Evaluation of Quantitative Affinity Chromatography by Comparison with Kinetic and Equilibrium Dialysis Methods for the Analysis of Nucleotide Binding to Staphylococcal Nuclease†

Ben M. Dunn*,‡ and Irwin M. Chaiken*

ABSTRACT: The elution of staphylococcal nuclease on thymidine 3′-(*p*-Sepharose-aminophenyl phosphate) 5′-phosphate (nucleotide ligand of nuclease covalently bound to Sepharose 4B) was studied in the presence of a variety of soluble nucleotide ligands. The elution volumes of nuclease vary proportionally with matrix-bound ligand concentration (at constant soluble ligand concentration), inversely with soluble ligand concentration (at constant matrix-bound ligand concentration), and inversely with dissociation constant of soluble ligand (at constant concentrations of soluble and matrix-bound ligand). The variation of elution volume was related to an expression which described the competition of soluble and matrix-bound ligand for nuclease binding. Using this expression, values for dissociation constants were derived for nucleotide ligands in both the soluble and bound form. The values for soluble ligand were found to correspond closely to those obtained by either equilibrium dialysis or kinetics of inhibition of nuclease activity. Furthermore, a close correspondence was found between values of dissociation constants for matrix-bound and soluble thymidine 3′-(*p*-aminophenyl phosphate) 5′-phosphate, thus defining the interaction of nuclease with the matrix-bound ligand as a process quite similar to that occurring in solution.

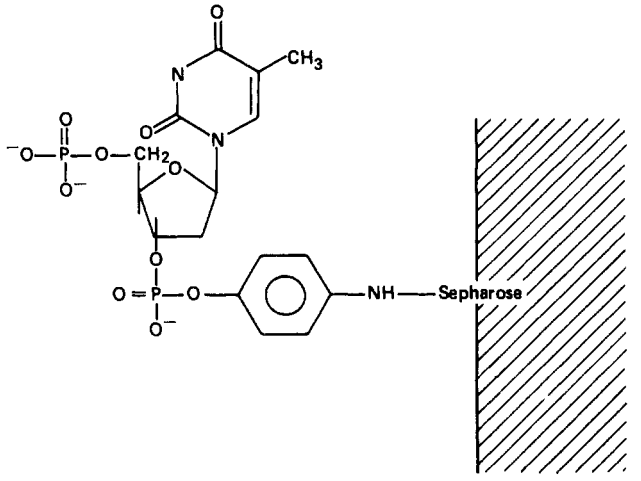
A unifying feature of most biochemical phenomena is the dependence upon association processes (Klotz, 1953, 1973, 1974). Current theories of catalysis attach great importance to the binding process and suggest that the initial steps of substrate activation derive from this specific association (Pauling, 1948; Wolfenden, 1972; Lienhard, 1973). Control processes in biology (Koshland, 1970; Hammes and Wu, 1971; Monod et al., 1963) depend on the association of a repressor to an operon, a feedback inhibitor to a biosynthetic enzyme, or a hormone to a specific receptor or other binding protein, to mention just a few examples. Methods currently employed for the direct analysis of ligand–protein interactions (Bush and Alvin, 1973) include equilibrium dialysis (Craig and King, 1955; Craig and Konigsberg, 1961; Colowick and Womack, 1969) (which depends on the retention of one component by a dialysis membrane), ultrafiltration (Blatt et al., 1968; Nazareth et al., 1974), gel filtration (Hummel and Dryer, 1962), and sedimentation (Schachman, 1963). In addition, spectroscopic techniques (Weber, 1965; Mildvan and Cohn, 1970; Sykes and Scott, 1972; Wetlaufer, 1962; Herskovits, 1967; Collins and Stark, 1969) may be employed to observe binding indirectly if one of the components undergoes a change in spectral properties upon complex formation. Steady-state kinetic analysis will yield refined information on the strength and relative position of interaction of substrates and inhibitors for enzyme-catalyzed reactions (Westley, 1969).

This paper deals in detail with a new technique for studying ligand–protein interactions based on affinity chromatography. Affinity chromatographic procedures have been widely accepted for the purification of biological macromolecules (Cuatrecasas et al., 1968b; Cuatrecasas and Anfinsen, 1971a,b; Cuatrecasas, 1972; Wilchek and Hexter, 1975; Jakoby and Wilchek, 1974). In principle, the method utilizes the specific interaction of an insolubilized ligand with a protein to effect the retention of that protein on a column bed of the matrix. In most cases, conditions are chosen to allow virtually complete binding of the protein so that the contaminants may be washed through the column. In a subsequent step, the now purified protein is eluted by disruption of the binding interaction by pH change or denaturation of the protein with chaotropic reagents. In some of the early studies in affinity chromatography, a requirement was demonstrated for a "spacer arm" to allow the protein steric access to the ligand (Steers et al., 1971). Recently, however, several groups (O'Carra et al., 1973) have suggested that this may result in nonspecific binding to the spacer arm rather than to the ligand. This finding has pointed out the need for careful scrutiny (Barry and O'Carra, 1973) of all affinity chromatography experiments. In particular, the basic assumption of affinity chromatography is that the binding to the insolubilized ligand will be virtually identical with that observed for binding to the free ligand in solution. This will be realized only in the optimal case, which Barry and O'Carra have termed "bio-affinity", wherein nonspecific binding and ion exchange interactions are not operative (Barry and O'Carra, 1973). We have pointed out (Dunn and Chaiken, 1974a) that, for cases of true specific affinity chromatography, added soluble ligands should compete effectively with the insolubilized ligand for binding to the affinity matrix and furthermore have presented results which suggest that under appropriate conditions elution volumes in affinity chromatography may be utilized to quantitate ligand–protein binding interactions. As a model, we have utilized the binding of staphylococcal nuclease to the affinity matrix thymidine 3′-(*p*-Sepharose-aminophenyl

† From the Laboratory of Chemical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received October 15, 1974.*

‡ Present address: Department of Biochemistry, The J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610.

phosphate) 5′-phosphate (Cuatrecasas et al., 1968b; Dunn and Chaiken, 1974a). In this paper, we present a detailed examination of the validity of quantitative analysis using affinity chromatography, including a comparison of predicted and observed results and an evaluation of the binding constants obtained.[1]



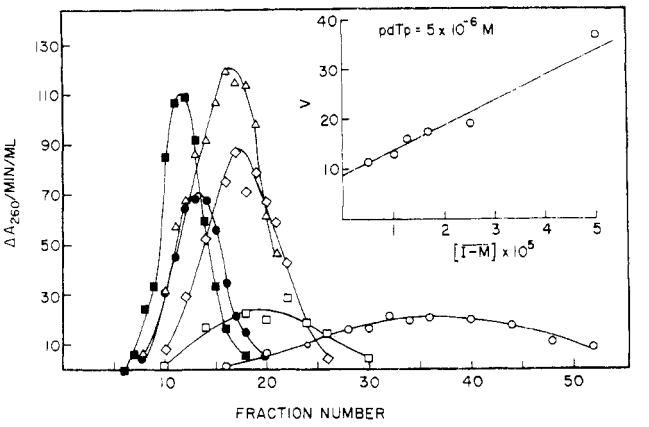


FIGURE 1: Composite plot of chromatography of nuclease on pdTpAP-Sepharose at varying bound ligand concentration. The affinity matrix was mixed with underivatized Sepharose 4B in the following proportions of milliliters of pdTpAP-Sepharose:milliliters of Sepharose 4B: 5.0 (○), 2.5:2.5 (□), 2:4 (◇), 1.5:4.5 (△), 1:4 (●), 0.5:4.5 (■). The mixtures were then used to prepare 5-ml columns of packed gel equilibrated with 5×10^{-6} *M* pdTp; 200-μg samples of nuclease were applied and eluted with 0.1 *M* ammonium acetate buffer containing 5×10^{-6} *M* pdTp. ΔA_{260} per min per ml is the activity in hydrolysis of salmon sperm DNA. Inset: plot of elution volume vs. concentration of immobilized ligand.

Experimental Section

Thymidine 3′,5′-bisphosphate (pdTp)[2] and thymidine 5′-monophosphate (5′-TMP) were obtained from Sigma. All other nucleotide derivatives were obtained from Ash Stevens, Inc. Nuclease was prepared by standard methods (Moravek et al., 1969) with subsequent functional purification by affinity chromatography with elution[3] by 0.5 *M* guanidinium chloride adjusted to pH 7.0. Active fractions were pooled and dialyzed against four changes of 0.1 *M* acetic acid followed by four changes of deionized water. Preparation of the affinity matrix (Cuatrecasas et al., 1968b), and DNase activity assay (Cuatrecasas et al., 1967) to determine the levels of nuclease in the chromatographic fractions have been described. Conditions for nuclease affinity chromatography on pdTpAP-Sepharose (0.9 × 15 cm column bed volume, 20 drop fraction size, and 40 drops/min flow rate) were as used previously (Dunn and Chaiken, 1974a). Unless otherwise noted, all experiments reported herein were carried out in a buffer consisting of 0.1 *M* ammonium acetate (pH 7.5) with 0.01 *M* $CaCl_2$.

^{14}C-labeled nuclease, a gift from Dr. Bruce Furie, was prepared as described (Furie et al., 1974) for the preparation of labeled fragments. The specific activity was 600 cpm/μg. Radioactivity was measured for aliquots (200 μl) in 5 ml of Bray's solution (Bray, 1960).

Quantitation of ligand substitution on the gel was accomplished by exhaustive acid hydrolysis using the procedure of Bartlett (Bartlett, 1959) followed by determination of phosphate by the Fiske-SubbaRow technique (Fiske and SubbaRow, 1925).

Equilibrium dialysis was carried out at ambient temperature using [^{14}C-*methyl*]pdTp essentially as described previously (Chaiken and Sánchez, 1972). The membrane used was 18 Nojax (Union Carbide) pretreated as before.

Results

Equation 1 has been derived (Dunn and Chaiken, 1974a) to correlate the data obtained from affinity chromatography experiments in the presence of added soluble ligands,

$$V = V_0 + \frac{(V_0 - V_m)\dfrac{[\overline{I\text{-}M}]}{K_{\overline{I\text{-}M}}}}{\left(1 + \dfrac{[I]}{K_I}\right)} \quad (1)$$

where V = elution volume, V_0 = volume at which protein elutes in the absence of interaction, V_m = void volume of column (determined by Dextran Blue elution), $[\overline{I\text{-}M}]$ = concentration of immobilized ligand, $K_{\overline{I\text{-}M}}$ = dissociation constant for interaction of protein with immobilized ligand, [I] = concentration of soluble ligand, K_I = dissociation constant for soluble binary complex. The suitability of eq 1 is confirmed by adherence of the data obtained to the predictions of this equation, indicated as follows.

Dependence on $[\overline{I\text{-}M}]$. Figure 1 shows the effect of diluting the affinity matrix with unsubstituted Sepharose 4B. The direct proportional dependence of V on $[\overline{I\text{-}M}]$ predicted by eq 1 is observed. Furthermore, extrapolation of the plot of V vs. $[\overline{I\text{-}M}]$ (inset to Figure 1) to $[\overline{I\text{-}M}] = 0$ yields a value for V_0 in excellent agreement with that obtained by elution of both Phenol Red in the same column and nuclease in a column of unsubstituted Sepharose packed in the same tube. From eq 1, the slope of the inset to Figure 1 is given by

$$\text{slope} = \frac{(V_0 - V_m)/K_{\overline{I\text{-}M}}}{\left(1 + \dfrac{[I]}{K_I}\right)} \quad (2)$$

Given a particular measured slope, a knowledge of either

[1] Portions of this material were reported at the 65th meeting of the American Society of Biological Chemists, Minneapolis (1974) (Dunn and Chaiken, 1974b).

[2] Abbreviations used are: pdTp, thymidine 3′,5′-bisphosphate; 5′-TMP, thymidine 5′-monophosphate; pdTpAP, thymidine 3′-(*p*-aminophenyl phosphate) 5′-phosphate; pdTpAP-Sepharose, thymidine 3′-(*p*-Sepharose-aminophenyl phosphate) 5′-phosphate; NPpdTp, thymidine 3′-phosphate 5′-(*p*-nitrophenyl phosphate); pdTpNP, thymidine 3′-(*p*-nitrophenyl phosphate) 5′-phosphate; dTpNP, thymidine 3′-(*p*-nitrophenyl phosphate); 5′-FPdT, thymidine 5′-fluorophosphate; dTpdT, thymidylthymidine; NphP, *p*-nitrophenyl phosphate; [^{14}C-*methyl*]pdTp, [^{14}C-*methyl*]thymidine 3′,5′-bisphosphate; nuclease, staphylococcal nuclease.

[3] Bohnert, J. L., and Taniuchi, H., personal communication.

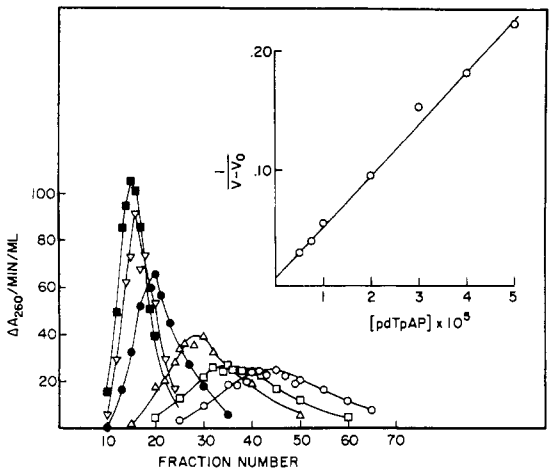


FIGURE 2: Composite plot of chromatography of nuclease on pdTpAP-Sepharose with varying concentration of soluble pdTpAP in the eluting buffer; 200-μg samples of nuclease were applied to columns equilibrated with 0.5×10^{-5} M (○), 0.75×10^{-5} M (□), 1.0×10^{-5} M (△), 2×10^{-5} M (●), 3×10^{-5} M (▽), and 4.0×10^{-5} M (■) pdTpAP each in 0.1 M ammonium acetate, and eluted with these buffers. Inset: plot of $1/(V - V_0)$ vs. [pdTpAP] according to eq 3, correlation coefficient $r = 0.997$. For the sake of clarity, the result obtained for [pdTpAP] = 5×10^{-4} M is included in the inset but the elution profile omitted from the main figure.

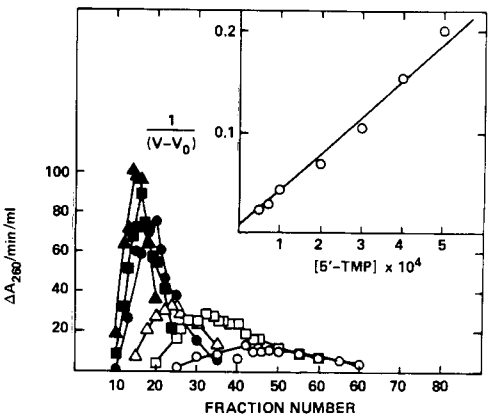


FIGURE 3: Composite plot of chromatography of nuclease on pdTpAP-Sepharose with varying concentration of 5′-TMP in the eluting buffer; 200-μg samples of nuclease were applied to column equilibrated with 0.75×10^{-4} M (○), 1.0×10^{-4} M (□), 2×10^{-4} M (△), 3×10^{-4} M (●), 4×10^{-4} M (■), and 5×10^{-4} M (▲) 5′-TMP. Inset: plot of $1/(V - V_0)$ vs. [5′-TMP], correlation coefficient $r = 0.994$.

$K_{\overline{I\text{-}M}}$ or $K_{\overline{I}}$ would allow the calculation of the other parameter. However, both parameters may not be derived from a single set of experiments. Use of a K_I determined independently by equilibrium dialysis (see below) yields a calculated value of $K_{\overline{I\text{-}M}}$ (2.6×10^{-6}) that agrees well with the value obtained in the following experiments.

Dependence on [I]. Equation 1 predicts an inverse dependence of elution volume on the concentration of soluble ligand, I. The composite plots of Figure 2 for pdTpAP and Figure 3 for 5′-TMP illustrate this dependence for these ligands. These data were obtained by running a series of columns with different levels of soluble inhibitor preequilibrated through the column. Equal amounts of nuclease were applied for each column run. The protein peak becomes broader as the retention increases when [I] is decreased.

Equation 1 may be rearranged to yield

$$\frac{1}{V - V_0} = \frac{1}{(V_0 - V_m)\dfrac{[\overline{I\text{-}M}]}{K_{\overline{I\text{-}M}}}} + \frac{[I]}{K_I(V_0 - V_m)\dfrac{[\overline{I\text{-}M}]}{K_{\overline{I\text{-}M}}}} \quad (3)$$

The insets to Figures 2 and 3 show that the data obtained give rise to linear plots of $1/(V - V_0)$ vs [I], with slope and intercept given by

$$\text{slope} = 1/K_I A \quad (4)$$

$$\text{intercept} = 1/A \quad (5)$$

where $A = (V_0 - V_m)([\overline{I\text{-}M}]/K_{\overline{I\text{-}M}})$. Then, intercept/slope $= K_I$. Thus, the data given in Figures 2 and 3 may be utilized directly to yield values of K_I. Such numbers have been collected in Table I for a variety of nucleotide inhibitors and substrates of staphylococcal nuclease. The intercept of the inset plots contains $K_{\overline{I\text{-}M}}$; thus, a knowledge of $[\overline{I\text{-}M}]$ and the column parameters V_0 and V_m allows the calculation of $K_{\overline{I\text{-}M}}$. These numbers are also tabulated in Table I. Thus, the experiments illustrated by Figures 2 and 3 may be utilized to provide both the binding constants for protein-soluble ligand interaction as well as the constant for protein-insoluble matrix interaction. The relationship of these numbers will be discussed below.

Table I: Dissociation Constants for Ligand Interaction with Staphylococcal Nuclease (pH 7.5, in the Presence of 10 mM $CaCl_2$).

Ligand	$K_I(M)$ Chromatography[a]	$K_I(M)$ Kinetics[b]	$K_{\overline{I\text{-}M}}$, ($M$)[g]
pdTpAP	2.3×10^{-6}	2.5×10^{-6}	1.1×10^{-6}
pdTp	2.5×10^{-6} [c]	5.9×10^{-6}	1.0×10^{-6}
5′-TMP	1.6×10^{-5}	2.8×10^{-5}	0.9×10^{-6}
NPpdTp	1.1×10^{-5}	6.3×10^{-6} [d]	0.6×10^{-6}
pdTpNP	4.1×10^{-6}	3.5×10^{-6}	2.6×10^{-6}
dTpNP	4.3×10^{-3}		1.5×10^{-6}
5′-FPdT	1.3×10^{-3} [e]		
dTpdT	5.6×10^{-4} [e]		
NphP	10^{-3} [f]		
Aniline	10^{-3} [f]		
None			1.2×10^{-6}

[a] Results (±30%) from competitive elution experiments. [b] Values are K_I's (±30%) determined from Dixon plots. [c] Value (±25%) derived from equilibrium dialysis is 2.5×10^{-6} M (Table II). [d] K_m value determined for enzymatic release of NphP (Dunn *et al.*, 1973). [e] Based on limited data. [f] 10^{-3} M was not effective in perturbing the elution volume. [g] Dissociation constant (±50%) for binding of nuclease to pdTpAP-Sepharose.

Elution with no Soluble Ligand or with Weak Ligands. At zero soluble inhibitor concentration, eq 1 reduces to

$$V = V_0 + (V_0 - V_m)\frac{[\overline{I\text{-}M}]}{K_{\overline{I\text{-}M}}} \quad (6)$$

Experiments were performed in which a sample of nuclease was applied and eluted with the standard starting buffer for very long times. An example of such an experiment is shown in Figure 4. It should be noted that the scales of the right-hand ordinate and abscissa of Figure 4 are not the same as in Figures 1-3. The sharp peak at fraction 14 is obtained by elution with a strong inhibitor, pdTp, and serves as a marker to indicate efficient elution. Since the peak obtained in the absence of pdTp is quite broad, the activities are correspondingly lower and hence these data are not as reliable as that obtained under conditions where the activities are higher. Nonetheless, a peak is clearly evident; using the elution volume obtained and other column parameters, one may calculate from eq 6 a value for $K_{\overline{I\text{-}M}}$ of 1.3×10^{-6}

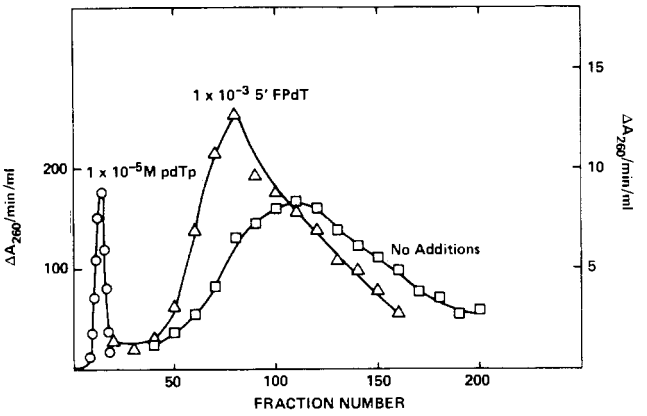


FIGURE 4: Composite plot of chromatography of nuclease on pdTpAP-Sepharose with 1×10^{-5} *M* pdTp in the eluting buffer (○) (left ordinate); with 1×10^{-3} *M* 5′-FPdT in the eluting buffer (△) (right ordinate); and with 0.1 *M* ammonium acetate buffer without added nucleotide (□) (right ordinate).

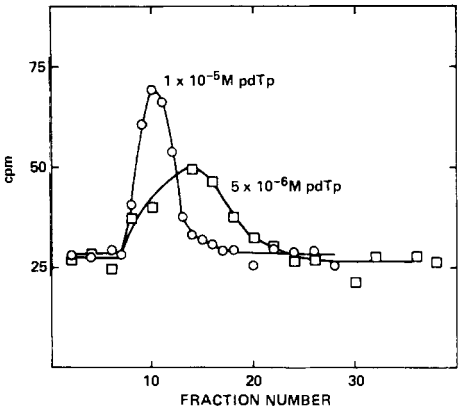


FIGURE 5: Elution of ^{14}C-labeled nuclease from pdTpAP-Sepharose by two concentrations of pdTp, 1×10^{-5} *M* (○) and 5×10^{-6} *M* (□). Cpm is counts per minute for a 200-μl aliquot of the fraction (10 drops) in 5 ml of Bray's solution.

M. This agrees well with $K_{\overline{I\text{-}M}}$ values obtained by other experiments with this matrix (see Table I).

In an attempt to determine if any element of the specificity for binding to the affinity matrix resides in the aromatic part of the insolubilized ligand, pdTpAP, elutions were performed with either 1×10^{-3} *M* *p*-nitrophenyl phosphate (NphP) or 1×10^{-3} *M* aniline in the starting buffer. In both cases the elution of nuclease was unchanged from that obtained without any added inhibitor. Experiments at higher concentrations of soluble ligand were not carried out since any effects found would have been in a concentration range where one would expect nonspecific adsorption to the protein surface and/or ionic strength effects.

For the weak ligands thymidine 5′-fluorophosphate (5′-FPdT) (Figure 4) and thymidylthymidine (dTpdT), we observed significant changes in the elution volume, suggesting binding to the active site. However, due to limiting amounts of material we were not able to obtain extensive data. Thus, the binding parameter values for these two species entered in Table I represent estimates from limited data.

Quantitation of Bound pdTpAP. In our earlier studies (Dunn and Chaiken, 1974a) we defined the concentration of covalently bound affinity ligand $[\overline{I\text{-}M}]$ based on the working capacity of the column to bind nuclease. An analysis of the bound material for phosphate content has now yielded a measure of the total amount of covalently attached material. From analysis of samples ranging from 0.1 to 0.45 ml of packed gel, an average of 0.41 μmol of phosphate/ml of packed gel was determined. Since there are two phosphate groups per inhibitor molecule, this yields a total concentration of insolubilized ligand, $[\overline{I\text{-}M}]$, of 2.1×10^{-4} *M*. This value may be compared with the value for $[\overline{I\text{-}M}]$, determined from the working capacity of the column and used in calculations of dissociations constants in this work, of 5×10^{-5} *M*. The percentage of insoluble ligand that is available for protein binding in our case is 24 ± 4%, which may be compared to values obtained earlier (Cuatrecasas et al., 1968b) of 20 and 35% for two preparations differing in initial amounts of ligand coupled. In those cases, the coupled ligand was estimated by the difference between the amount of ligand added for coupling to activated Sepharose and the amount of soluble ligand recovered after this coupling. Apparently, a considerable portion of the insolubilized ligand is unavailable for binding to the protein.

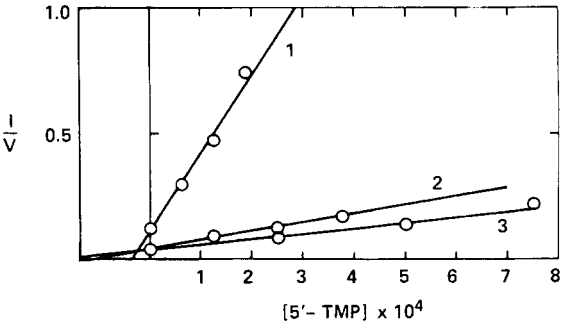


FIGURE 6: Dixon plot of the reciprocal of the velocity of hydrolysis of NPpdTp with 6×10^{-7} *M* nuclease at pH 7.5, 25°, 0.1 *M* ammonium acetate vs. concentration of 5′-TMP: (1) 1.25×10^{-5} *M*, (2) 1.25×10^{-4} *M*, and (3) 2.50×10^{-4} *M* NPpdTp. Average correlation coefficient $r = 0.994$.

Dependence on Protein Concentration. In order to apply the quantitative affinity chromatography technique to other systems where tighter binding is observed, it will be necessary to work at lower protein concentrations. The effect on the system studied here of using lower protein concentration was tested by employing a ^{14}C-labeled nuclease (see Experimental Section). This material, carbamylated at the α-NH_2, is active catalytically and binds to the affinity column. Data obtained at two different pdTp concentrations are presented in Figure 5, illustrating the same dependence of elution volume and peak width on varying concentrations of soluble ligand as that observed in Figures 2 and 3. In these experiments, 4 μg of nuclease are applied in each experiment as opposed to the 300 μg applied in all experiments reported above. In other experiments, the labeled material (4 μg) was mixed with cold nuclease (30–300 μg). Here, the peaks of radioactivity and catalytic activity were shown to be coincident. In these experiments we have detected a slight dependence of the elution volume on the amount of protein applied to the column. In general, 30 μg of nuclease eluted several fractions *earlier* than 300 μg of nuclease.

Kinetic Experiments. Binding of inhibitors was examined under the same conditions as the chromatography experiments by studying the inhibition of thymidine 5′-*p*-nitrophenyl phosphate 3′-phosphate hydrolysis by some of the ligands used in the affinity experiments. A representative Dixon plot (Dixon, 1953) is shown in Figure 6 with the value of K_I obtained from the intersection of the three straight lines. The K_I values derived in the inhibition exper-

Table II: Dissociation Constants for pdTp Binding to Nuclease Obtained by Equilibrium Dialysis.

pH	$CaCl_2$	$K_I(M)$ (±25%)
7.5[a]	10 m*M*	2.5×10^{-6}
	None	3.8×10^{-5}
8.8[b]	10 m*M*	8.8×10^{-7}
	None	4.3×10^{-5}

[a] 0.1 *M* ammonium acetate. [b] 0.1 *M* Tris-HCl.

iments are listed in Table I under the heading "Kinetics."

Equilibrium Dialysis Experiments. Binding of pdTp to nuclease was measured independently by equilibrium dialysis. Dissociation constants were calculated from data at a variety of [^{14}C-*methyl*]pdTp concentrations, either by the Scatchard plotting technique (Scatchard, 1949) or by averaging K_I values obtained at individual ligand concentrations. The value obtained in 0.1 *M* ammonium acetate (pH 7.5) in the presence of 10 m*M* $CaCl_2$, is given in Table II and may be compared with the K_I value determined by affinity chromatography for pdTp given in Table I. Table II also contains K_I values derived at pH 8.8 with $CaCl_2$, and at both pH 7.5 and 8.8 in the absence of $CaCl_2$.

Discussion

A schematic view of the interactions under investigation is given in Figure 7. This diagram illustrates the complexes involved and the competitive nature of the binding. The data presented in this paper are sufficient to confirm the overall adherence of this system to the simple scheme, given in our earlier paper, which leads to eq 1. In particular, comparison of the binding constants for the various inhibitors (Table I) obtained by the affinity chromatography method with values obtained by the more classical techniques of inhibition kinetics or equilibrium dialysis show very good agreement. This would seem to indicate that the binding to the affinity matrix is fully reversible and does not impose any limitations on the mobility of the protein by virtue of entrapment or precipitation phenomena. In comparing potential limitations of the different techniques for measuring binding, it should be noted that the kinetic technique depends on an accurately measurable activity and the dialysis technique depends on the availability of a radioactively labeled ligand or other suitable handle for quantitation of ligand. The affinity chromatography technique depends on the availability of a matrix-bound ligand and on the ability to detect protein specifically in the elution from a column.

A central question in this study concerns the comparison of the binding processes for protein with free ligand vs. protein with immobilized ligand. This may best be answered by comparison of the values of K_I and $K_{\overline{I\text{-}M}}$ derived from experiments with thymidine 5′ phosphate 3′-(*p*-aminophenyl phosphate) (pdTpAP). This is the species coupled to Sepharose to prepare the affinity column. The agreement between the experimental values for K_I and $K_{\overline{I\text{-}M}}$, 1.6×10^{-6} and 1.1×10^{-6} *M*, respectively, suggests that the same fundamental forces are involved in binding the ligand in both cases. Thus, the matrix would seem to have little effect on this process. This is consistent with the expectation that the primary interaction occurs at the 5′ end of the molecule and that the binding should not involve deep penetration of the ligand into the protein structure. Also, the apparent reversibility of binding to the matrix is in accord with a binding mode identical with that observed in solution.

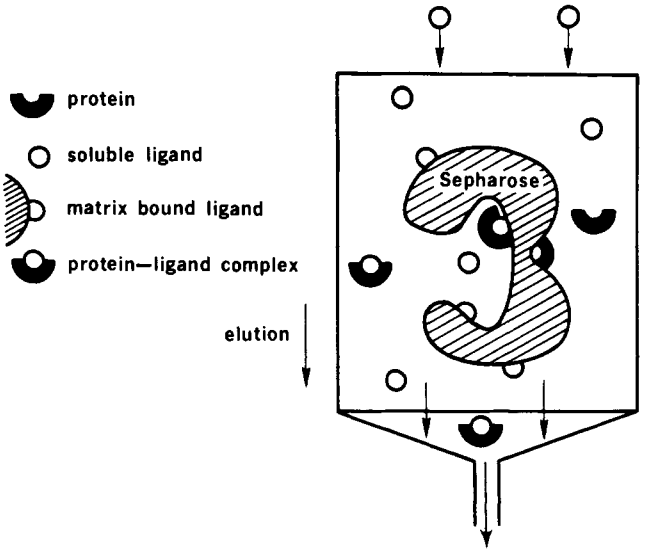


FIGURE 7: Schematic view of affinity chromatography of a protein illustrating the direct competition between matrix-bound ligand and soluble ligand.

A further check on the matrix binding process is provided by a comparison of the $K_{\overline{I\text{-}M}}$ values obtained independently from elution with six different ligands. The values range from 0.6 to 2.6×10^{-6} *M* with an average of 1.3×10^{-6} *M*. The scatter in the $K_{\overline{I\text{-}M}}$ values appears to be independent of the nature of the substituent on the thymidine. The general agreement of these numbers suggests that the nature of protein–matrix interaction is independent of the presence of the other ligand. Since nuclease has a large active site, multiple binding phenomena are possible and have been observed for small ligands by studies of kinetics of NP-pdTp hydrolysis (Chaiken, 1971; Dunn et al., 1973). However, the earlier observations of multiple binding phenomena were concerned with concentration ranges not approached in this study.

In contrast to the constancy of the $K_{\overline{I\text{-}M}}$ values, the K_I values obtained vary a great deal with substitution. The requirement for a 5′ phosphate is evident in comparing K_I for pdTpNP (4.1×10^{-6} *M*) and dTpNP (4.3×10^{-3} *M*). Furthermore, the thymidine dimer dTpdT lacking a 5′-phosphate has a K_I of 5.6×10^{-4} *M*, whereas the phosphorylated analog pdTpdT has, at pH 8.8 in borate buffer, a K_I of 6.3×10^{-7} *M* (Cuatrecasas et al., 1968a). The 5′-fluorophosphate analog shows very weak binding (cf. to 5′-TMP). Cuatrecasas et al. (Cuatrecasas et al., 1969) reported hydrolysis of this compound at pH 8.8 but gave no value for K_m, apparently due to the weak binding. This binding through the 5′-phosphate is in complete agreement with the X-ray crystallographic evidence presented by Cotton, Hazen, and coworkers for pdTp binding to nuclease (Cotton et al., 1973).

Because of the reports of binding to spacer arms, we sought evidence for the influence of the aminophenyl group of the affinity ligand, which serves as an arm in this case. First, it should be noted that the presence of the arm provides no significant enhancement of the binding (cf. K_I for pdTp with K_I for pdTpAP). Second, we attempted the specific elution of nuclease with aniline and NphP. These two species were ineffective in elution of the protein from the column at a concentration of 10^{-3} *M*. Thus the binding constant for presumed interaction of these species must be greater than 10^{-2} *M*. At this concentration, many organic

molecules bind indiscriminately to the surface of proteins and there is some evidence for association of NphP with nuclease (Dunn et al., 1973). Thus the specific elution of nuclease by other ligands in Table I derives from their nucleotide structure and not from their aromatic ester group. In general, the earlier results (Cuatrecasas et al., 1969) on ligand binding are supported by the findings of this study.

The elution of the enzyme from the affinity column in the absence of added competitive ligands deserves further comment. This finding illustrates that, for the column employed for these measurements, the useful "fractionation range" is from fractions 10 to 110 for fractions of 10 drops each. The K_I will be the concentration that is sufficient to elute the protein at tube 60. However, it should be emphasized that more accurate data are obtained at concentrations of soluble ligand above K_I, simply due to the sharper peaks obtained. Although there have been many published reports of affinity columns which effect only a retardation of the protein (Barry and O'Carra, 1973) it has been generally assumed that a truly effective affinity matrix would bring about absolute retention of the protein on the column. Indeed, it is frequently observed that a given affinity matrix is so effective that the elution of the protein is impossible. For the pdTp-aminophenyl-Sepharose employed in this study it has been reported (Cuatrecasas et al., 1968b) that exhaustive washing (50 bed volumes) of the column did not elute any protein. The results of Figure 4 suggest that, in fact, nuclease can be eluted from pdTpAP-Sepharose by buffer alone. It should be noted that the conditions used in the earlier study (0.05 M borate buffer (pH 8.0) containing 0.01 M $CaCl_2$) are slightly different from those used here (0.1 M ammonium acetate (pH 7.5) containing 0.01 M $CaCl_2$) and give rise to smaller ligand dissociation constants. A fivefold decrease in $K_{\overline{I\text{-}M}}$ would increase the elution volume, V, from eq 6, by 20 column volumes or, in our case, 40 bed volumes (400 fractions). The nuclease elution we have observed upon washing the pdTpAp-Sepharose with starting buffer suggests the possibility of premature elution of protein during initial buffer washes in protein purification experiments using affinity chromatography, with the resultant probability of diminished yields. It is obvious that conditions must be chosen that are appropriate for the experiments to be performed. Thus conditions that are optimal for purification may not be ideal for quantitation of ligand binding by the methods described in this paper.

Nichol et al. (1974) have made the important point that the concentration of the protein species (in their terminology [A]) will be undetermined in the zonal analysis type of experiment which we have described here. They suggest a procedure of frontal analysis, in which sufficient A is added to yield a plateau of concentration in the elution profile. The additional term they introduce to account for the material balance of species X or in our terminology, $\overline{I\text{-}M}$, is $1 + ([E]/K_{\overline{I\text{-}M}})$. This must be close to one in our system since we see little or no dependence of elution volume on the concentration of the protein species. Thus, as [E] is smaller, then $[E]/K_{\overline{I\text{-}M}}$ becomes vanishingly small. This implies that the experiments may be carried out at a lower protein concentration to yield the simplification we have derived. This is analogous to the study of enzyme kinetics at low enzyme concentration to avoid the additional terms that arise when the concentration of the Michaelis complex E·S is significant. The derivation of Nichol et al. is a general expression applicable to all systems. However, experimentally, we feel it can be more useful to study this phenomena in the low range of protein concentration.

The equilibrium involved in affinity chromatography is composed of the "on" and "off" rate constants for binding:

$$E + \overline{I\text{-}M} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \overline{E{\cdot}I - M}$$

$$k_1[E][\overline{I\text{-}M}] = k_{-1}[\overline{E{\cdot}I - M}]$$

$$\frac{[E][\overline{I\text{-}M}]}{[\overline{E{\cdot}I\text{-}M}]} = \frac{k_{-1}}{k_1} = K_{\overline{I\text{-}M}}$$

The forward rate constant is most likely near the diffusion-controlled limit for this system. For binding of 3′-CMP to RNase A, Hammes and Walz have measured a forward rate constant of $4.2 \times 10^7\ M^{-1}\ sec^{-1}$ (Hammes and Walz, 1969; Hammes, 1968). If one assumes that this value is a reasonable estimate for k_1, in the case of binding to staphylococcal nuclease one can calculate therefore, for a $K_{\overline{I\text{-}M}}$ of $1.3 \times 10^{-6}\ M$, a k_{-1} of $53\ sec^{-1}$. This is fast enough so that the system is in rapid equilibrium and thus that the protein eventually appears in the effluent. For cases where the binding constant is significantly smaller, i.e., $10^{-9}\ M$, the calculated rate constant for dissociation of the $\overline{E{\cdot}I\text{-}M}$ complex would be correspndingly smaller, i.e., $0.042\ sec^{-1}$. At this point the kinetic effect would serve to effectively prevent the elution of protein *in an experimentally feasible time.* Addition of soluble ligands will have no effect at this point, since the dissociation step is unimolecular. Thus reports of failure to observe elution of certain proteins from their affinity matrices by irrigation with buffer containing strong inhibitors are probably due to this overwhelming kinetic effect. It is also possible that in some cases the protein peak is so broad as to be undetectable. This analysis suggests that for successful application of this affinity chromatography technique, columns should be prepared utilizing a ligand of intermediate binding strength so that the system will be freely reversible in the time scale of a chromatographic experiment.

The equilibrium dialysis method used here allowed dissociation constants for pdTp to nuclease binding to be obtained under variant conditions of pH and calcium concentration. As shown in Table II, there is only a slight increase in binding affinity at pH 8.8, but a marked dependence on the presence of Ca^{2+} for high affinity. Both of these phenomena are predicted from previous studies with nuclease substrates and inhibitors (Cuatrecasas et al., 1968c). These results reinforce the comparability of data obtained by the dialysis approach with that obtained by affinity chromatographic analysis. Inasmuch as the dialysis method has the advantage of yielding the stoichiometry of binding, whereas the chromatographic method allows wide latitude in the ligand species studied, it is expected that the two techniques could be applied in concert to answer particular problems, for nuclease and other favorable proteins as well.

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