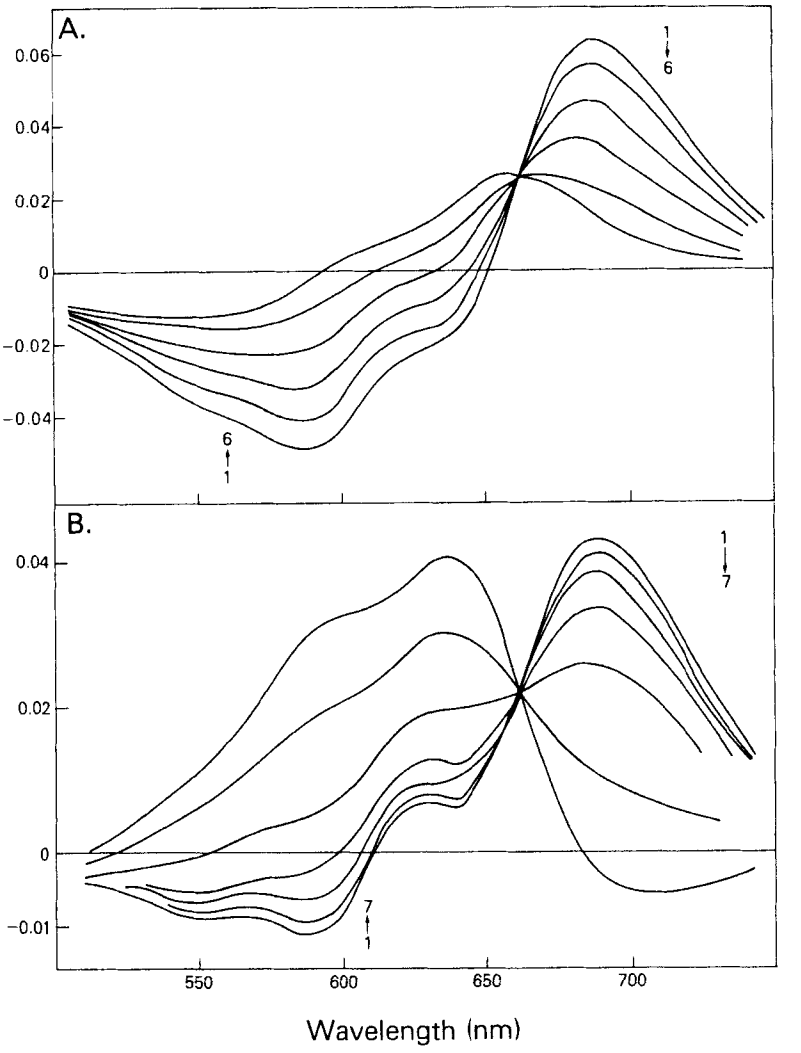


FIGURE 2. (A) Difference spectra of Cibacron Blue F3GA in dioxane-H_2O mixtures vs. water. Spectra 1 through 6 were obtained by first measuring the difference spectrum of the dye (14.84 μM) in 96.77% dioxan-3.23% H_2O (v/v) vs. in water and then adding small aliquots of water to both reference and sample cells. The dye concentration and dioxan composition (by volume percentage), respectively, are as follows: curve 1, 14.84 μM, 96.77%; curve 2, 14.74 μM, 96.15%; curve 3, 14.65 μM, 95.54%; curve 4, 14.56 μM, 94.94%; curve 5, 14.47 μM, 94.34%; curve 6, 14.38 μM, 93.75%. (B) Difference spectra of Cibacron Blue F3GA in dioxan-H_2O mixtures vs. 0.1 M potassium phosphate buffer (pH 7.4). The dye concentration (in both sample and reference cells) and the dioxan composition (volume percentage) in the sample cells are as follows: curve 1, 14.84 μM, 96.77%; curve 2, 14.79 μM, 96.46%; curve 3, 14.74 μM, 96.15%; curve 4, 14.70 μM, 95.85%; curve 5, 14.6 μM, 95.24%; curve 6, 14.49 μM, 94.49%; curve 7, 14.38 μM, 93.75% (From Subramanian, S., *Arch. Biochem. Biophys.*, 216, 116, 1982. With permission.)

effect in the spectrum, i.e., positive peak at 690 nm and negative trough at 580 nm). This shielding of the charge on the dye molecule further reduces the solvating ability of water and consequently stacking is favored (which produces hypochromism).

The difference spectra of the dye in aqueous butanol and aqueous dioxan (Figure 3) are suggestive of a nonspecific hydrophobic (nonpolar) interaction of the organic solvent com-

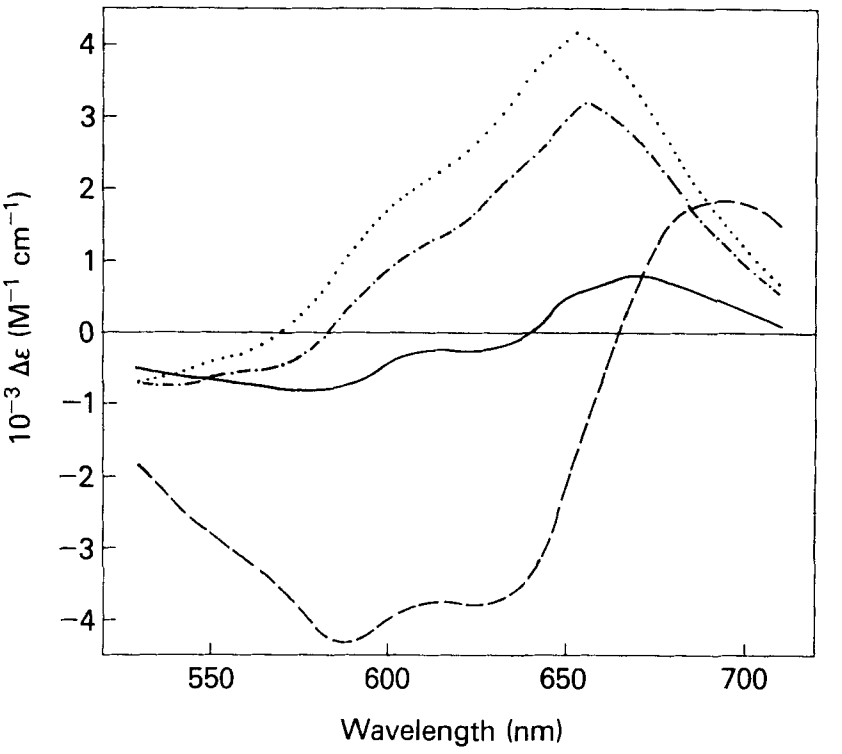


FIGURE 3. Difference spectra of Cibacron Blue in aqueous binary solvent mixtures vs. in H_2O. (- - -) 2.8 *M* NaCl vs. H_2O; dye concentration, 30 μ*M*. (——) 7 *M* urea vs. H_2O; dye concentration, 8.2 μ*M*. (-·-·-) 50% (v/v) aqueous dioxan, dye concentration, 15 μ*M*. (···) 91% t-butyl alcohol-9% H_2O (v/v) vs. H_2O; dye concentration, 10.9 μ*M*. Note that the spectra have been normalized with respect to the dye concentrations. (From Subramanian, S., *Arch. Biochem. Biophys.*, 216, 116, 1982. With permission.)

ponent with the aromatic moieties of the dye chromophore, inducing a red shift in the absorption spectrum which manifests as a positive peak at 655 nm in the difference spectrum. Since nonpolar solvents disrupt dye aggregation, the resulting hyperchromic effect is seen as a shoulder at 610 nm in the difference spectrum. Thus, the difference spectrum of the dye in binary aqueous solvents characterized by a positive peak at ~655 nm and a shoulder at ~610 nm is assigned a tentative nomenclature of a "nonpolar" or "apolar" spectrum.

The difference spectrum in high salt (3 *M* NaCl) characterized by a positive peak at ~690 nm and double minima in the 630- to 585-nm region is classified as "ionic" or "electrostatic" spectrum. The "ionic" spectrum manifests in electrolyte solutions as well as in high dioxan-low-water solvent medium (Figure 2). In the latter case the dielectric constant is so low that ion pairing between the dye anion and its counterion is induced, mimicking the situation in high salt medium.

The interaction of the dye with lysine, arginine, oligolysines, poly-L-lysine, poly-L-arginine, and protamine produces difference spectra which are similar[19] to the "ionic" spectra obtained with NaCl. Besides, the intensity of the effect as measured by $\Delta A_{685 \text{ to } 585 \text{ nm}}$ is almost the same for 3 *M* NaCl as it is for poly-L-lysine with an equivalent lysine concentration of 30×10^{-6} *M*. This argues for cooperative ionic effects in the basic polypeptide chain in interacting with the negatively charged dye molecules. It is not surprising that there would be specific selectivity of ionic interactions between polycations and Cibacron Blue since the interactions between spermine and DNA and also histones and DNA are known to be specific. Spermine, a polyamine tetracation, also interacts strongly and specifically with CB producing a typical "ionic" spectrum.[21]

A knowledge of such basic interactions between CB and other small molecules, nonpolar and ionic, is hepful in characterizing the interactions between the dye and the proteins. Such an attempt is made in Table 1 where the protein-dye interactions as studied by difference spectroscopy are assembled. The red shifts observed in the interaction between proteins and CB were previously attributed[17] to differences in the orientation of the dye when bound to the protein. But it is now clear from the extensive studies of the dye in different environments[19,21] that the difference spectrum of the protein-dye complex can almost be taken as a "finger-print" of the nature of the dye-protein interaction.

Basically the interaction is classified as either "hydrophobic" (apolar) or "electrostatic". However, caution must prevail in any serious interpretation. In some cases, the dye can interact with the protein in both modes, and the spectrum will reflect this in a composite manner. It is obvious when the interaction is classified as "electrostatic", the protein has arginine or lysine side chains at the dye-binding site interacting with the dye sulfonate groups. Likewise when the interaction is described as "hydrophobic", the dye binding site must be replete with nonpolar side chains. A further aid in identifying the interactions would be the medium used to elute the bound proteins from Blue Sepharose columns. If salt can elute a protein but ethylene glycol cannot, it is indicative of an electrostatic interaction. Conversely, a hydrophobic interaction can be inferred if ethylene glycol or glycerol elutes the protein when salt could not.* A mixed type of interaction is to be assumed if either salt or ethylene glycol or a combination of the two can elute the protein.

In a few cases, the protein-dye difference spectrum is virtually nonexistent which does not always mean the dye does not bind to the protein. If it is known that the particular protein binds to Blue Sepharose column, but does not produce a significant difference spectrum, it is likely that the dye-binding site in the protein has an "aqueous" environment, i.e., preserving the aqueous nonpolar-ionic balance in the presence of the protein as compared to the dye in aqueous medium alone. In some other cases, multiple binding of the dye gives rise to a composite spectrum. It is possible to resolve these into "specific" and extraneous binding. In certain instances like phosphofructokinase, and the holoenzyme flavocytochrome b_2, a hypochromic difference spectrum having the same shape as the absorption spectrum of the dye has been noticed. Such effects could arise if the binding site contains aromatic side chains against which the aromatic moieties of the dye molecule stack. Where possible,

* Glycerol is used to stabilize enzyme activities and also to preserve the native structure of proteins. The mechanism by which glycerol acts has recently been investigated by Gekko and Timasheff.[21a,b] It is pointed out that the chemical potential of glycerol increases in the presence of the protein and thus contact between glycerol and protein is minimized and the native structure of the protein is stabilized. Nonspecific interactions between glycerol and protein are still possible at high concentrations. An analysis of the free energies of transfer of hydrocarbons from alcohols to water indicates[21c] that glycerol is not a very good solvent for hydrocarbons but still it is a better solvent than water. The legitimate question then arises as to why ethylene glycol and glycerol are used as hydrophobic eluants in preference to ethanol or methanol. The answer lies in the protein stability. Ethanol or methanol interacts with the proteins and denatures them. However, ethylene glycol and glycerol stabilize the native conformations of proteins probably through participation in the composite solvent structure.

In the elution of proteins from Blue Sepharose columns, ethylene glycol and glycerol may not interact directly with the proteins but may interact with the dye component of the Blue gel by virtue of their lower dielectric constants than water (H_2O 78.5; ethylene glycol 37.7; glycerol 42.5 — all at 25°C). Spectral evidence indicates that this may be the case. The difference spectra of Cibacron Blue in 50% (v/v) aqueous dioxan[19], 50% (v/v) aqueous ethylene glycol[17], and 50% (v/v) aqueous glycerol (unpublished results from this laboratory) are almost identical, indicating nonpolar interactions of the organic cosolvent molecules with the dye.

The high concentration required of these solvents to elute the proteins from Blue Sepharose columns is perhaps due to the fact that ethylene glycol, and glycerol are only weakly nonpolar. Nevertheless, they appear to be useful solvents for eluting proteins bound to Blue Sepharose columns in the hydrophobic mode. Thus, the mechanism of elution of proteins from Blue gel columns by glycerol (or ethylene glycol), while far from being certain, can be ascribed to the nonpolar-type interactions between dye ligand and glycerol which presumably disrupt the dye-protein hydrophobic interactions.

the binding site has been identified (in Table 1) if the effect of coenzyme and/or substrate molecules on the difference spectrum is known. An attempt was made to correlate the tendency of a protein to bind the dye in an electrostatic mode with its isoelectric point. The presumption here is that the higher the isoelectric point, the greater the preponderance of basic amino acid side chains in the protein and consequently greater interaction between the cationic side chains and the dye sulfonate groups. The relationship, if any, could not be verified unequivocally with the available isoelectric point information for the proteins listed in Table 1. Also, the presumption could be invalid as far as the binding site is concerned, although it is applicable to the total protein.

B. Circular Dichroism

Edwards and Woody[16,20] studied the circular dichroic (CD) spectra of CB bound to lactic dehydrogenase (LDH), alcohol dehydrogenase (ADH), adenylate kinase, phosphoglycerate kinase, and aldolase. The free dye is optically inactive but when bound to these enzymes, circular dichroism is induced. It was found that the CD spectra of the dye bound to the dehydrogenases are quite different from one another. Thus, no unique conformation of the dye is required for binding to the dehydrogenases and kinases. However, for closely related proteins like horse liver ADH and yeast ADH, the conformation of the bound dye is quite similar. Saturating concentrations of coenzymes eliminate the induced CD spectra for the dehydrogenases and kinases mentioned above. The coenzyme addition had no effect on the CD spectrum of aldolase-CB complex. Also in the case of aldolase, the CD spectra of Cibacron Blue, Blue Dextran, and Blue Dextran Sepharose are quite different from one another. This may be a reflection of the restrictions on the conformation of the chromophore when both the triazine ring and the anthraquinone ring are coupled to polysaccharide matrices.

C. X-Ray Study of a Dehydrogenase-Dye Complex

The binding of CB to orthorhombic crystals of horse liver alcohol dehydrogenase was studied[22] by X-ray diffraction to 0.37 nm resolution. It was found only the *para* isomer was bound to the enzyme and the *meta* isomer of the dye neither bound well nor inhibited the enzyme activity. Although the difference between the meta and the para isomers is just the position of the sulfonate group in the terminal benzene ring, the interaction of the sulfonate group with a positively charged group on the enzyme may contribute to the binding energy only if it is at the right locus. Consequently, if the para position is crucial, the meta isomer will bind only weakly. The difference Fourier map indicated that the dye bound to the enzyme in the general extended conformation, in the proximity of the ADP-ribose binding site. The anthraquinone ring binds in a wide hydrophobic pocket which normally binds the adenine part of the coenzyme. The sulfonate on the attached benzene ring is found in a position to interact with Arg-271. The triazine ring binds to the region where the pyrophosphate moiety of the coenzyme binds bringing the chlorine atom close to the nicotinamide ribose binding site. Thus far the dye segment matched the ADP-ribose binding site. The terminal ring is bound in the cleft between the coenzyme and catalytic domains with a possible interaction of the sulfonate group with Arg-369. We found that the binding of the dye to liver ADH produced a difference spectrum resembling the "ionic" spectrum, with a positive peak at 670 nm and negative trough at 575 nm (unpublished results). The spectrum could be eliminated by addition of NADH. Most probably, a weak hydrophobic interaction is also involved. The X-ray study was the first solid evidence of the location of the specific binding of CB to an enzyme. It was also speculated[22] that Blue Dextran would also bind at the same site without any difficulty since the triazine ring of the dye occupied a site close to the surface of the enzyme molecule.

Table 1
INTERACTION OF CIBACRON BLUE F3GA WITH ENZYMES AND PROTEINS AS STUDIED BY VISIBLE DIFFERENCE SPECTROSCOPY

Enzyme or protein	Difference spectral data (nm) λ_{max}	λ_{min}	Suggested interaction[a]	Site of interaction[b]	Ref.
Dihydrofolate reductase (chicken liver)	700	580	Electrostatic	Dihydrofolate	77
Dihydrofolate reductase (*L. casei*)	645	690	Hydrophobic	Dihydrofolate + NADPH	70, 77
Dihydrofolate reductase (*S. faecium*)	670	570	Composite	—	70
Glyceraldehyde 3-phosphate dehydrogenase	680	580	Electrostatic	NADH	17, 150
(rabbit muscle)	660	—	Hydrophobic (?)	—	35[c]
Malate dehydrogenase (pig heart)	680	580	Electrostatic	NADH	150
Alcohol dehydrogenase (horse liver)	670	575	Composite	NADH	150
Alcohol dehydrogenase (yeast)	650	560	Hydrophobic	NADH	150
Lactic dehydrogenase (beef heart)	650	550	Hydrophobic	NADH	150
	665	—	Composite (?)	NADH	17
Lactic dehydrogenase (rabbit muscle)	650	550	Hydrophobic	NADH	150
	660	—	Composite (?)	NADH	17
Isocitrate dehydrogenase (pig heart)	670	—	Composite	—	33
Glucose 6-phosphate dehydrogenase	700	590	Electrostatic	—	70
Glutathione Reductase[d]	—	—	Stacking (?)	—	70
Phosphoglycerate kinase (yeast)	680	—	Electrostatic	3 Phosphoglycerate, ATP	17
Phosphoglycerate kinase (*L. plantarum*)	680	580	Electrostatic	3 Phosphoglycerate, ATP	40
Phosphoglycerate kinase (*E. coli*)	680	580	Electrostatic	3 Phosphoglycerate, ATP	41
Phosphofructokinase[d]	—	—	Stacking	ATP	18
Flavokinase (mung bean)[e]	680	575	Electrostatic	—	138
	630	—	Hydrophobic	—	138
	585	—			138
NAD kinase (pigeon liver)[f]	670	575	Electrostatic (?)	Nonspecific	28
	660	565	Composite	NAD, ATP	28
NDP kinase (pig heart)	680	580	Electrostatic	ATP (?)	148
Pyruvate kinase[g]	—	—	Hydrophobic	—	45
Hexokinase (yeast)	680	—	Electrostatic	—	140
Serine transhydroxymethylase (monkey liver)	670	—	Electrostatic (?)	Tetrahydrofolate	62
Cytochrome b_5 reductase	680	585	Electrostatic	Flavine (?) Adenine (?)	100
Flavocytochrome b_2 (Baker's yeast)	680	575	Electrostatic	Flavine (?)	99
Flavin free	—	—	Stacking	Adenine (?)	99
Holoenzyme[d]				—	99
Aldolase (rabbit muscle)	680	—	Electrostatic	Fructose 1,6-diphosphate	65, 72
RNA polymerase (yeast)	680	580	Electrostatic	ATP	83
RNA polymerase (*Azotobacter vinelandii*)	675	575	Electrostatic	—	84
Phosphoglucomutase (*Leuconostoc*)	680	580	Electrostatic	3-Phosphoglycerate	68
Glutamine synthetase (*E. coli*)					
Tense	660	—	Hydrophobic	—	149
Relaxed	650	—	Hydrophobic	—	
Aspartate transcarbamylase (*E. coli*)					
Catalytic subunit	668	580	Composite	ATP, carbomoyl phosphate	134
Regulatory subunit	688	588	Electrostatic	ATP, CTP	134
Phospholipase A_2	675	580	Electrostatic	Lipid substrate	74
Dopamine monooxygenase	676	580	Electrostatic	Fusaric acid	135

Table 1 (continued)
INTERACTION OF CIBACRON BLUE F3GA WITH ENZYMES AND PROTEINS AS STUDIED BY VISIBLE DIFFERENCE SPECTROSCOPY

Enzyme or protein	Difference spectral data (nm) λ_{max}	λ_{min}	Suggested interaction[a]	Site of interaction[b]	Ref.
β-lactamase (OXA-2)	680	580	Electrostatic	Substrate (?)	103
Ricin A chain	685	580	Electrostatic	—	75
Troponin (whole)	670	580	Electrostatic (?)	—	107
Troponin (I subunit)	660	—	Hydrophobic (?)		107
Troponin (T subunit)	660	—	Hydrophobic (?)	Nonspecific	107

Note: In most cases, the spectra were obtained either in phosphate or tris buffer at neutral pH.

[a] The suggested interaction is based on the spectra of the dye in ionic and nonpolar media as studied in simple systems.[19,21] If the spectral characteristic is between electrostatic and hydrophobic, it is termed "composite".
[b] The site of interaction is described as the ligand binding site on the enzyme. This ligand displaced the dye and diminished or eliminated the difference spectrum.
[c] Blue Dextran was used instead of the free dye.
[d] In these cases, the spectrum consisted of a general hypochromic spectrum centered at the wavelength of the dye absorption spectrum maximum, i.e., 610 nm.
[e] In water, the spectrum was "electrostatic", but in 0.1 *M* phosphate buffer it was "hydrophobic".
[f] The spectrum in presence of ATP or NAD was "electrostatic", but in the absence of NAD or ATP it was "hydrophobic".
[g] Minor perturbations in the 600- to 650-nm region.

D. Thermodynamics

The only thermodynamic study of binding CB to an enzyme until now involves the enzyme cyclic nucleotide phosphodiesterase from bovine heart.[23] CB is a competitive inhibitor for this enzyme and the inhibition constant, K_i, was measured as a function of temperature. K_i increased with increasing temperatures in the range of 0 to 30°C, suggesting that hydrophobic interactions are not largely responsible for the binding of the dye. Values of thermodynamic parameters for the binding were determined from the temperature dependence of the K_i value. At 298°K the values are $\Delta G° = -37.7$ kJ mol^{-1}; $\Delta H° = -31.6$ kJ mol^{-1}; and $\Delta S° = 20$ J deg^{-1} mol^{-1}. Using recognized thermodynamic forces[24] for the analysis of these thermodynamic values, it can be concluded that the dye binding in all likelihood is assisted by a combination of electrostatic interactions (which are characterized by negligible $\Delta H°$ values, but significant positive $\Delta S°$ values) and stacking interactions (which contribute negative $\Delta H°$ and negative $\Delta S°$ values). Hydrophobic interactions contribute positive values for $\Delta H°$ and $\Delta S°$, while hydrogen bonding interactions would make both $\Delta H°$ and $\Delta S°$ negative. Since hydrophobic interactions have been ruled out on the basis of the temperature dependence of the inhibition constant and since there are not many hydrogen bonding groups in the dye, it seems very likely that the dye-enzyme interactions are mainly electrostatic and stacking. Direct calorimetric measurements to provide thermodynamic parameters for a number of enzyme-CB complexes would further help characterize the nature of such interactions, although thermodynamics cannot provide the details on a microscopic scale.

E. Kinetics

CB has been known to inhibit a large number of enzymes[2,25,26] on a competitive basis with respect to nucleotides, substrates, and several effectors. Both Blue Dextran and the free dye have been known to inhibit several enzymes, although Blue Dextran was shown[25] not to inhibit yeast hexokinase and bovine liver dihydrofolate reductase. In general, the

inhibition of enzymatic activity by the free dye results from the dye binding directly at the coenzyme site or substrate site and rarely by affecting a conformational change in the enzyme. It was also demonstrated[26] that the portion of the dye molecule necessary for effective inhibition of nucleotide binding in several enzymes does not have to possess the triazine ring or the terminal benzene sulfonate ring. It is most likely that the tail end of CB is nonspecific and binds only when the catalytic site is large enough and properly oriented to accommodate it.

Whatever be the mechanism of inhibition of an enzyme by CB, it is important to make sure that the inhibition is reversible. Irreversible inhibition usually results from a covalent modification of a group on the protein by the active chlorine in the triazine ring. In most cases, the inhibition is reversible. So it was surmised[27] that the heterogeneous composition of commercially available CB might be responsible for the covalent modification. For instance, Weber et al.[27] found that yeast phosphoglycerate kinase was inhibited completely at 100 μM of the crude dye, but even 1 mM of purified dye did not inhibit the enzyme to any measurable extent. A purification procedure was given[27] and the component which has an R_f value of 0.77 in thin layer chromatography was shown to inhibit the phosphoglycerate kinase, while the component with $R_f = 0.57$ was shown not to inhibit. Similarly, isoleucyl t-RNA synthetase from *E. coli* was also inhibited[27] by the crude product but not by the purified product.

The dye Cibacron Blue F3GA is a mixture of *meta* and *para* isomers (see Figure 1); the isomeric position of the sulfonate group in the terminal phenyl ring is reckoned with respect to the -NH- bridge. Cibacron Blue 3GA, which is the form currently manufactured by Ciba Geigy (see Reference 135), is the *ortho* isomer. Although all the three isomers bind to dopamine β-monooxygenase,[135] slight differences in the difference spectra of the dye isomer-enzyme complexes have been noticed. This difference probably arises from the different positions of the sulfonate group and its relative contribution to the binding. However, in most preparations of blue gels, it is the mixture of meta and para isomers that is used for immobilization. This, by itself, is not expected to cause any significant change in the binding tendencies of proteins. The irreversible inhibition mentioned in the previous paragraph was caused by a minor impurity in the dye. In other cases, the inhibition is caused by covalent modification of the enzyme by the pure dye through attachment of the chlorotriazinyl ring to the enzyme *(vide infra)*. Since, in blue gel preparations, the dye is coupled covalently to dextran or Sepharose, such inhibition anomalies are not expected to manifest in the chromatographic procedure. Nevertheless, it would be desirable to use a purified, homogeneous dye sample in preparing the blue gels.

A preparation of the dye purified by precipitation from methanol solution with diethyl ether was still bound tenaciously to NAD Kinase[28] when used to elute the enzyme from the Blue Sepharose column. The bound dye was not removable by dialysis, gel filtration, or charcoal adsorption and was probably bound irreversibly. With the enzyme UDP-glucose 4-epimerase, competition between NAD and the dye for the pyridine nucleotide binding site was demonstrated.[29] In addition, the dye was bound irreversibly to the reconstituted apoenzyme which prevented NAD binding to form the holoenzyme. In this case, it was clear that the irreversible inhibition was caused by the dye proper and not by an impurity through nonspecific interaction. As a further step, Witt and Roskoski[30] purified the dye by the procedure given by Weber et al.[27] and still obtained irreversible inactivation of the C subunit of c-AMP-dependent protein kinase. The holoenzyme is resistant to dye inactivation. However, addition of c-AMP converts the enzyme to a form susceptible for irreversible inhibition. So the pure dye is capable of causing irreversible inactivation of certain enzymes. In order to be sure, all studies with the dye should ascertain: (1) purity of the reagent, (2) whether the inhibition is noncovalent or covalent, and (3) whether the purified dye is freshly dissolved since, upon storage, the dye seems to produce other components.[27]

IV. PURIFICATION AND SEPARATION OF PROTEINS ON BLUE GEL AFFINITY COLUMNS: PROTEIN-CHROMOPHORE INTERACTION

This section will deal with the purification of several proteins with the aid of Blue gel affinity columns. In many studies, Blue gel chromatography is used as an important intermediate step in the isolation of a desired protein or enzyme. The purification factor has been increased significantly with the inclusion of this step. In other cases, without the Blue gel step, purification was impossible. Invariably, elution of the desired enzyme was achieved by a natural ligand for the enzyme. Where there is no natural ligand for the enzyme, high salt or ethylene glycol has been used with great success. Salt gradients have been helpful in the separation of several components. Even pH gradients have been used in some cases. The elution of an enzyme by its own natural ligand has been generally termed "biospecific" desorption[3,5] inasmuch as the binding to the dye chromophore (although by itself is not biospecific) is disrupted by the natural ligand of the enzyme under consideration. The review by Dean and Watson[4] is an excellent compilation of several proteins with regard to the nature of the Blue gel adsorbent, and the eluting medium. In the following sections, we will cover the proteins and enzymes, group by group classified in terms of commonality of function. The interactions will be emphasized and any peculiar features pointed out. The concept of the specificity of the dye chromophore for the "NAD binding domain" will be discussed and its limitations analyzed. Several important applications will also be indicated.

A. Dehydrogenases and Kinases

Of the 2000 or so enzymes currently cataloged, 620 require a nucleotide coenzyme. Dehydrogenases and kinases have thus been the most widely studied class of enzymes, in terms of kinetics and mechanism. Correspondingly, a large number of them have been purified recently using several forms of immobilized Cibacron Blue. The classic case of the separation of four enzymes[9] from a crude yeast extract with high elution specificity is shown in Figure 4. The crude extract was applied to a Blue Sepharose column. Most of the contaminating proteins did not bind to the column. A pulse of 5 mm NAD eluted alcohol dehydrogenase, while a subsequent pulse of NADP eluted glucose-6-phosphate dehydrogenase. A change in pH by +2 units eluted hexokinase and another pulse of 10 mM NAD at a higher pH eluted glyceraldehyde-3-phosphate dehydrogenase. Although in all cases the binding of the enzymes to the column occurred through the nucleotide site, a judicious use of the eluting agents achieved good separation in fairly good yields.

A large-scale purification of horse liver alcohol dehydrogenase (ADH) was made[31] by applying a prefractionated horse liver extract to a Blue Sepharose column in acetate buffer at pH 5.5. Elution was achieved with buffer containing 1 m*M* NAD at pH 5.9. Pure ADH binds to Blue Sepharose in 0.015 *M* phosphate/0.05 *M* KCl at pH 6.4. Ninety pecent of the bound enzyme could be eluted at pH 8.3 with 0.1 *M* $NaHCO_3$/0.25 M NaCl. This indicates that both structure-specific and ionic interactions occur between the enzyme and the blue ligand. A 40-fold purification of ADH from cotton seed extracts in a single step was accomplished[32] on a Blue Sepharose column and recovered completely with 5 m*M* NAD.

Rat liver lactic dehydrogenase was eluted[8] from BDS either by ~0.2 *M* NaCl or by 1 m*M* NADH. NAD alone could not elute the enzyme. Cibacron Blue was found to be a strong competitive inhibitor with respect to NADP for human heart isocitrate dehydrogenase.[33] NADP, ATP, and nicotinamide mononucleotide elute the enzyme from BDS and so does 50% glycerol, thus suggesting nonspecific hydrophobic interactions with BDS at the coenzyme binding site. Glyceraldehyde 3-phosphate dehydrogenase (GPDH) from several lactic acid bacteria was adsorbed on BDS and eluted with KCl.[34] ADP, AMP, and glyceraldehyde 3-phosphate were ineffective in eluting GPDH, while NAD(H), NADP(H), or

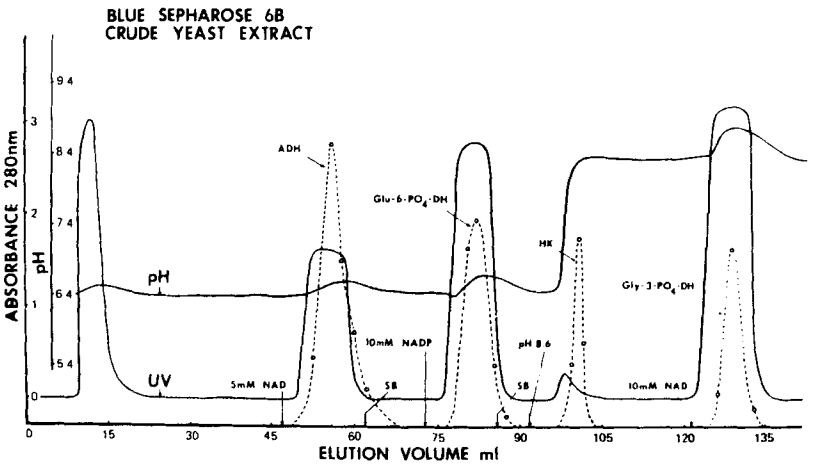


FIGURE 4. Elution of enzymes absorbed on Blue Sepharose CL-6B by the use of specific eluting agents. Dried Baker's yeast was lysed in 1 *M* dibasic sodium phosphate at 37°C for 3 hr. The extract was centrifuged at 13,700 × g for 1 hr, filtered through cheesecloth, and precipitated using 75% saturated ammonium sulfate. The extract was centrifuged and the precipitate dissolved in 20 m*M* Tris/HCl (pH 6.4) containing 5 m*M* $MgCl_2$, 0.4 m*M* ethylenediaminetetraacetic acid, and 2 μ*M* 2-mercaptoethanol. The solution was desalted using Sephadex G-25 and 223 A_{280} units of the resultant solution applied to a 1.6 × 5 cm bed of Blue Sepharose CL-6B. After the initial protein peak eluted off the column, enzymes were eluted in the pH 6.5 buffer or the same buffer adjusted to pH 8.6 with the addition of cofactors. Alcohol dehydrogenase (ADH), glucose 6-phosphate dehydrogenase (glue-6-PO_4DH), hexokinase (HK), and glyceraldehyde 3-phosphate dehydrogenase (gly-3-PO_4-DH) were eluted with 5 m*M* NAD, 10 m*M* NADP, pH 8.6, and 10 m*M* NAD, respectively. The dashed lines represent activity assays for the particular enzymes indicated. (From Haff, L. A. and Easterday, R. L., *Theory and Practice in Affinity Techniques*, Sundaram, P. V. and Eckstein, F., Eds., Academic Press, New York, 1978, 23. With permission.)

ATP eluted at 1 m*M* concentration. A critical region of the enzyme active site is apparently involved in the interaction with BDS. Rabbit muscle GPDH was found to bind more to the BDS column if glyceraldehyde 3-phosphate was also present.[35] The substrate is known to dissociate the enzyme from a tetramer to dimers and monomers. The dissociated forms of the enzyme bind preferentially to BDS. It follows that GPDH molecules couple to the BDS matrix through binding sites not occupied by NAD, since both apo- and holoenzymes are bound equally well.

6-Phosphogluconate dehydrogenase (PGDH) from human erythrocytes was purified[36] 3000-fold on a BDS column and eluted with 1 m*M* NADP. The thermophilic PGDH from *Bacillus stearothermophilus* was also purified[37] on Blue Agarose, eluted by 0.5 m*M* NADP or a KCl gradient, but not by 6-phosphogluconate. It is obvious the interaction of the Blue chromophore is at the nucleotide site. When the column temperature was increased from 0 to 55°C, the interaction between the enzyme and the column strengthened, as evidenced by the higher KCl concentration required to elute. Naturally the thermophilic enzyme would be expected to bind the nucleotides (and hence the dye) tighter at higher temperature. Inosine 5′-monophosphate dehydrogenase[38] was purified on Blue Sepharose, using a KCl gradient for elution with an overall recovery of 62%. It was observed that preincubation of the enzyme sample with the gel column was necessary to increase the recovery upon salt elution.

An artificial mixture of five isozymes of beef lactic dehydrogenase (LDH) was fractionated[2] on a Blue Sepharose column by eluting the isoenzymes with a concave gradient of NADH

ranging from 0 to 1 m*M* as shown in Figure 5. The separation technique exploits the differential affinity of the heart (H_4) and muscle (M_4) LDHs for NADH. The hybrid isozymes with varying numbers of H and M subunits would be expected to have NADH affinities intermediate between those of H_4 and M_4 species with the result that the isozymes were well separated. Using an ideal affinity gel of immoblized NADH, no isozyme separation could be achieved with a soluble NADH gradient since the relative effectiveness of NADH as an eluting agent would be the same for each isozyme.

Kawaii et al.[39-42] found that the Blue dye inhibition of phosphoglycerate kinase (PGK) was competitive with respect to 3-phosphoglycerate, but noncompetitive with respct to ATP in *Lactobacillus* and *E. coli* PGK. The yeast PGK, however, was found to be inhibited by the dye in a competitive manner with respect to both ATP and 3-phosphoglycerate.[43] The *L. plantarum* PGK, yeast PGK, and *E. coli* PGK were all bound to BDS columns and eluted by 1 m*M* 3-phosphoglycerate or ATP.[39,41,43] While there are minor variations in kinetic terms, it is clear that the PGK from all these species binds the Blue chromophore at an overlapping site (at the active site region). The spectral perturbations also indicate electrostatic interaction. It is thus mainly the phosphate moiety binding region of the enzyme which is responsible for binding the dye.

Pyruvate kinase from human erythrocytes,[44] *Neurospora crassa*,[45] and human liver[46] was bound to BDS and eluted only by fructose 1,6-diphosphate (FDP). The dye also acts as a noncompetitive inhibitor with respect to the substrates.[45] FDP is an allosteric effector and could elute the enzyme by affecting a conformational change[44] in the enzyme. However, it is more likely that the enzyme binds to the column at the FDP allosteric site. In contrast, fructose 1,6-bisphosphatase from chicken liver[47,48] binds to BDS not through the substrate (FDP) site, but at the allosteric AMP site. Only AMP (but not FDP) elutes the enzyme from the BDS column. Thus, we see it is not an absolute configuration that is critical for binding, but an overall optimization of interactions.

Phosphofructokinase (PFK) from yeast bound to Blue Sephadex[7] could not be eluted with even 20 m*M* ATP, while the enzyme from the BD-enzyme complex in polyacrylamide gel was eluted with 2 mm ATP. It was found[49] that CB inhibits yeast PFK competitive with respect to ATP at the substrate site. PFK from several strains of homolactic bacteria[50] was adsorbed by BDS and completely eluted by 1 m*M* ATP or 1 *M* KCl. It appears that PFK binds to the Blue chromophore through the ATP site and the method of immobilization of the chromophore may determine elution capability of ATP.

Three homodimeric creatine kinase isozymes (A_2, B_2, and C_2) of the green sunfish[51] were purified on Blue Sepharose by loading at 0.05 *M* Na phosphate at pH 5.8 and eluting at the same buffer concentration but at a pH of 8.0. Herein lies a case of nonspecific ionic binding where elution is achieved by changing the acidic-basic groups balance in the enzyme by shifting to a higher pH.

NAD kinase[28] from pigeon liver did not adsorb on BDS, but it bound to Blue Sepharose. The elution pattern was also strange, i.e., 1 *M* KCl, 5 m*M* NAD, 2 m*M* NADH, or even 10 m*M* Mg ATP could not elute the enzyme. Elution was achieved only by 0.5 m*M* of the free dye. The free dye inhibited the enzyme competitively with respect to NAD. The specificity of the enzyme for the dye itself is not understood clearly at this time. The pig heart nucleotide diphosphate kinase (NDP Kinase) was purified[52] on Blue Sepharose column using ATP to elute, with a 500-fold purification.

Cheng and Domin[53] effected separation and purification of nucleoside monophosphate kinases, and nucleoside diphosphate kinase from nucleoside kinases from human erythrocytes. The nucleoside kinases were not at all retained on the Blue Sepharose column. Obviously, the nucleoside binding site of the enzyme does not have the same groups as the nucleotide kinases have in their substrate binding sites. The NDP kinase was eluted with 2 m*M* Mg ATP, while AMP kinase and CMP kinase were eluted with a tris chloride gradient.

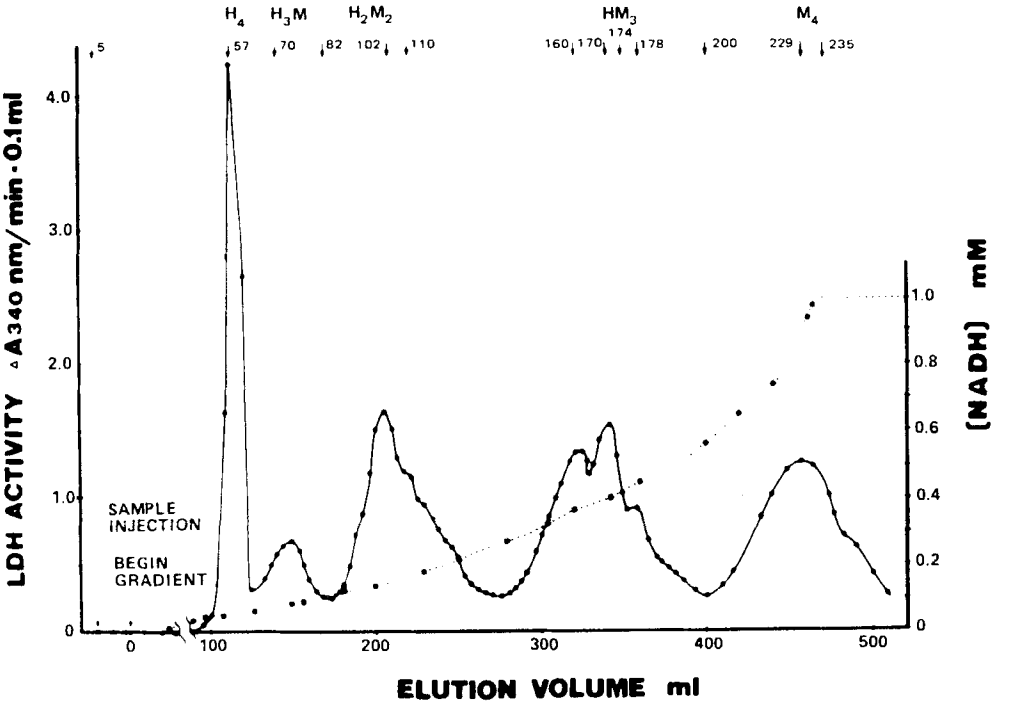


FIGURE 5. Separation of lactic dehydrogenase isoenzymes on Blue Sepharose CL-6B using a gradient of NADH. 3.0 mg of purified heart lactic dehydrogenase and 4.5 mg of purified muscle lactic dehydrogenase (beef, Sigma Chemical Company) were separately desalted on a Sephadex G25F gel bed into 0.1 *M* sodium phosphate, pH 7. 0.35 mg of heart lactic dehydrogenase and 0.40 mg of muscle lactic dehdrogenase were mixed, quickly frozen, and slowly thawed. The mixture was injected into a 1.6 × 6 cm bed of Blue Sepharose CL-6B with a dye substitution of 6.2 μmol/mℓ gel. The isoenzymes were eluted using a 250-mℓ concave gradient from 0 to 0.5 m*M* NADH in 0.1 *M* Tris/HCl (pH 7) — 1 m*M* mercaptoethanol. Samples were eluted at a flow rate of 0.5 mℓ/min. 1-mℓ fractions were collected and assayed for LDH activity as described in *Worthington Biochemicals* catalog. (From Haff, L. A. and Easterday, R. L., *Theory and Practice in Affinity Techniques*, Sundaram, P. V. and Eckstein, F., Eds., Academic Press, New York, 1978, 23. With permission.)

A protein kinase[54] of bacteriophage T7 was purified on Blue Sephadex using NH_4Cl gradient from 0 to 0.5 *M*. A cyclic GMP-dependent protein kinase[55] was retained on the BDS column and eluted by 0.2 *M* KCl with a 90% recovery. Mg nucleotides neither prevented the enzyme from binding, nor did they elute it from the column. Apparently the dye chromophore binds at the cyclic GMP site. This type of selectivity of the allosteric site for dye binding may result from the inaccessibility of the active site in the absence of bound c-GMP. On the other hand, the c-AMP-dependent protein kinase[30,56] from bovine brain was bound to BDS column only when c-AMP was present and it was eluted by 5 m*M* Mg ATP. In the absence of c-AMP, the protein kinase holoenzyme does not bind to BDS presumably because the catalytic-regulatory subunit combination is inaccessible for dye binding.

B. Other Nucelotide-Dependent Enzymes

Phosphodiesterase (PDE) from snake venom was separated[57] from nonspecific phosphatase and 5′-nucleotidase on Blue Sepharose. The latter two passed through the column unadsorbed. The retained PDE was eluted with 30 m*M* phosphate. c-AMP phosphodiesterase[23]

from bovine heart was retained on Blue Sephacryl S-200 and eluted by cyclic AMP or 1 *M* KCl. Similarly, the bovine brain cyclic nucleotide PDE[58] was retained on BDS and eluted by c-AMP or c-GMP or 1 *M* NaCl. In these instances, the phosphodiesterases bind to the free as well as immobilized dye at the catalytic site. Robinson et al.[59] purified a cyclic nucleotide phosphodiesterase from bovine brain particulates, by extracting them with Triton X-100 and adding an anionic detergent like deoxycholate and chromatographing the extract on BDS. Without the addition of deoxycholate or sodium dodecyl sulfate, the enzyme does not bind to BDS since Triton X-100 prevents the interaction of the enzyme with BDS.

In the case of multinucleotide-dependent enzymes like IMP dehydrogenase and adenylosuccinate synthetase, the former enzyme is bound to Blue Sepharose and completely eluted by IMP alone.[60] NAD also elutes the enzyme but only weakly. The dye binding site most likely spans the IMP site, but may also extend into the NAD site. Adenylosuccinate synthetase, however, can be eluted only by IMP + GTP + L-aspartate, i.e., all the three substrates are required to displace the dye chromophore. NADPH-dependent 5,10-methylenetetrahydrofolate reductase can be displaced from BDS column by 1 *M* KCl but not by coenzyme.[61] But aldehyde reductase (NADPH-dependent) from rat liver was adsorbed on BDS or Blue Sepharose[61] and eluted by NADPH. NADH is a heterotropic effector for monkey liver serine hydroxymethyl transferase. CB inhibits this enzyme competitively with respect to tetrahydrofolate.[62] NADH does not desorb the enzyme from Blue Sepharose column. The above cited cases point out that the dye chromophore does not necessarily bind at the NAD(H) site whether it is an active site or allosteric site. Although the NAD(H) site is the most likely choice, other sites have preference if they present optimal interactions or when the NAD(H) site is not made available by the enzyme to the dye chromophore for lack of a conformational change or other factors.

The question then remains with what common feature or structural locus does the blue chromophore interact? Initial experiments with Blue Dextran suggested nonspecific binding. However, a large number of dehydrogenases and kinases were bound to the immobilized blue gel columns and were displaced by the coenzymes although there were notable exceptions. Some generalizations were in order and that is how the specificity for the "dinucleotide fold" set in.

C. Dinucleotide Fold

Stellwagen[3,63] was the first to recognize a structural similarity between Cibacron Blue and NAD. Comparison of a space-filling model for the dye and NAD as it binds to several dehydrogenases in its extended conformation revealed distinct structural similarities. The anthraquinone and the terminal phenylsulfonate rings of the dye can be oriented like the adenine and nicotinamide rings of NAD, respectively. The two internal rings of the dye mimic the two ribofuranose rings of NAD. Two of the dye anionic sulfonate groups are positioned similarly to the two anionic phosphates of NAD. The description of enzyme structures to high resolution by X-ray crystallographers has led to the recognition that enzymes which catalyze reactions involving NAD (i.e., mainly dehydrogenases) possess a common architectural supersecondary structure variously called the "NAD-binding domain", the "Rossman fold", or simply the "dinucleotide fold". This domain[64] involves a continuous 150-residue segment of a polypeptide chain arranged in a β-sheet core composed of six parallel β-strands connected by α-helical loops above and below the plane of the sheet. There are variations to the number, direction, and connectivity of α-helices and β-strands, but the overall supersecondary structure is similar in most cases. NAD binds at the carboxy terminus of the β-sheet. Since it was first reported that it was the Blue chromophore of Blue Dextran that interacted with phosphofrucktokinase,[7] subsequent chromatographic[63] and spectrophotometric[17] measurements indicated that the Blue chromophore could bind at the

nucleotide-binding site of the dehydrogenases and kinases. Confirming this idea, several enzymes were inhibited by the dye competitively with respect to the nucleotides, and the coenzymes reduced or eliminated the difference spectral signal of the enzyme-dye complex.[17] In addition, the coenzymes displaced the enzymes from the BDS column. A survey was made[63] of several proteins known to contain the "dinucleotide fold" and it was found that all those proteins which possessed the NAD domain were bound to the BDS column and displaced by the appropriate nucleotide ligand at very low concentration (usually in the millimolar range). The concentration of the nucleotide required for elution was about two orders of magnitude smaller than the concentration of NaCl required to elute the enzyme. In a number of cases including phosphogluconate dehydrogenase, phosphofructokinase, fructose diphosphatase, and DNA polymerase whose supersecondary structures were not known, Stellwagen and co-workers[63] found these enzymes were bound to BDS and eluted by low concentrations of the ligand nucleotides. This led Stellwagen to believe that BDS affinity chromatography can be used to readily identify proteins that possess the dinucleotide fold and to improve purification methods for those proteins that have the fold. In principle, all that is required to establish the presence of the fold is a crude cellular extract in a low ionic strength medium and an assay for the protein of interest. If the biological activity is missing from the crude extract after passage over BDS column and if specific ligands eluted the protein more effectively than NaCl, it is very likely that the protein possesses the dinucleotide fold. This criterion was applied later by Stellwagen and co-workers to predict the distribution of the NAD domain among all enzymes in the glycolytic pathway.[65] Phosphorylase *a*, by this criterion, should possess a dinucleotide fold. It was subsequently found[66] by X-ray crystallography that one region in the enzyme has the same topology as the NAD domain which accommodated the dye or adenosine but not AMP. The dye additionally binds at the allosteric AMP site as well. This finding was neither a proof nor invalidation of the Stellwagen hypothesis. Based on chromatographic experiments, Thompson et al.[63] concluded that dihydrofolate reductase and hexokinase do not possess the dinucleotide fold. They also propounded that an allosteric site if bound to BDS should have a dinucleotide fold, and that a full intact dinucleotide fold (rather than remnants of a dinucleotide fold like that found in apoflavodoxin and subtilisin) is necessary to bind to BDS.

D. Merits and Demerits of the Dinucleotide-Fold Hypothesis

The dinucleotide-fold hypothesis was a generalization pronounced after studying a variety of proteins and as such it is applicable to a large number of enzymes. It is a useful guideline for a proper choice of the affinity matrix for separation and purification of a number of proteins. There is no basic flaw in the rationalization of the structural homology between NAD and the Blue dye. However, the generalization was rather sweeping and in several other studies was interpreted as a dogma. At the time the hypothesis was proposed there seemed to be enough ground for it. As more studies were made, exceptions to the hypothesis became widespread.

There were several deficiencies in Stellwagen's conclusions:[3]

1. The use of BDS as a standard immobilized Blue gel was not appropriate, as it turned out later[67] that a number of enzymes which did not bind to BDS, bound to Blue Sepharose, thereby eroding the validity placed on BDS.
2. The concept of nucleotide requirement was rendered null in the case of phosphoglycerate mutase which does not require a nucleotide but all the same eluted from BDS column by 2,3-diphosphoglyerate or even NADPH.[68]
3. A number of proteins like hemogloin found not to bind to BDS[3] were actually bound[9] at a lower pH.

4. Dihydrofolate reductase (DHFR) from *S. faecium* did not bind to BDS and hence was declared not to have a dinucleotide fold. Actually it is found[69] to have a variation of the NAD domain and DHFRs from other species are now known to bind to Blue Sepharose columns.[70]

Specifically, phosphoglucomutase and phosphoglucose isomerase bound to Blue Sepharose,[67] although they did not bind to BDS.[3] Phosphoglucose isomerase could be eluted with 2 *M* KCl or 2 m*M* fructose 6-phosphate, while phosphoglucomutase bound to Blue Sepharose only below pH 7.0 and was eluted by glucose 1-phosphate.[67] Stellwagen[71] predicted that aldolase should have an NAD domain in the amino acid sequence 147 to 299 based on a sequence analysis and homology with lactic dehydrogenase. But Grazi et al.[72] pointed out that each subunit of aldolase binds more than 3 molecules of the dye and that modification of the residue lys-227 (which is supposedly in the NAD domain) does not alter dye-binding characteristics. This diluted the dinucleotide-fold hypothesis for aldolase significantly.

Edwards and Woody[16,20] found that the induced CD spectra of the dye with alcohol dehydrogenase were opposite in sign to those with lactic dehydrogenase and the CD spectrum of aldolase-dye was not diminished by coenzymes although they eliminated the spectra for alcohol and lactic dehydrogenases. They reasoned that CB is not a highly specific analog of nucleotides, but that any ensuing specificity is just a by-product, in view of limited conformational freedom of the dye as well as the protein.

Wilson[25] found that Blue Dextran would not inhibit yeast hexokinase or bovine liver dihydrofolate reductase (DHFR). The enzymes did not bind to the BDS column either. But the free dye inhibited yeast hexokinase. The inhibition of DHFR by the dye was competitive with respect to dihydrofolate indicating that the dye is not nucleotide-site specific. Thus it is the dye binding and not adsorption to BDS column that should be a guiding factor in evaluating the suitability of other Blue gel columns for purification of enzymes. It must also be recognized that the dye may be a universal substitute ligand for nucleotides, cyclic nucleotides, dihydrofolate/folate, and a host of other aromatic-anionic molecules.

Beissner and Rudolph[26] concluded that the anthraquinone ring and the attached phenylsulfonate ring portion of the dye are enough to cause inhibition of several nucleotide-requiring enzymes, thereby removing the structural analogy between the dye and NAD as the basis for functional similarity. Yeast hexokinase does not contain a dinucleotide fold; nevertheless, the enzyme is inhibited by the dye or fractions of the dye molecule. So, the premise that an enzyme has to possess a dinucleotide fold in order to bind CB is not valid even for nucleotide-requiring enzymes. Not even the precise position of the groups in the dye seems to be a requirement since it was found[14] that a structural isomer of CB such as Cibacron Brilliant Blue, and Procion Brilliant Blue (which lacks the terminal ring of CB) when coupled to Sepharose are equally effective in binding enzymes. The coupling mode probably plays a more important role than the position of the groups.

The L-arabinose binding protein from *E. coli* was shown not to bind CB even though it possessed a dinucleotide fold.[73] In a similar vein, both pyruvate kinase and triosephosphate isomerase have the same type of folding with eight parallel β strands and α helices, but only the former and not the latter enzyme binds to Blue gel column. Phospholipase A_2 binds the free dye and it also binds to BDS column.[74] NAD(H) does not displace the enzyme from the column, but the water-soluble dihexanoyl phosphatidylcholine does. Similarly, the A chain of ricin binds the free dye which could not be displaced by any nucleotide, although the A chain is known to interact with ribosome.[75] Albumin is another classic example for specific dye binding[76] without having a nucleotide requirement.

To sum up, the current status of the linkage of the requirement of dinucleotide fold with dye binding by an enzyme is undergoing a steady transition. Stellwagen has realized, "Ci-

bacron Blue F3GA has suffered the fate of many biological inhibitors namely to appear less specific as its use becomes more widespread'' (quoted in Reference 4). This is not to deny the structural homology between NAD and CB. Caution must prevail in overextending the analogy. It is still true in a large number of cases that NAD or other nucleotides and the dye are mutually replaceable. But it is also true that the dye can engage in specific hydrophobic or specific electrostatic interactions as the situation demands. It was proposed[77] that the dye CB is capable of binding to any protein possessing a cluster of aromatic and aliphatic apolar groups and/or geometrically spaced positively charged groups for proper interaction with the aromatic rings and/or sulfonate groups of the dye molecule. The specificity of the blue dye, when it obtains, for the nucleotide binding sites is thus a special case of the above-mentioned requirements. The sanctity of the dinucleotide fold itself is now in question as to whether it represents a divergent evolution or it is just the most convenient way of folding for proteins (convergent evolution). To this end, it is important to recognize the specific interactions and utilize the dye for separation and purification of proteins rather than use it as a conformational probe for the presence of the ''nucleotide binding domain'' in a given protein.

E. Reductases

The enzyme 3-hydroxyl 3-methylglutaryl coenzyme A reductase[78] was purified over 1000-fold from a rat liver microsomal extract on a BDS column. Elution was achieved either with 0.5 *M* KCl or 5 m*M* NADPH in buffer. Ionic interactions seem to be significant in retaining the enzyme on the column. The ribonucleotide reductase from mammalian Erlich tumor cells was separated[79] into two fractions on a BDS column — a tris fraction which passed through without binding to the column and a dye fraction which was eluted from the column with 0.3 m*M* free dye. Each fraction by itself was inactive but when pooled together regained activity. Apparently the dye chromophore binding site is at the interface of the two subunits which separate upon loading to the column. The dye fraction, strangely enough, could not be eluted with ATP, NADH, or 1 *M* NaCl.

The plant enzyme NADH:nitrate reductase from corn leaf or squash cotyledon binds poorly to the BDS column.[80] It was eluted with 0.1 m*M* NADH to achieve purification by a factor of 630. The specificity of the dye chromophore for the folate binding site is demonstrated in the case of dihydropterin reductase[81] and dihydrofolate reductase.[70,77] Dihydropterin reductase from beef kidney was eluted from a BDS column completely by 1 m*M* folate or methotrexate in addition to being eluted by NAD(H), and 1 *M* NaCl. The presence of tetrahydropterin strengthens the binding of the enzyme to BDS column. This indicates that there is significant leeway at the active site of the enzyme for the dye.

L. casei dihydrofolate reductase (DHFR) binds to Blue Sepharose and is eluted by NADPH, dihydrofolate, or methotrexate.[70] There is a partial elution by 50% glycerol and complete elution by 2.5 *M* NaCl. The binding site for the dye chromophore overlaps the NADPH and dihydrofolate subsites at the active site. It was again shown[70] that DHFR from *S. faecium* does not bind to BDS column confirming Stellwagen's observation.[3] *L. casei* DHFR does not bind to Blue Dextran when either NADPH or methotrexate is present, indicating the occupation of an overlapping site by Blue Dextran. The comparative study of the binding of the blue dye to DHFR from *L. casei* and chicken liver[77] indicates the danger of extrapolating the properties of an enzyme from one species to another. Figure 6 details the difference spectra obtained for CB binding to DHFR from *L. casei* and chicken liver and the effect of NADPH and methotrexate on the spectra. The dye seems to bind in an almost totally ''electrostatic mode'' at the dihydrofolate binding site of the chicken liver enzyme and is displaced from the enzyme only by dihydrofolate, folate, or methotrexate and *not* at all by NADPH. In contrast, the binding of the dye to *L. casei* DHFR is characterized by a totally

"apolar interaction" at a site partially overlapping the NADPH site and the methotrexate/dihydrofolate site. NADPH can only partially displace the dye, and methotrexate is more efficient than NADPH. Both NADPH and methotrexate are needed for a total displacement of the dye from the bacterial enzyme.[77]

In view of the similar three-dimensional structures of the chicken liver and *L. casei* dihydrofolate reductases, it is surprising that the dye chooses to bind at two totally different microenvironments in the two enzymes producing totally dissimilar spectra. One reason for the ionic interactions with chicken liver enzyme and apolar interactions with *L. casei* enzyme is that the isoelectric points are 8.4 and 6.02 for the chicken liver and the *L. casei* enzymes, respectively.[77] However, the active sites in both enzymes have basic amino acid groups. The reason for avoiding these groups in favor of nonpolar groups in *L. casei* enzyme while favoring them in the chicken liver enzyme for the dye interaction must lie in the composition of the hydrophobic pocket in each enzyme. This is another example of optimization of dye-protein interactions, be they hydrophobic or electrostatic.

F. Enzymes of Polynucleotide Metabolism

A number of enzymes of polynucleotide metabolism such as RNA polymerases,[82-84] tRNA-synthetases,[85-88] polynucleotide phosphorylase,[89] polynucleotide kinase,[90] and DNA polymerase[91] have been reported to be inhibited by CB or use it as an affinity ligand. In most of these cases, the dye chromophore is known to interact at the polynucleotide binding site while the mononucleotide site is occupied in a few cases. This dichotomous preference of the dye chromophore for either the mono- or the polynucleotide site should not be surprising since the dye is a trianion and could function like an oligonucleotide. However, the inhibition at the polynucleotide site is not merely due to the polyanionic character of the dye since the inhibition is much more effective (on an equivalent anion basis) than heparin or poly-L-glutamate, as evidenced in the case of estradiol receptor complexes.[92]

When the dissociated subunits of DNA-dependent RNA polymerase from *B. subtilis* were applied to BDS column, only the β subunit binds.[82] It was eluted by 0.6 *M* KCl and this is the subunit directly involved in phosphodiester bond formation. CB inhibited yeast RNA polymerase I noncompetitively[83] with respect to both the template (DNA) and ATP, at a site different from the active site. Inhibition was not prevented by preincubation with DNA and ATP. The enzyme was bound on the BDS column and was eluted in the active form by 0.4 *M* NaCl, with a 50% recovery. The DNA-directed RNA polymerase from *Azotobacter vinelandii* was also inhibited noncompetitively with respect to ATP, UTP, or the template.[84] The chain elongation was prevented by CB by "occluding at the RNA product site".[84]

Among several tRNA-synthetases studied, the ilu-tRNA synthetase from *E. coli* was purified on a BDS column by eluting with 2 m*M* ATP or 100 m*M* NaCl.[88] In this instance, the dye chromophore displayed a specificity for the nucleotide site. However, yeast cytoplasmic val-tRNA synthetase was eluted from the BDS column with 1 μ*M* of the cognate tRNA (i.e., $tRNA_2^{val}$) with total homogeneity.[85] Similarly, aspartyl-tRNA synthetase and arginyl-tRNA synthetase from yeast were readily displaced[87] from BDS column by their cognate tRNAs but not by ATP or a mixture of ATP and the cognate amino acid, indicating the preference of the polynucleotide binding site of these enzymes for the dye chromophore. Tryptophanyl-tRNA synthetase of *E. coli* can form a complex[86] with BDS, in the presence or in the absence of Mg^{+2}. In its absence the complex is dissociated by either ATP or cognate tRNA. However, in the presence of Mg^{+2}, only $tRNA^{trp}$ can dissociate the complex but not ATP. Thus, the presence of Mg^{+2} can direct the dye discriminatingly to the polynucleotide site. The selective elution by cognate tRNAs of the individual tRNA synthetases from immobilized blue columns provides a rapid and efficient method of purification of the enzyme under consideration. The desorption is "biospecific", but the adsorption involves a complementary characteristic.

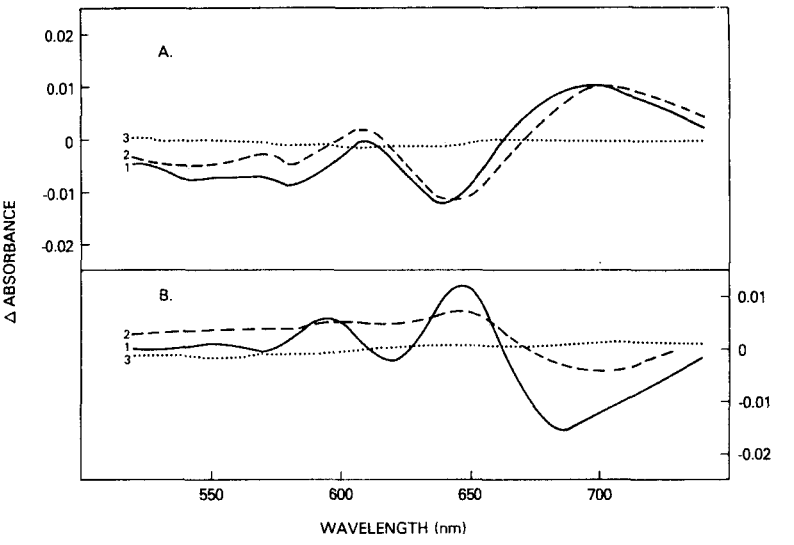


FIGURE 6. Difference spectra of Cibacron blue bound to chicken liver (A) and *L. casei* (B) dihydrofolate reductases. (A) Curve 1: difference spectrum obtained upon mixing 1 ℓ of 30 μM Cibacron blue in buffer with 1 mℓ of 17.7 μm of chicken liver dihydrofolate reductase in the split sample cell vs. mixing 1 mℓ of 30 μ*M* Cibacron blue in buffer with 1 mℓ of buffer in the split reference cell; Curve 2: spectrum obtained after subsequent addition of NADPH to both cells to a final concentration of 50 μ*M*: curve 3: methotrexate (final concentration 40 μ*M*) added to both cells. (B) Curve 1: difference spectrum obtained upon mixing 1 mℓ of 30 μ*M* blue dye in buffer with 1 mℓ of 18.7 μ*M* of *L. casei* dihydrofolate reductase in the split sample cell vs. mixing 1 mℓ of 30 μ*M* blue dye in buffer with 1 mℓ of buffer in the split reference cell; curve 2: after addition of NADPH (final concentration of 25 or 250 μ*M*) to reference and sample cells; curve 3: after addition of methotrexate (final concentration 22 μ*M*). (From Subramanian, S. and Kaufman, B. T., *J. Biol. Chem.*, 255, 10587, 1980. With permission.)

It was established that polynucleotide phosphorylase of *E. coli* could not be eluted by any of the nucleoside diphosphates, but low concentrations of the polymeric substrates[89] could elute the enzyme from BDS column. Clearly the mononucleotide site of the enzyme was not involved in the binding. Similarly, ATP was a poor eluant for T4 polynucleotide kinase.[90] The free dye inhibited the kinase competitively with respect to ATP. *E. coli* DNA polymerase I could be eluted from the BDS column with deoxynucleoside triphosphates, while the T4 DNA polymerase could not be.[91] The dye was a competitive inhibitor for the deoxynucleoside triphosphate in the host enzyme but not in the T4-infected enzyme. The polynucleotide binding site occupation by the dye chromophore is a novel case of cooperative binding indulged in by the dye as previously demonstrated in the formation of spermine-CB complex.[21] An autolysin, murein transglycosylase[93] was also inhibited by the free dye at the polynucleotide binding site of the enzyme and was purified by elution from a Blue Sepharose column by single-stranded DNA.

G. Interferons

The clear-cut case of a hydrophobic vs. electrostatic interaction of a protein with the blue dye chromophore was demonstrated for interferon.[94] Human fibroblast interferon bound completely and was not displaced from Blue Dextran Agarose by 1 *M* NaCl; however, 50%

ethylene glycol completely eluted it, indicating the hydrophobic nature of the interaction. Leukocyte interferon, on the other hand, could be recovered from the same column by increasing the ionic strength of the medium, indicating primarily the electrostatic nature of the binding. Neither of the interferons could be displaced from the column with nucleotides or aromatic amino acids. When the free dye was immobilized directly to CNBr-activated agarose, the strength of binding of the fibroblast interferon was decreased but the leukocyte interferon did not bind at all.[94] This indicates the importance of the amino and sulfonate groups in the anthraquinone ring for effective binding of some proteins.

Interferon induced by poly(IC) in human muscle skin fibroblasts eluted in the void volume with Blue Dextran on a Sephadex G-200 column.[95] The protein was not separated from Blue Dextran by ATP or NAD, but 2 *M* KCl effected a 92% recovery of the protein. This indicates electrostatic interaction although in another study[94] the fibroblast interferon was found to display a hydrophobic interaction. This discrepant finding probably has its origin in the source of preparation of the protein.

In another study,[96] the bulk of the human leukocyte interferon (83%) was eluted by 0.5 *M* NaCl from a Blue Sepharose column, whereas most of lymphoblastoid interferon (68%) was desorbed by 1.0 *M* NaCl. A 1000-fold purification was effected. The interferon induced in mouse C-243 cells with Newcastle disease virus was adsorbed by the BDS column and eluted by poly(I) or poly(U).[97] The desorption was specific for the polynucleotides and not necessarily a charge effect since oligo(U) at a molar concentration 20 to 100 times greater than the poly (U) used did not desorb the protein. Poly(I) or poly(U) also offered protection against thermal denaturation of interferon. These observations point to a direct interaction of interferon with polynucleotides and suggest that interferon has a polynucleotide binding site. The unifying factor, of course, is the induction of interferon by poly(I)·poly(C) or poly(A)·poly(U). Kenny et al.[98] also purified human fibroblast interferon on a Blue Sepharose column by eluting with 50% ethylene glycol.

Caution must prevail in the interpretation of elution pattern of interferons from Blue gel columns. All interferons carry varying amounts of glycosylation and the degree of glycosylation in different types and in different preparations may alter the resultant hydrophobicity of the protein. The polynucleotide elution of the mouse interferon may be specific to that source or it may be a "memory" effect of the induction source.

One of the advantages of Cibacron Blue is that it could be used as an affinity ligand at pH 2 to 3 without losing its ionic advantage (in view of the very low pK values of the sulfonic acid groups) if a suitable matrix stable at this pH is available. This may be important in isolating leukocyte interferon obtained by induction with Newcastle disease virus which is then inactivated by adjusting the pH to a value of 2 to 3.

H. Flavoproteins

Apoflavodoxin was shown[63] not to bind to BDS although its structure could be superimposed on the NAD domain of dehydrogenases. Flavin-free cytochrome b_2 was, however, shown[99] to bind to BDS and eluted by higher ionic strength, dilute ethylene glycol, and low concentrations of flavin mononucleotide (FMN). The holoenzyme was not bound by BDS. Obviously the binding to the column is through the flavin site, which is characterized as both hydrophobic and ionic. This site is not available in flavocytochrome. However, the free dye inhibits the holoenzyme, showing a mixed type inhibition with respect to lactate. The inhibition was not relieved by purine nucleotides, but was sensitive to salt and ethylene glycol. The difference spectra of the enzyme-dye complex indicate that while the apoenzyme produces an "ionic" spectrum, the holoenzyme displays only a hypochromic effect. It is likely that the dye binding site in the holoenzyme is a weak extraneous site which is not strong enough to interact with BDS. Part of the weakness of this interaction may stem from

the BDS itself as noted before.[67] In contrast to flavocytochrome b_2, both the holo- and apoenzyme cytochrome b_5 reductases were bound to the BDS column, the holoenzyme rather weakly and the apoenzyme strongly.[100] The holoenzyme was eluted with NADH, while the apoenzyme required both NADH and FAD or high salt for elution. Apparently, the holoenzyme binds to the Blue gel through the NADH site. This belief is reinforced by the inhibition of NADH oxidation by the free dye. The difference spectrum for the holoenzyme-dye complex was characteristically "ionic".

I. Endonucleases

The restriction endonucleases *Bam* HI, *Pal* I, and *Bgl* I together with *Bgl* II were retained[101] on Blue agarose when sonicated crude extracts of the organisms were applied to the column, and eluted with a 0 to 0.5 *M* linear gradient of NaCl. The purified enzymes were free of nucleic acids and other nucleases. Using CNBr-activated Sepharose to couple CB, George and Chirikjian[102] fractionated several type II — specific restriction endonucleases, including *Bam* HI, *Hinf* 1, *Pst* 1, and *Xba*, using a salt gradient to elute. The enzymes eluted at different salt concentrations reflecting the extent of ionic interactions in each case.

J. β-Lactamases

β-lactamases do not require any nucleotide cosubstrates. The plasmid-mediated OXA-2 β-lactamase (R46) was adsorbed[103] on Blue Sepharose and eluted by 28 m*M* benzyl penicillin, a substrate for the enzyme, or 1.2 *M* NaCl. ATP and NADH were not able to elute the enzyme. In kinetic studies, the free dye inhibited competitively with respect to benzyl penicillin, with a K_i value of 1.2 μ*M*. The difference spectrum indicated an "ionic" type interaction. The enzyme binds the dye chromophore at the active site, and benzyl penicillin is able to displace the dye from Blue Sepharose. The β-lactamase from culture supernatant of *Streptomyces cellulosae* was purified 1450-fold using Blue Sepharose chromatography as one of the steps. The enzyme has an isoelectric point of 9.5. The enzyme was loaded on the column at pH 7.5 and eluted with a NaCl gradient of 0 to 1.0 *M*. It was noted[104] that the enzyme interacted with NADP, but not with NAD or ATP. It seems very likely that the interaction with NADP involves only the adenosine and the 2′-phosphate (just like the dye segment of 1-amino anthraquinone 2-sulfonate), and does not necessarily involve the whole nucleotide.

K. Muscle Proteins

Kobayashi et al.[105] purified myosin from bovine thyroid extract. Five millimeters of ATP eluted the ATPase activity and to confirm this, it was also found that Blue Dextran inhibited ATPase activity. Myosin subfragment 1 (S-1) was obtained by tryptic digestion of myosin filaments from rabbit skeletal muscle and was observed[106] to bind to an Affi-Gel Blue column *not* involving the ATP site. The bound S-1 fragment hydrolyzed Mg ATP and Ca ATP. It was eluted with a pulse of 0.5 *M* KCl. The free dye inhibited the ATPase activity of S-1. Either 4 or 5 dye molecules per mole of subfragment 1 were observed to bind of which 3 or 4 could be removed by 1 *M* salt without relieving inhibition. The inhibition caused by one dye molecule per subfragment 1 is reversible and is facilitated by the presence of Mg ADP and Mg ATP, suggesting that the inhibition is indirect. It was also found that the whole molecule of CB (and not a part of it) is necessary for inhibition.

Troponin was purified by Reisler et al.[107] on the Blue agarose column. The elution medium contained 0.5 *M* KCl. Adsorption of troponin to Blue gel column occurs through the T subunit since subunits I and C pass through the column unadsorbed. The dye binding site at the T subunit involves the T-C interface but not the T-I interface. Troponin is also eluted

by 50% glycerol. The interactions seem to be both electrostatic and hydrophobic in nature. The difference spectra with λ_{max} at 660 nm (Table 1) confirm such a composite interaction.

Toste and Cooke[108] found that rabbit skeletal myosin itself was not bound to Blue Sepharose in view of its low solubility in low salt buffers. G- and F-actin were not bound either. The bound subfragment 1 could be eluted either by high salt or the free dye at 1 m*M*. Nucleotides do not elute S-1, but 20 m*M* TES buffer at pH 9.0 does. This adduces to the ionic nature of the interaction. Heavy meromyosin (HMM) binds to the column better than S-1. When Acto-S1 is loaded on the column in the absence of Mg ATP, 100% of actin and 70% of S1 pass through unadsorbed. When Mg ATP (1 m*M*) is present, the actin-S1 complex is dissociated and S1 is bound to the Blue gel column. Among the light chains, chain 2 is bound, but not 1 and 3. The free dye inhibits ATPase activity of myosin. Blue Sepharose chromatography was thus found to be an excellent final step for purifying S1 and myosin light chains. Actin and the heavy chain fragments were easily removed.

L. Serum Proteins

Blue gel affinity chromatography has been effectively used in the fractionation and purification of plasma and serum proteins. For example, cold ethanol precipitation of plasma to separate albumin loses about 30% albumin in the Cohn fraction IV precipitate. Hanford et al.[109] showed that by processing this precipitate on a Blue Sepharose Column an additional 3.3 kg albumin per 1000-ℓ batch of ethanol-precipitated plasma could be obtained. Travis and Pannell[11] demonstrated that plasma applied to a BDS column equilibrated with 50 mm tris buffer at pH 8 in presence of 0.5 *M* NaCl effectively removed albumin from the plasma enabling the albumin-free plasma to be used as starting material for the isolation of many other plasma proteins. This procedure was improved later[110] when Blue Sepharose CL-4B was used instead of BDS. BDS was known to bind albumin poorly[110] probably due to the nonavailability of the amino group of the chromophore. It was shown[111] that 96% of the total plasma proteins was adsorbed at pH 4 by Blue Sepharose. Elution was carried out with a pH gradient from 5.85 to 11. The major difference between Blue Sepharose and ion-exchange celluloses is that the elution from the former with a pH gradient did not correlate with the pI values of the eluted proteins.

Using a composite elution technique, Gianazza and Arnaud[112] demonstrated they could separate 27 plasma proteins by successive elution with starting buffer at pH 7, then buffer plus 0 to 1 *M* NaCl, and finally 0.5 *M* NaSCN. Figure 7 depicts this fractionation. The locations of the 27 different proteins were determined by fused rocket immunoelectrophoretic analysis. It was also found[112] that metalloproteins like transferin and ceruloplasmin did not interact strongly with the gel. The correlation between elution order and pI and also between elution order and logarithm of molecular weight was weak, thus ruling out exclusion-diffusion mechanism for the elution of unbound protein. A combination of higher pH and lower salt had the same efficiency of a higher salt concentration for the elution of a particular protein.[113] At pH 7.0 and 1 *M* NaCl, only albumin and some lipoproteins were retained by the column. Ionic interactions, mediated by pH changes, prevail in general although in the case of haptoglobin, hydrophobic binding was also noticed. As evidence, haptoglobin bound to the Blue gel column at 25° but not at 4°C. This is compatible with the concept that hydrophobic interactions diminish with a decrease in temperature.

Using Blue Sephadex, Birkenmeier and Kopperschlager[15] found that while a high degree of dye substitution retained a large number of serum proteins on the column, α-1 acid glycoprotein passed through the column unbound at pH 6.0. α-1 Proteinase inhibitor was slightly retarded compared to α-1 acid glycoprotein in the breakthrough fraction which was purified further on DEAE-cellulose. Likewise, α_2M and haptoglobin were eluted with low ionic strength buffer when the dye concentration was 2 μmol/mℓ of the gel,[110] but could be eluted only with a salt gradient when the dye concentration was 4.5 μmol/mℓ of the gel.[112]

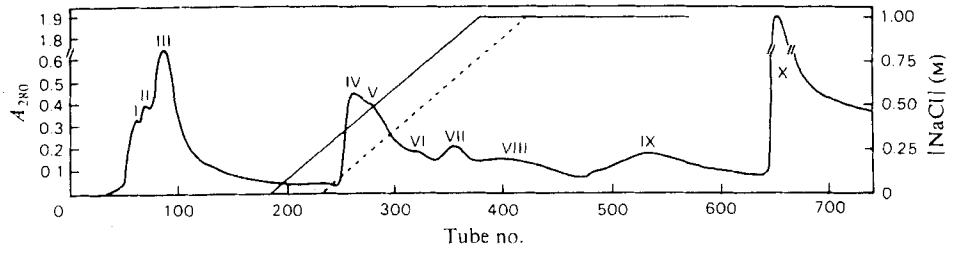


FIGURE 7. Elution profile of plasma proteins from immobilized Cibacron Blue F3GA. Plasma (80 mℓ) was run on a 400-mℓ column at room temperature. The equilibration buffer was 30 m*M* H_3PO_4/Na_3PO_4, pH 7.0. Fractions (4.8 mℓ) were collected. At tube 185, a linear NaCl gradient (0 to 1.0 *M*, total volume of 1.0 ℓ) was applied. The broken line indicates the development of the gradient, taking into account the void volume of the column. At tube 380, the elution was continued with 1.0 *M* NaCl. Starting at tube 575, the column was washed with 0.5 *M* NaSCN. (From Gianazza, E. and Arnaud, P., *Biochem. J.*, 201, 129, 1982. With permission.)

Human serum complement components were eluted from Affi-Gel Blue column over a narrow NaCl gradient[114] without contamination by albumin or I_gG. Human blood clotting factor was isolated (2000-fold purified) from human citrated plasma in the breakthrough fracion on BDS.[115] Blue Agarose was not as effective as BDS. Human α-2-macroglobulin eluted early from Blue Sepharose and was separated[116] from other components by gel filtration on Ultrogel AcA 22.

Human α-1-antichymotrypsin passed through Blue Sepharose unbound[117] at pH 8.0 although it was bound at pH 7.0. α-Fetoprotein from several fetal sera[118,119] was bound to Blue Agarose column at a low ionic strength of 0.02 *M* Na phosphate at pH 7.1 and eluted by phosphate buffered saline[118] or 1.4 *M* NaCl.

The key to successful separation of the various fractions of serum proteins appears to lie in the proper modulation of the ionic strength and pH in the loading and eluting media. Sequential use of the same column with different protocols is thus useful in the separation of a wide range of serum proteins. Although there appears no specificity in the binding of nonalbumin proteins, a remote "pseudoligand" affinity is seen.

Frontal analysis chromatography[13] and affinity gel electrophoresis[120] were used to determine the affinity constants of several mammalian albumins. Pretreatment of defatted albumins with bilirubin does not change the dissociation constants for any of the albumins, but pretreatment with palmitate increased the dissociation constant in all cases.[120] This suggests the involvement of fatty acid binding sites of albumins in binding to the Blue gel columns. The affinity constants were also a function of the matrix[13] with Sepharose matrices (cross-linked or not) giving higher affinity constants for albumin than Sephadex or cellulose matrices. This matrix dependence is independent of the degree of dye substitution, i.e., a cellulose matrix even with high degree of dye substitution gave low values for albumin affinity constants. The human albumin had much higher affinity than other mammalian albumins,[121] with Blue Sepharose as the matrix.

The vitamin B-12 transport protein, transcobalamin II, complexes with the dye in the apo- and the holoforms.[122] Electrostatic forces seem solely responsible for the binding, since 50% glycerol is unable to elute the protein from Affi-Gel Blue. The fact that the protein-dye complex was stable even at pH 9.0 (2.5 units above the isoelectric point of the protein) does not argue against[122] ionic interactions, since one or two positively charged groups at the active site are enough for a strong ionic interaction.

Leatherbarrow and Dean[76] used epoxy-activated Sepharose 6B to couple the dye via its free amino group after deactivating the reactive chlorine group. They studied the binding of several mammalian albumins and found the human serum albumin was bound to a far greater extent than others. Several lines of evidence, including nuclear magnetic resonance

studies, indicated that the bilirubin site in human albumin was involved in dye binding while it was not so with rabbit, horse, sheep, or bovine albumins. Addition of palmitate does not significantly diminish human albumin binding to the column, while it has weakened the binding of other albumins. Bilirubin diminished human albumin binding but not that of others. An apparent dichotomy exists here — human albumin binding the dye at bilirubin site and others at the fatty acid sites. At higher pH (~9.0) all binding is weakened, indicating the predominance of ionic interactions.

M. Miscellaneous Proteins and Enzymes

Most chromatin proteins from Ehrlich ascites tumor cells, both histones and nonhistones, interact[123] strongly with Blue agarose column at a moderate ionic strength of 0.14 *M* NaCl. The strength of the interaction between a given protein and the immobilized ligand is demonstrated by the concentration of NaCl required to elute that protein. Poly (ADP-ribose) polymerase (90% pure) was obtained by eluting with 0.4 *M* NaCl. Histone H1 was eluted at 1 *M* NaCl, H2A and H2B at 2 *M* NaCl, and H_3 and H_4 at 1 *M* NaCl plus 6.75 *M* urea. It is known that histones interact with DNA in a predominantly electrostatic manner and the interaction with CB is likewise electrostatic.

The photoreceptor pigment phytochrome from etiolated rye seedlings bound to Blue agarose with a high degree of hydrophobic interaction.[124] Purine nucleotides do not elute the protein but flavin nucleotides do. Blue dextran agarose was found to bind only the far-red active form and not the red-active form. 1,25-Dihydroxy vitamin D_3 receptor[125] from chick intestinal mucosa was purified 800-fold on BDS; elution was achieved with 0.2 *M* KCl. The eukaryotic initiation factors[126] eIF-4A and eIF-4D were bound to Blue Sepharose and eluted at two different salt concentrations. These factors do not bind to heparin-Sepharose, so a partial specificity for the dye chromophore is implied.

Ahmad et al.[127] purified arylsulfatases A and B from human urine, rat liver, rat brain, and sheep brain on Blue Sepharose. The A enzyme was not bound at all, while the B enzyme was eluted with tris buffer at pH 7.4. The A enzymes typically have pI values between 3 and 4 and hence at pH 6 they were not bound, as expected. A similar procedure was adopted in the separation of arylsulfatases A and B from human leukocytes.[128] The purification of glyoxalase I was achieved[129] by elution with 2 m*M* S-hexylglutathione from a BDS column. The dye-chromophore and the inhibitor compete for the same site. Affi-Gel Blue column retained the thiosulfate transferase[130] from bovine liver homogenate which was eluted by $Na_2S_2O_3$, the substrate for the enzyme. Carboxypeptidase G from *Pseudomonas* ATCC 25301 was eluted from Blue Sepharose by p-amino benzoyl glutamate (33%); the remaining enzyme was eluted by 1 *M* NaCl.[131] Alkaline phosphatase[132] was eluted by 50 m*M* phosphate and ATP citrate lyase[133] from rat liver was eluted by ATP but not NAD. Diverse substrate-specific and ionic interactions have been demonstrated in the foregoing instances.

Aspartate transcarbamylase from *E. coli*[134] binds CB at more than one site. With the catalytic subunit, the difference spectrum has a composite character. At the catalytic site, the dye is displaced by ATP or carbamoyl phosphate. With the regulatory subunit, the spectrum is distinctly ionic and the dye is displaced by ATP or CTP. With the catalytic subunit, the dye inhibits activity noncompetitively with respect to the substrate carbamoyl phosphate. The dye and nucleotides bind competitively at the regulatory subunits.

Dopamine β-monooxygenase[135] from bovine adrenal medulla was bound on Blue Sepharose and eluted by 0.2 *M* NaCl. The ortho and meta iosmers generated a typical ionic spectrum with the holoenzyme. The holoenzyme has eight binding sites per tetramer, while the copper-depleted enzyme has four sites per tetramer. The β-hydroxydecanoyl thioester dehydrase[136] from *E. coli* could not be eluted with NAD or ATP, but only by chaotropic 1 *M* KCNS from Blue Sepharose. This chaotropic elution indicates a hydrophobic interaction between the enzyme and the immobilized dye (*vide infra*, salt effects).

Catalase and carbonic anhydrase with pI values of 5.8 and 5.3, respectively, emerged with the buffer when applied to Blue Sepharose column at pH 6.9 (tris buffer 20 m*M*), while hemoglobin and lactic dehydrogenase bound to the column.[137] Hemoglobin was then eluted, free of catalase and carbonic arhydrase, with 0.1 *M* triethanolamine acetate buffer at pH 8.0. This was useful when oxyhemoglobin was used to measure oxygen consumption of biological samples without interference from catalase and glycolytic enzymes.

A plant flavokinase[138] from mung bean seed did *not* bind to Blue Sepharose in the absence of orthophosphate. When loaded in the presence of 0.5 *M* phosphate, the enzyme was bound and eluted by AMP, ADP, NAD, or even PP_i. In the absence of phosphate, the dye binds to the enzyme producing a weak ionic perturbation in the spectrum. In presence of phosphate, due to a conformational change, the dye binds at a different site in an apolar mode. the free dye was shown[139] to inhibit protein synthesis in several cell-free extracts by binding to the initiation factor and inhibiting the ternary complex formation between eIF-2, GTP, and the initiator tRNA.

From the foregoing list of several proteins, it is seen that the dye binds to the proteins in multifarious modes. The binding is nonspecific in some cases but a strict specificity, whether it was for a substrate, nucleotide, or another inhibitor, was maintained in other cases. In general, the feature that stands out is the diversity and versatility of the dye chromophore.

N. Metal Ion and Salt Effects

Bivalent metal ions, particularly Zn^{+2}, promote irreversible inactivation of yeast hexokinase by CB at a site competitive with both ATP and D-glucose.[140] The dissociation constant for the hexokinase-dye complex decreased in the presence of the metal ions suggesting the formation of tight ternary complex involving dye, metal ion, and enzyme. However, the Zn^{+2}-mediated inactivation is inhibited by ATP, presumably due to competition for the metal ion between ATP and the dye. The authors[140] suggest irreversible inactivation is due to a tight ternary complex; however, covalent modification cannot be ruled out. It was also observed that Zn^{+2} promotes the binding of hexokinase to the Blue gel. This could arise by a strengthening of the hydrophobic interaction at the expense of electrostatic interaction mediated by a charge neutralization of the dye by the metal ion.

Low concentrations of Zn^{+2}, Mg^{+2}, and Al^{+3} enhance the binding of certain enzymes like carboxypeptidase G2, alkaline phosphatase, hexokinase, and ovalbumin (which does not bind in the absence of metal ion) to immobilized Blue dye columns.[141] The metal ions may form a bridge between the dye and the protein to form a stable ternary complex or the cations may neutralize the negative charges on the dye thereby facilitating a strong hydrophobic interaction between the dye and the protein which was prevented in the absence of the metal ion.

The ability of neutral salts in eluting proteins from BDS follows the viscosity B-coefficients of the salts.[142] The relative potencies are independent of the protein or the immobilized ligand. The eluting ability of a salt is inversely proportional to its B-coefficient (KSCN $\gg$ LiCl). The salts do not act on the protein in an electrostatic fashion, but weaken the hydrophobic interaction between the protein and the dye by altering the structure of the aqueous solvent, i.e., a chaotropic salt disrupts hydrophobic interaction indirectly through the solvent.

O. Other Applications

CB and other triazine dyes have been converted to their 6-aminohexyl derivative and then coupled directly to glycolsilylated-silica or γ-glycidoxy propyl trimethoxy silane-activated silica via the triazine ring.[143,144] These silica-dye adsorbents have been used for the resolution of protein mixtures. Depending on the nature of the immobilized dye, required flexibility can be achieved. High pressure liquid chromatography has been combined with the affinity

gel technique for an efficient, high-speed, fully automated resolution of dehydrogenases and kinases, isoenzymes, and others from simple or complex mixtures. The separation of a mixture of hexokinase and phosphoglycerate kinase has been affected by using 10 m*M* ATP + 25 m*M* glucose for the former and 10 m*M* ATP + 10 m*M* 3-phosphoglycerate for the latter.[144]

Watson et al.[145] noticed that Procion Red HE-3B columns retarded NADP-dependent dehydrogenases more efficiently than NAD-dependent dehydrogenases, while Blue Sepharose did the reverse. Thus the two dyes can be used in a complementary manner, for sequential separation of the two classes of dehydrogenases.

The BDS column has been used by Thompson et al.[146] to convert holoenzymes to apoenzymes. For example, NAD binds very tightly to glyceraldehyde 3-phosphate dehydrogenase, and charcoal treatment results in considerable loss of protein and biological activity. When applied to the BDS column and eluted with 1 *M* NaCl, the eluate contained 90% of the protein and 97% of the applied enzymatic activity.

Affinity gel electrophoresis using CB-agarose-polyacrylamide gels has been useful in determining dissociation constants in analytical investigations.[147]

V. CONCLUSION

Cibacron Blue has been transformed from being a synthetic dye to a "universal pseudoaffinity ligand" through an inadvertent finding of Blue Dextran association with some proteins in gel filtration studies. Since then it has been used in the immobilized form to isolate and purify hundreds of enzymes. The dye is not specific for the "dinucleotide fold"-containing proteins, but specificity is obtained in a lot of cases where the environmental conditions are totally determinant. In some instances, the dye engages in stereoselective electrostatic interactions. The relative conformational inflexibility together with the geometrically fixed sulfonate groups of the dye are suitable for specific ionic interactions in a complementary situation. By arbitrarily choosing the loading and eluting conditions, a lot of proteins and enzymes could be isolated and purified on Blue gel columns which could not be purified or fractionated by other means. The serum proteins are a good case in point. The dye draws its diversity and versatility from the proper mix of the hydrophobic and ionic groups. By appropriate modification, the dye can be and has been fine tuned for more specific needs. It is probably appropriate to say that the dye has only begun a long and pioneering journey in the exploration of newer techniques of protein isolation and purification.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. W. E. Goldstein for his encouragement of the effort. Ms. Terri Lucterhand did an excellent job in expeditiously typing the manuscript.

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