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Affinity chromatography in nonionic detergent solutions

(Triton X-100/sodium dodecyl sulfate/blue dextran-Sepharose/cyclic nucleotide phosphodiesterase)

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ABSTRACT Anionic dye affinity chromatography is commonly unproductive in the presence of nonionic detergents used to extract particulate proteins. Using lactate dehydrogenase as a model protein, Cibacron blue F3GA as a model dye, and Triton X-100 as a model detergent, we find that the dye is encapsulated in nonionic detergent micelles, rendering the dye incapable of ligation with the enzyme. However, the dye can be liberated from the micelles without altering the nonionic detergent concentration by addition of an anionic detergent, such as deoxycholate or sodium dodecyl sulfate, forming mixed anionic/nonionic micelles that displace the anionic dye. Encapsulation of the anionic detergents prevents their activity as protein denaturants. These observations have been successfully translated to the dye affinity chromatography of a detergent extract of brain particulate cyclic nucleotide phosphodiesterase.

The purification of membrane-bound enzymes, especially those requiring nonionic detergent treatment for release from the membrane, has posed severe problems in the application of classical protein purification techniques to these enzymes. Immobilized anionic dyes have been found to be very useful for the purification of a wide variety of soluble enzymes, but the biospecific dye–protein interaction is also seriously impaired in the presence of detergents. Accordingly, we have sought to determine the source of this impairment in order to utilize traditional purification procedures in general and dye–protein affinity chromatography in particular in the presence of detergent. In developing this study we have taken advantage of the environmental spectral sensitivity and strong catalytic competitive inhibition exhibited by mobile anionic dyes. This report describes the nature of the impairment of dye–protein interactions produced by detergent, procedures to circumvent the impairment, and their successful application to enzyme purification in the presence of detergent.

MATERIALS AND METHODS

Rabbit muscle lactate dehydrogenase (EC 1.1.1.27), pig heart citrate synthase (EC 4.1.3.7), rattlesnake venom 5′-nucleotidase (EC 3.1.3.5), calf intestinal mucosa adenosine deaminase (EC 3.5.4.4), sodium pyruvate, NADH, acetyl coenzyme A, oxaloacetic acid, polyoxyethylene 10 cetyl ether (Brij 56), and blue dextran were obtained from the Sigma. Triton X-100 was purchased from Mallinckrodt, sodium deoxycholate from Fisher, NaDodSO$_4$ from BDH (Poole, England), 3′,5′-cyclic adenosine monophosphate from P-L Laboratories, and tetraiodofluorescein (I$_4$Fl) from Eastman Kodak. A purified sample of Cibacron blue F3GA (Color Index 61211, the blue dye of blue dextran) was kindly provided by H. Bossard (Ciba-Geigy, Basel, Switzerland). Blue dextran-Sepharose was prepared by the method of Ryan and Vestling (1).

The catalytic activity of lactate dehydrogenase was measured spectrophotometrically at 340 nm with solutions containing 0.2 M Tris·HCl buffer at pH 7.4, 1 mM sodium pyruvate, 100 μM NADH, and 0.025 unit of enzyme in a total volume of 1 ml. The catalytic activity of citrate synthase reaction was measured as described by Srere *et al.* (2). The presence of detergent produced no observable changes in the thiophenolate color yield.

Visible absorbance spectral measurements of dye solutions in 0.1 M Tris·HCl buffer at pH 7.4 were made at room temperature by using a Cary model 17 spectrophotometer. The concentrations of Cibacron blue F3GA and blue dextran solutions were determined using an extinction of 13.6 mM^{-1} cm^{-1} at 610 nm, and I$_4$Fl was determined by using an extinction of 83.5 mM^{-1} cm^{-1} at 526 nm. The partition coefficient, K_p, for dye between micelles and solvent was determined by:

$$K_p = \frac{[\text{dye}]_{\text{micelle}}}{[\text{dye}]_{\text{solvent}}} = \frac{\Delta\epsilon_{\text{obs}}/\Delta\epsilon_\infty}{1 - \dfrac{\Delta\epsilon_{\text{obs}}}{\Delta\epsilon_\infty} V_{\text{micelle}}}$$

in which $\Delta\epsilon_{\text{obs}}$ is $\Delta A_{\text{obs}}/[\text{dye}]_{\text{total}}$ and $\Delta\epsilon_\infty$ is obtained by plotting $(\Delta\epsilon_{\text{obs}})^{-1}$ versus $(\text{detergent})^{-1}$. The $\Delta\epsilon_\infty$ for I$_4$Fl has a value of 48 mM^{-1} cm^{-1} and for the blue dye, a value of 4.0 mM^{-1} cm^{-1} under the conditions used. The volume of micelles in solution, V_{micelle}, was calculated by:

$$V_{\text{micelle}} = (\bar{v})([\text{detergent}]_{\text{total}} - \text{CMC}).$$

Triton X-100 has a partial specific volume, $\bar{v}$, of 0.908 ml/g (3) and a critical micelle concentration, CMC, of 0.3 mM (4).

Particulate cyclic nucleotide phosphodiesterase (EC 3.1.4.1) was prepared from frozen beef brain. All procedures were done at 4°C. Brain tissue was suspended (1 kg in 2 liters) in 100 mM Tris·HCl, pH 7.4/250 mM sucrose/2 mM MgSO$_4$/1 mM EDTA/1 mM 2-mercaptoethanol/0.3 mM phenylmethylsulfonyl fluoride and dispersed by three 10-sec homogenizations in a large Waring Blendor. The mixture was centrifuged for 20 min at 13,000 × *g* and the supernatant was discarded. The pellet was suspended in 2 liters of 100 mM Tris·HCl, pH 7.5/5 mM MgSO$_4$/5 mM 2-mercaptoethanol/1 mM EDTA/2.5% (vol/vol) Triton X-100/1.25% (wt/vol) Brij 56 and stirred for 2 hr. This mixture was centrifuged at 13,000 × *g* for 1 hr; the supernatant was made 5% (wt/vol) in polyethylene glycol 6000, stirred for 1 hr, and centrifuged for 20 min. The supernatant was brought to 12% (wt/vol) polyethylene glycol 6000, stirred for 1 hr, and centrifuged for 20 min. The pellet was dissolved in 50 mM Tris·HCl, pH 7.5/5 mM MgSO$_4$/5 mM 2-mercaptoethanol/2% (wt/vol) Triton X-100. This solution is termed "the crude extract." Cyclic nucleotide phosphodiesterase activity was measured spectrophotometrically as described by Dedman and Means (5). Protein concentration was measured by the biuret procedure described by Gornall *et al.* (6) or by the method of Lees and Paxman (7), where appropriate.

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Abbreviation: I$_4$Fl, tetraiodofluorescein.

RESULTS

For our initial studies we chose lactate dehydrogenase as a model protein because of its direct enzymic assay procedure, Cibacron blue F3GA as a model ligand because it binds selectively to the catalytic site as a competitive inhibitor with high affinity (8), and Triton X-100 as a representative nonionic detergent. Solutions containing up to 1% Triton neither activated nor inhibited the catalytic activity of the enzyme (Fig. 1). However, the inhibition of catalytic activity resulting from the presence of Cibacron blue F3GA or blue dextran was relieved by addition of Triton X-100. It should be noted that relief of dye inhibition only occurred above the critical micelle concentration of Triton X-100 (0.02%), that the extent of relief exhibited by a fixed concentration of detergent was inversely proportional to the dye concentration, and that virtually no inhibition was observed at Triton concentrations normally used to extract enzymes from particulate preparations. Similar results were obtained with citrate synthase instead of lactate dehydrogenase, I_4Fl instead of Cibacron blue F3GA, and Brij 56 instead of Triton X-100. Accordingly, the results shown in Fig. 1 probably are representative of the interaction of proteins with anionic dyes in nonionic detergent solutions.

One interpretation of the results shown in Fig. 1 suggests that the free dye concentration in solution is significantly diminished by encapsulation of the dye in detergent micelles. In order to study the interaction between nonionic detergents and dyes directly, we measured a series of absorbance spectra in the visible region. For these studies we chose I_4Fl (9–11) because its greater extinction allowed us to use a concentration range free of complications from dye–dye association.

Addition of increasing concentrations of Triton X-100 only above its critical micelle concentration produced an abrupt spectral red shift, generating a series difference spectra having a single maximum at 543 nm and an isosbestic point at 532 nm. A plot of the change in extinction at the difference spectral maximum exhibited a sigmoidal dependence on the concentration of detergent, beginning at the detergent critical micelle concentration (Fig. 2). Such a sigmoidal dependency is characteristic of a saturable complexation. Analysis of the transition shown in Fig. 2 in terms of the distribution of I_4Fl between micelles and aqueous solvent gives a partition coefficient of $1.89 \pm 0.07 \times 10^3$. The absorbance maximum of solutions of Cibacron blue F3GA and blue dextran are also red-shifted significantly in the presence of Triton X-100 but only at detergent concentrations above the critical micelle concentration. As shown in Fig. 2, the difference spectral titration profile for each of these chromophores indicates that they are also encapsulated in detergent and give a partition coefficient of about 10^3. It should be noted that the detergent concentration range over which the spectral transition occurs parallelled the detergent concentration range over which relief of dye inhibition occurred (Fig. 1).

Dye affinity chromatography is likewise impaired in detergent solutions above the critical micelle concentration. Lactate dehydrogenase was quantitatively bound to blue dextran-Sepharose and specifically eluted by 1 mM NADH in chromatographic equilibration solvents containing either 0 or 0.1% Triton X-100 (Fig. 3). By contrast, the enzyme exhibited only a weak affinity for the immobilized blue dextran in equilibration solvents containing 2% Triton X-100, a detergent concentration commonly used to extract and process particulate enzymes. The more facile elution of the enzyme by 1 mM NADH in the presence of 0.1% Triton compared with the absence of detergent is in keeping with the partition of blue

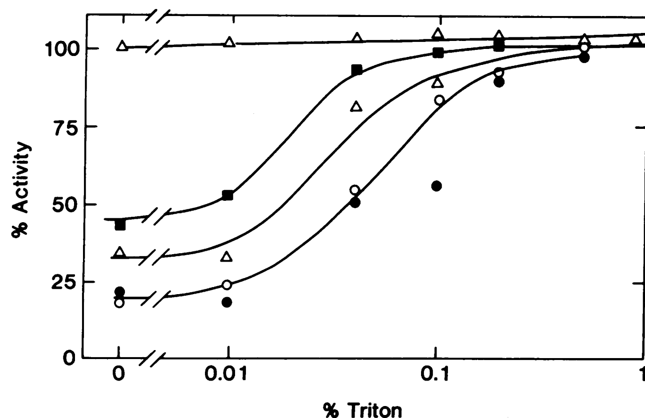

FIG. 1. Effect of Triton X-100 on dye inhibition. The catalytic activity of rabbit muscle lactate dehydrogenase was measured in the absence (△) or in the presence of 9.7 μM (■), 24 μM (△), or 48 μM (●) blue dextran or 46 μM Cibacron blue F3GA (○) and the indicated concentrations of Triton X-100. One hundred percent activity refers to the catalytic rate measured in the absence of blue dextran, Cibacron blue F3GA, and Triton X-100.

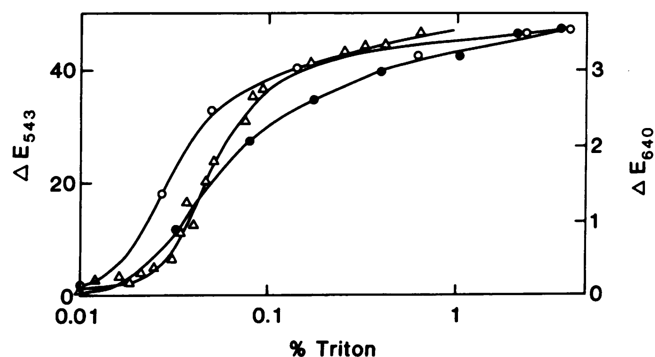

FIG. 2. Difference-spectrum titrations: change in extinction of 10 μM I_4Fl measured at 543 nm (△) or 56.5 μM Cibacron blue F3GA (○) or 60 μM blue dextran (●) measured at 640 nm resulting from the addition of the indicated concentrations of Triton X-100.

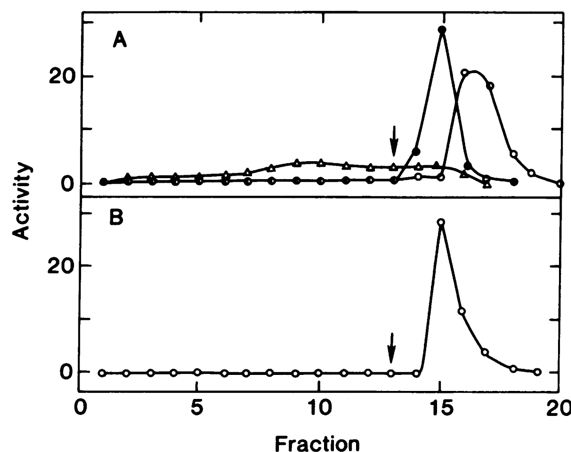

FIG. 3. Chromatography of muscle lactate dehydrogenase on blue dextran-Sepharose. All chromatography was done using a 1.2 × 1.8 cm column of blue dextran-Sepharose equilibrated with 0.2 M Tris·HCl buffer (pH 7.4) containing the indicated concentration(s) of detergent(s). One milliliter of the equilibration solvent containing 33–48 enzyme units was applied to the column (fraction zero) followed by 25 ml of the equilibration solvent and then (at the arrow) 10 ml of equilibration solvent containing 1 mM NADH. Each eluate fraction contained 2 ml. The units of activity are μmol of substrate converted per min per fraction. At least 80% of the applied enzyme units was recovered. (*A*) Equilibration solvent contained 0% (○), 0.1% (●), or 2% (△) Triton X-100. (*B*) Equilibration solvent contained 2% Triton X-100 and 1% deoxycholate.

dextran between micelles and solvent. About one-third of the blue dextran should remain exposed to the solvent in 0.1% Triton (Fig. 2). Because [blue dye] ≫ [enzyme] in the chromatographic measurements, sufficient dye should be available to bind the enzyme. However, the decreased concentration of exposed dye would accelerate the elution of the enzyme by mobile ligand as seen in Fig. 3. The results in Fig. 2 further predict that the concentration of exposed dye in 2% Triton would be very small, accounting for the weak retention of the enzyme by the column equilibrated with solvent containing 2% Triton.

In principle, dye affinity chromatography could be done in relatively high detergent concentrations by using a very large, highly substituted column or by substantial dilution of a detergent extract prior to its application to a modest-sized chromatographic column. However, the first option is impractical and the second option usually results in massive aggregation of the protein of interest, often accompanied by a loss of biological function. Accordingly, we sought a procedure to permit successful execution of dye affinity chromatography in the presence of relatively high detergent concentrations using modest-sized columns.

Our approach was to diminish the solubility of dye in detergent micelles by addition of an anionic detergent such as deoxycholate which, if incorporated into Triton micelles, should lower the solubility of the anionic dyes by charge repulsion. This expectation indeed was realized (Table 1). In contrast to the nonionic detergent Triton X-100, the anionic detergent deoxycholate was a potent inhibitor of lactate dehydrogenase catalysis: >95% of the catalytic rate was inhibited in assay solutions containing 1% deoxycholate. However, in the presence of both deoxycholate and Triton X-100, the intrinsic catalytic rate of the enzyme was largely restored, suggesting the formation of mixed Triton/deoxycholate micelles which are not injurious to catalysis.

The inhibition of catalysis exhibited individually by blue dextran and by deoxycholate appeared to be additive when both these reagents were present in the assay solution. However, assay solutions containing all three reagents—blue dextran, deoxycholate, and Triton X-100—exhibited a catalytic rate characteristic for blue dextran inhibition, consistent with the premise that the anionic dye is not incorporated into mixed anionic micelles. Fig. 4 predicts that the activity in 50μM blue dextran/0.25% Triton/1% deoxycholate would remain about 40%. Because the observed activity in 1% deoxycholate was <5% and that of 50 μM blue dextran was 34%, the Triton must have absorbed the deoxycholate and not the blue dextran. Also, deoxycholate facilitated inhibition of the enzyme by the anionic dyes Cibacron blue F3GA and I_4Fl in the presence of Triton X-100, indicating a general rather than a specific effect. As predicted from the difference spectra shown in Fig. 2, inhibition by blue dextran returned at lower concentrations of deoxycholate.

Table 1. Lactate dehydrogenase activity

Additive	Relative catalytic rate
None	100
50 μM blue dextran	34
0.25% Triton X-100	103
0.7% deoxycholate	35
0.7% deoxycholate/0.25% Triton X-100	88
50 μM blue dextran/0.25% Triton X-100	94
50 μM blue dextran/0.7% deoxycholate	5
50 μM blue dextran/0.7% deoxycholate/0.25% Triton X-100	40

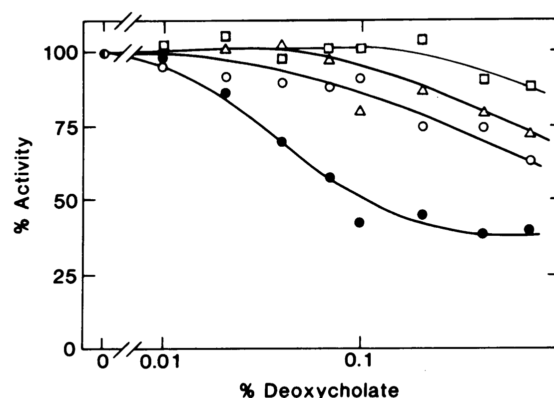

FIG. 4. Recovery of dye inhibition by addition of deoxycholate. The catalytic activity of rabbit muscle lactate dehydrogenase was measured in the presence of 0.25% Triton X-100 and either no dye (□), 50 μM I_4Fl (△), Cibacron blue F3GA (○), or blue dextran (●) and the indicated concentrations of deoxycholate. One hundred percent activity refers to the catalytic rates measured in the presence of 0.25% Triton X-100, the presence (or absence) of the dye, and the absence of deoxycholate.

Although addition of deoxycholate to Triton X-100 solutions liberated the dye for protein interaction, deoxycholate also removed divalent cations from solution, a property detrimental to those enzymes whose stability requires such cations. We then considered that an anionic detergent whose polar moiety was a sulfonic acid would be preferable in that magnesium salts of such compounds are quite soluble. $NaDodSO_4$ in the presence of Triton X-100 not only restored dye inhibition of lactate dehydrogenase catalysis but also did not itself inhibit the enzyme activity presumably due to the formation of mixed Triton X-100/$NaDodSO_4$ micelles (Fig. 5*A*). That the *ratio* of the two detergents is the important consideration in generating the desired response is illustrated in Fig. 5*B*. Both the intrinsic catalytic activity and dye inhibition are conserved at a 5:1

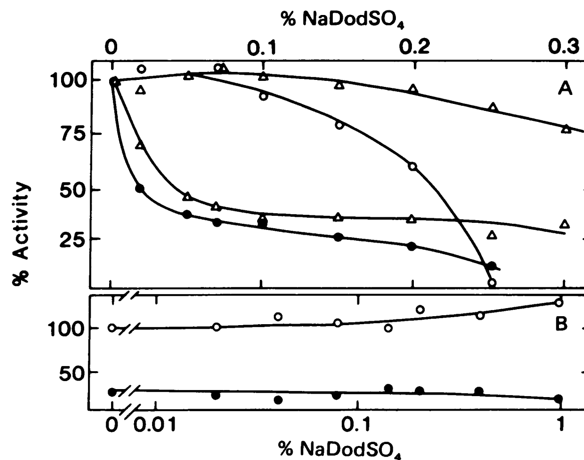

FIG. 5. Recovery of dye inhibition by addition of $NaDodSO_4$. (*A*) The activity of rabbit muscle lactate dehydrogenase was measured in the presence of 0.25% Triton X-100 (○), 0.25% Triton X-100 containing 50 μM blue dextran (●), 0.50% Triton X-100 (△), or 0.50% Triton X-100 containing 50 μM blue dextran (△) and the indicated concentrations of $NaDodSO_4$. (*B*) The catalytic activity of rabbit muscle lactate was measured in a constant ratio of Triton X-100/$NaDodSO_4$ of 5:1 over the indicated absolute concentration range of $NaDodSO_4$ in the presence (●) or absence (○) of 50 μM blue dextran. In both panels, 100% activity represents the catalytic rate in the absence of detergent and blue dextran.

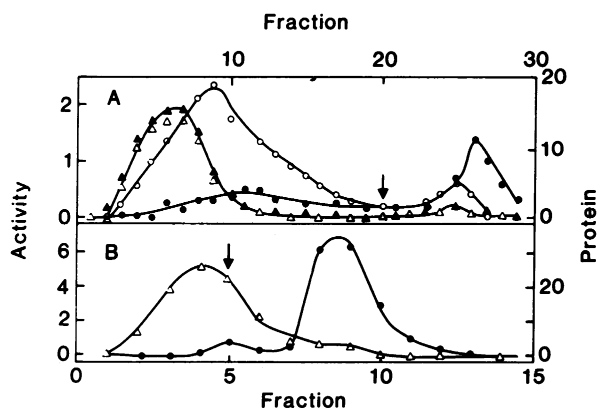

FIG. 6. Chromatography of a detergent extract of a particulate fraction from bovine brain. All chromatography was on blue dextran-Sepharose columns equilibrated with 50 mM Tris·HCl, pH 7.5/100 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$/5 mM 2-mercaptoethanol/2% Triton X-100. An aliquot (5 ml) of the extract in the equilibration solvent containing 95 mg of protein and 13 units of phosphodiesterase enzymic activity was applied to a column (zero fraction). The column was washed with equilibration solvent and then with equilibration solvent containing 0.5 M KSCN beginning at the arrow. Enzymic activity (○, ●) has the units ΔA_{265} min per fraction; protein concentration (△, ▲) has the units mg per fraction. (*A*) A 1.2 × 2.2 cm column was used and 1.5-ml fractions were collected. In separate experiments the equilibration buffer contained 0% $NaDodSO_4$ (○, △) or 0.5% $NaDodSO_4$ (●, ▲). (*B*) A 1.2 × 4.4 cm column was used and 3-ml fractions were collected. The equilibration solvent contained 0.2% $NaDodSO_4$. The increase in conductivity of the eluant resulting from the emergence of KSCN occurred at fraction 8. Seven percent, 48%, and 93% of the enzymic activity applied in equilibration solvents containing 0%, 0.5%, and 0.2% $NaDodSO_4$, respectively, emerged in the KSCN eluate.

(vol/vol) ratio of Triton X-100 to $NaDodSO_4$ up to an absolute concentration of 1% of the latter which by itself is an excellent protein denaturant.

The translation of the observations noted above into chromatographic practice is shown in Fig. 3. In contrast to the weak affinity of lactate dehydrogenase for immobilized blue dextran in the presence of 2% Triton, addition of 1% deoxycholate to 2% Triton X-100 solutions facilitated both quantitative retention of the enzyme by immobilized blue dextran and its biospecific elution by 1 mM NADH solutions.

Although lactate dehydrogenase is a convenient model protein, it may be argued that it is not representative of a particulate enzyme solubilized by complexation of nonionic detergent. Accordingly, we examined the chromatographic behavior of detergent-solubilized particulate cyclic nucleotide phosphodiesterase from beef brain. Only 7% of the enzyme activity in a crude extract containing 2% Triton X-100 was retained by an immobilized blue dextran column equilibrated with the enzyme solvent (Fig. 6*A*). However, addition of 0.5% $NaDodSO_4$ to both the crude enzyme extract and equilibration buffer (each containing 2% Triton X-100) resulted in retention and subsequent elution of 48% of the enzymic activity by thiocyanate, indicating improvement in column performance by 1 order of magnitude due solely to the presence of mixed micelles. The failure of this system to retain all of the phosphodiesterase activity was due an inadequate column volume—i.e., insufficient immobilized ligand for the amount of protein applied. A column having twice the volume under the same conditions gave retention and elution of >90% of the phosphodiesterase activity in the crude extract with a 10-fold increase in specific catalytic activity (Fig. 6*B*). The low K_m for cyclic AMP, 14 ± 6 μM, and the catalytic unresponsiveness to added excess calmodulin indicate that the isolated phosphodiesterase is the particulate and not the cytoplasmic form of the enzyme.

DISCUSSION

The advantages afforded by immobilized anionic dye affinity chromatography for enzyme purification are fourfold: (*i*) economy of time and expense in column preparation, (*ii*) retention of a wide range of enzymes by a single column, (*iii*) selective elution of a desired activity by the choice of mobile ligand, and (*iv*) the nonbiodegradability of the immobilized ligand. These columns can be used to advantage early in enzyme purification procedures to provide up to 1000-fold enhancement in specific enzymic activity with excellent yields. It was thus disappointing that immobilized dye columns were initially found to be unproductive for the purification of particulate enzymes solubilized in nonionic detergents.

Our results indicate that encapsulation of the anionic dyes in nonionic detergent micelles is the source of the problem. This conclusion is in keeping with previous studies using dyes for spectral determination of detergent critical micelle concentrations (12, 13). What was not appreciated is that a protein having a significant affinity for dye—e.g., muscle lactate dehydrogenase having a K_d of 0.1 μM—cannot compete effectively with detergent micelles for free dye due to the large partition coefficient of the dye for micelles. At equilibrium in the absence of detergent the concentration ratio of free (P) and dye complexed (PD) protein is given by the expression

$$\frac{(P)}{(PD)} = \frac{K_d}{(D)} = \frac{K_d}{D_T - (PD)}$$

in which D_T, (D), and K_d represent the total dye concentration, the free dye concentration, and the dissociation constant of the protein·dye complex. In the presence of a concentration of detergent above the critical micelle concntration this ratio becomes

$$\frac{(P)}{(PD)} = \frac{K_d(K_p + 1)}{D_T - (PD)}$$

in which K_p is the partition coefficient of the dye.

The ratio $(P)/(PD)$ is increased by $K_p + 1$ in the presence of detergent. In the case of blue dextran and Triton X-100, the ratio is increased by about 10^3. Although several operational solutions to the problem of dye encapsulation are available, we are of the opinion that formation of mixed nonionic/anionic detergent micelles to liberate the anionic dyes is particularly advantageous in that (*i*) it facilitates use of dye affinity chromatography early in a purification procedure to take advantage of its full selective potential, (*ii*) no dilution of detergent extracts is required, avoiding the need to process cumbersome volumes, and (*iii*) no reversible or irreversible changes in either biospecific function or state of polymerization of the desired enzyme need be risked by decreasing detergent concentration below its critical micelle concentration.

Although the vast majority of protein in a crude brain nonionic detergent extract is not retained by blue dextran-Sepharose in the absence of added anionic detergent (Fig. 6*A*), we have observed that at least one enzyme is retained by the column under these conditions, nucleoside diphosphate kinase (EC 2.7.4.6). This enzyme is eluted as a homogeneous preparation by using solvents containing 0.3 mM GTP and 2% nonionic detergent Brij 56 (the extraction detergent). We believe this unique situation results from the very strong affinity of the kinase for the blue dye which minimizes the effect of dye partition.

Because most biochemical ligands are ionic (frequently anionic) and have some aromaticity or apolarity, we suggest that

the observations made here with anionic dyes may pertain to affinity chromatography in general. In addition, ion exchange chromatographic materials consisting of ionic groups immobilized to inert supports via apolar spacers may also experience detergent encapsulation. A simple test of this phenomenon would entail repeating a fractionation attempt that was unproductive in nonionic detergent with the added presence of an ionic detergent of the appropriate charge.

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