Hydrophobic affinity chromatography of nucleic acids and proteins

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ABSTRACT

5' tritylated oligonucleotides binding hydrophobically to low trityl cellulose/sepharose (<15µM Tr/ml) retain their hydrogen-bonding specificities for complementary sequences. This, constitutes a novel mode of attaching affinity ligands to solid supports, is more convenient than existing methods, and proceeds with 100% yield. The salt, dielectric constant and temperature dependence of these non-covalently anchored ligands permits the isolation of a variety of RNAs including fibroin mRNA. Medium trityl sepharose (15-40µM Tr/ml) has a high binding specificity for poly A and poly A containing mRNA, equivalent to dT cellulose. Most proteins, including nucleic acid enzymes, bind to these columns and retain enzymatic activity, thus mimicking enzymes attached covalently to solid phases. A number of in vivo counterparts to this hydrophobically determined specificity are noted, as are homologies to nitro-cellulose filters.

INTRODUCTION

Current methods used to attach medium to long chain synthetic DNA ligands (1) covalently to columns (2,3) result in fairly low yields and some side products. These methods were worked out, generally, with shorter and more easily synthesized oligomers in mind. The method of Wagner et al (3) gave low yields and introduced positive charges on the affinity resin (4). Besides, the use of a 5' or 3' phosphate as the ligand linkage point is not too convenient, in that the usual method of synthesizing longer oligomers involves a 5'trityl group, which, after deblocking, leaves a 5'hydroxyl (5). Addition of a ribonucleotide to the 3' end of the synthetic DNA, periodate oxidation and CNBr mediated attachment (6) was also explored, but yields were low.

Since TC columns are useful for the isolation of tritylated intermediates during the chemical synthesis of DNA (7) and, since
most nucleic acid chemical syntheses involve trityl-like-derivatives, usually at the 5' end (5), we carried out a variety of prototype experiments, involving simple tritylated oligomers and complementary sequences (diagram 1, top). By anchoring the 5'-tritylated (sticky) end of a synthetic oligomer to TC hydrophobically, one might purify complementary sequences without resorting to covalent attachments. Unfortunately, poly A bound unexpectedly to the highly tritylated columns then available (diagram 1, bottom). However, it later appeared that the original hydrophobically-supported hybridization concept was tenable using weakly tritylated resins (diagram 1, top).

This report details: 1) the specificity and mechanism of poly-nucleotide binding to TRs; 2) the use of 5'tritylated oligomers as affinity ligands, operating by means of H-bonds; 3) the binding of proteins to TRs. Upon reviewing the literature with regard to poly A (8,9), DNA (10) or viral RNA (11) binding to cellulose or to aromatic cellulose derivatives many precedents were found. The specificity of these columns, was rather weak in that both poly U and poly A were bound. The role of stacking forces in this phenomenon was clearly suggested (8).

MATERIALS AND METHODS

TrCl, MTrCl and DMTrCl were from Aldrich Chemical Co.; cellulose powder (CF 11) was from Whatman; sepharose CL-4B was from Pharmacia, as were phenyl and n-octyl sepharoses. Neither of the latter derivatives (~40μM/ml) bound poly A (21). Most homo- and hetero-polynucleotide were from Sigma Chemical Co., otherwise, they were synthesized by us with PNP'ase as described below. All synthetic deoxypolymers were made according to established triester methods (5,12); 3H-FmRNA (5,000 CPM/μg) and 3H-rRNA (2980 CPM/μg) isolated from B. mori (13) were supplied by Dr. P. Lizardi. dT cellulose (T₃) was from Collaborative Research, Waltham, Mass.; M. luteus lyophilized cells were from Sigma; E. coli 5 and 16s RNA were from Miles. All detergents were from Tridom Chemical Co., Hauppauge, N.Y. All proteins including enzymes were from Sigma, except vpd'ase, BAP and pDNase which were from Worthington and PNP'ase (primer dependant) from Miles.
Synthesis of trityl sepharose. Conditions for TC synthesis have been described (14). The procedure detailed below for sepharose/agarose is milder and more convenient; it may also be used for cellulose: an aqueous suspension of sepharose CL-4B (180 ml pack vol.) was made anhydrous by filtering with ethanol/pyridine and azeotropically evaporating excess (~700 ml) pyridine to a final volume of 210 ml. To 29 ml portions of this suspension were added TrCl (0.25 to 1.6g). At designated times, aliquots were taken, washed (ethanol) to remove excess TrCl and the μMTr/ml bed vol. determined by suspending a known volume of resin in H$_2$O and mix-

![Diagram 1. Schematic summary of hydrophobic modes of binding. Top shows how a trityl group covalently attached to a low (<15μMTr/ml) trityl cellulose/sepharose resin can associate with the corresponding 5'-trityl group of an oligomer which, in turn, is still free to H bond with complementary nucleic acid sequences. The 5'-tritylated ligand may be preannealed to its cognate sequences in free solution prior to TR application or may anneal to them during the course of the chromatography. The binding, both hydrogen and hydrophobic, occurs optimally at high salt conditions; at low salt the H bonds alone will be disrupted, allowing the complementary polynucleotide to be eluted. The bottom portion shows the putative stacking type bonds between trityl groups in medium/high (>15μMTr/ml) trityl cellulose/sepharose and poly A. Unlike the stronger trityl to trityl hydrophobic bonds described above, these type bonds while stable enough at high salt are broken at low salt concentrations and the poly A elutes from the column.](image-url)
ing it with an equal volume of 70% HClO₄, using a molar extinction coefficient of 35,200 (14) for the trityl group. In general, 1.2g TrCl added to 20 ml of the suspension gave low TS resin (<15µM Tr/ml) after ~45', medium TS resin (15-40µM Tr/ml) after 2-3 hrs and high TS (>40µM Tr/ml) in 12-20 hrs of reaction at room temperature. TRs are very stable, some being used for 7 years without any apparent capacity loss; dT cellulose and poly U sepharose (to a lesser degree) are also stable, but with extended use nuclease damage and attendant capacity loss might be incurred. The benzoyl ester linkage in benzoyl cellulose or derivatives is reported to be somewhat unstable (8,9). A 3 ml bed vol of TR costs <$10; current catalogue prices for a comparable dT cellulose column are <$125.00.

**Column Procedures.** Resins were packed into Isolab "quik-sep" columns, fitted with a 25 ml extension top. Column dimensions for a typical 3 ml bed vol: 0.9 (i.d.) x 4.5 cm; fractions of ~3 ml were taken, with a flow rate of ~1 ml/min at room temp. After the HS-LS elution sequence, columns were usually (except those described in figs. 2,3 and Table 2) washed with 0.1N NaOH and/or ~40% ethanol to remove very strongly bound ("irreversible") material. With dT cellulose and low-medium TC/TS ~5% "irreversible" binding was seen; with high TRs up to 25%. Probably owing to the polyaromatic lignin (15) content of cellulose (8) and the closer packing of its fibers, more "irreversible" binding was seen with TC than with TS columns.

PNP'ase was isolated from M. luteus as a 45-57% (NH₄)₂SO₄ cut (F₁₁₁;16) and incubations done as described elsewhere (16). A 3' poly A tail was added on to 5'- ³²P-poly C (17) using methods similar to those published recently (18). The 5'-poly C-3'-poly A copolymer was purified over dT cellulose and TC columns before use.

Poly A containing mRNA was isolated using established methods (19) from a variety of sources: bovine and murine liver, Hela and Vero cells and WI38 human fibroblasts (normal and SV40-infected) grown in tissue culture. The latter cells were prelabeled with ³²P₀₄ for 20 or 48 hrs before harvest in PO₄-depleted medium; cytoplasm was supplied by Dr. M. Krause (20). Sucrose gradients of 10-30% in 0.1M NaCl, 0.5 mM Tris pH 7.4, 1 mM EDTA and 0.1% DEPC were done in an IUC SB-283 rotor at 26,000 RPM for 15-18 hours using 5 & 16s rRNA markers.

Up (2',3' isomers) as the free acid was methoxytritylated
using conventional methods (5). Solvent extraction separated the 2' and 3' products. Their individual identification was helped by doing an alternate synthesis using only the 3' isomer as starting material, followed by solvent extraction (21.). The 5' tritylation of poly U or poly C was achieved after removing the 5' phosphate groups with BAP, using hydrophobic cations (e.g. dododecyltrimethylammonium; (22)) for solubility.

RESULTS

There is a striking resemblance between the polyribonucleotide binding specificity of TS and that of dT cellulose or poly U sepharose as far as the natural bases (A,C,G,U) are concerned (lines 1,2, table 1). Medium TS and dT cellulose have about the same capacity (footnote 2). Footnote 3 indicates that tetramers of A will bind to high TS; the minimum size binding to medium TS or to dT cellulose is ~ a 12-mer (21). Footnote 4 indicates that A enriched heteropolymers also bind; identical experiments with dT

<table>
<thead>
<tr>
<th>polynucleotide</th>
<th>% bound at HS²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. poly A³⁴</td>
<td>100⁵</td>
</tr>
<tr>
<td>2. poly C, poly G, poly U⁶</td>
<td>0%</td>
</tr>
<tr>
<td>3. 5'-³²P poly C-3'poly A copolymer</td>
<td>100%</td>
</tr>
<tr>
<td>4. native DNA</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>5. heat denatured DNA</td>
<td>100%</td>
</tr>
</tbody>
</table>

1. Same patterns seen with TC as well, except greater tendency for poly U to bind at HS. Experiments run as described in Methods, using 5-10 A260u of polynucleotide applied to a TS column (1½-2 ml bed vol, using medium TS).
2. Bound compounds were released quantitatively by changing to LS, unless noted otherwise. Relative binding capacities of low, medium and high TS columns (2 ml vol) for poly A are: zero, ~15 and ~200 A260u respectively. Binding capacity drops by ~50% at 80°.
4. Poly (AG) and poly (AC) each containing 60-70% A bind ~50%; analogous heteropolymers with <50% A content do not bind. Similar results seen with dT cellulose.
5. Addition of 0.1% SDS to HS solution abolishes all binding. By contrast, this detergent has no effect on poly A's binding to dT cellulose.
6. Each applied separately. Poly X and poly I also applied; former doesn't bind, the latter binds to the extent of ~80%.
7. Required 3.5 M urea to elute.
cellulose indicate a similar imprecision of binding. Hence, among the 4 major bases and compounds compared, it may be said that TS and dT cellulose have equivalent specificities and also the same (low) degree of imprecision. Line 3 indicates that the 5', 32p-poly C-3'-poly A copolymer, roughly designed to simulate poly A-ended-mRNA, does indeed bind. Identical binding is seen on dT cellulose.

Figure 1A, top and bottom, elution profiles indicate that ~5% of hot phenol extracted (19) and ethanol precipitated rabbit liver RNA binds to both dT cellulose and TS columns. Reciprocal crossover experiments in part B and again in part C (top and bottom) suggest equivalent binding specificities between the 2 columns. Similar patterns are seen (using 3H uridine as label) in RNA extracted from Hela cells, polio virus-infected Hela cells, WI38 human fibroblasts, both normal and SV-40 infected and murine liver (21). Sucrose gradient analysis of the non-binding HS RNA fractions generally show 3 peaks corresponding to 4-5S,18S and 28S rRNA.

Native and denatured forms of DNA behave very differently (table 1, lines 4,5); this resembles their respective behaviors on (the apparently structurally unrelated) NO2 cellulose. In general, TC exhibits very similar specificities to TS, except that there is a more pronounced tendency for poly U to bind to it, probably due to traces of polyaromatic lignins in cellulose. It is noteworthy that within the diverse category of lignins, some groupings have striking resemblances to trityl (15).

A detailed comparison of poly A binding to dT cellulose and to TRs over a wide range of conditions including: different types of detergents (footnote 5, table 1), a variety of neutral Hofmeister-type salts, different pH and temperature conditions, shows pronounced differences between the two (21). This, in turn, suggests corresponding differences in the mechanisms whereby poly A is bound by the respective columns. Hence, the similarity between dT cellulose and TRs in binding poly A at HS and releasing it at LS is probably only coincidental when viewed in the context of more detailed comparative experiments (21). The implication of these experiments is that, in contrast to the well known H bonded associations of dT cellulose, poly A binds to TRs by means of distinctive hydrophobic, base-stacking forces.
Figure 1. Isolation of poly A containing mRNA on trityl sepharose and dT-cellulose columns. RNA was phenol extracted (19) from rabbit liver. 40 A_260 units was applied each to medium trityl sepharose (top, A) and dT-cellulose (bottom, A) columns of ~ 3 ml bed volume. Both columns were equilibrated with HS. The RNA was applied in 10 ml HS; elution with HS continued till fraction 5; fraction size 5 ml each. Further fractions were collected with deionized H_2O of 2 ml each; these fractions contained ~ 5% of applied A_260 units. Water fractions of each were pooled made to HS and reapplied in a mutual crossover experiment as shown in part B, top and bottom. Same elution pattern was followed as above. Nearly 100% of the applied A_260 units were eluted in the water fractions. Water fractions from B were pooled made to HS and reapplied in crossover fashion as shown in part C, top and bottom. Same elution followed as above. Nearly 100% of the applied A_260 units (poly A^+ mRNA) emerged in the water fractions.

Figure 2 indicates a rather different type of experiment is possible, using a 5' tritylated oligonucleotide and TC/TS columns of low trityl content (<15μM Tr/ml). Part A indicates that poly A has no binding tendency at HS to these low TRs; also, that the DMTr-(Tp)_16 ligand, anchored non-covalently by means of hydrophobic bonds, remains bound to the column throughout the HS-LS wash-
Figure 2. Poly A isolation on low trityl sepharose columns using hydrophobically anchored DMTr(Tp)$_{16}$ as ligand. (A) 5.6 A$_{260}$ u of poly A in 2 ml HS solution was added to a low (15μM Tr/ml; 2 ml bed vol.; equilibrated with HS) TS column, eluted with HS, then with LS. Subsequently 8 A$_{260}$ u of 5'-DMTr(Tp)$_{16}$ in HS was applied to the same column reequilibrated with HS. Elution was continued with HS, LS and finally 50% ethanol. Fractions were checked both for A$_{260}$ and A$_{500}$ (the latter being the Amax of the DMTr group in 35% HClO$_4$, with a molar extinction coefficient of ca 45,000). (B) After flash evaporating the ethanol from above solution, it was made to HS and reapplied to the same column. Poly A (5.7 A$_{260}$ u in HS) was next applied (as fraction 1) and washed in with HS followed by LS solutions as above. Fractions of ca 3.3 ml were collected; recovery of poly A was quantitative. Subsequent washing of the column with 50% ethanol gave full recovery of the tritylated ligand. Alternatively, the column may be reequilibrated with HS and used for other poly A isolations without removing the ligand.

Hence, by preattaching DMTr(Tp)$_{16}$ to the TR, via trityl to trityl stacking bonds only (diagram 1, top), one can simulate a covalently attached oligo dT group on dT-cellulose and thus isolate poly A by means of conventional H bonds, by going through the usual HS-LS sequence (figure 2, part B). In table 2 this approach has been broadened to include a variety of A-T, A-U and C-G complementary homopolymers, as well as FmRNA and a
Table 2. Isolation of various polynucleotides on trityl sepharose columns using complementary 5'-tritylated ligands.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DMTr(Tp)(_{16})(10)+poly A(20)</td>
<td>12 A(_{260}) u poly A bound(^2)</td>
</tr>
<tr>
<td>2. MTrpoly U (8)+poly A (6)</td>
<td>5 A(_{260}) u poly A bound(^2)</td>
</tr>
<tr>
<td>3. DMTr(Tp)(_{16})(6)+poly A(4)+poly C(16)</td>
<td>only poly A bound</td>
</tr>
<tr>
<td>4. DMTr(Tp)(_{16})(6)+poly A(4)+poly G(16)</td>
<td>only poly A bound</td>
</tr>
<tr>
<td>5. DMTr(Cp)(_{6})(4)+poly G(5)</td>
<td>40% poly G bound(^2,3)</td>
</tr>
<tr>
<td>6. DMTr(Cp)(_{15})(10)+poly G(9)</td>
<td>50% poly G bound(^2,3,4)</td>
</tr>
<tr>
<td>7. DMTr(ACCAGC)(_2)(8)+F(_mRNA)(5000CPM)</td>
<td>80% binding(^5)</td>
</tr>
</tbody>
</table>

1. Columns run as described in methods; low TS columns (not bind-poly A) used in most experiments. Unless stated otherwise, tritylated ligands were prebound to the column and the complementary polynucleotides then added in HS. Numbers in parentheses refer to A\(_{260}\)u added.

2. Equivalent results obtained whether tritylated ligand prebound to the column or preheated with polynucleotide at 65\(^\circ\) in LS for 5', quick cooled to room temperature and applied in HS.

3. Medium TS used.

4. Upon reapplication of same poly G in HS, another 3 A\(_{260}\)u stuck; flow rate much slower 2nd passage.

5. Experiments involved preheating FmRNA at 50\(^\circ\) in LS for 4 minutes, adding tritylated ligand, quick cooling to 5\(^\circ\), making up to HS and applying to the column which was run at 5\(^\circ\). Controls were run with B. mori rRNA which has a 50% G/C content relative to 57\(^\circ\) for FmRNA (13). Less than 5% FmRNA binds if it is not predenatured.

*Table continued...*
variety of conditions, chosen so as to maximize both binding specificity and capacity. Subsequently the resultant H-bonded duplex, tritylated at one of the two 5' ends, may be recovered with full efficiency (table 2, footnotes 2 & 5) upon passage through a TR.

This trityl-group-supported method of nucleic acid chromatography, also appears to hold in the case of proteins, in that tritylated ligands as short as a mononucleotide bind efficiently to their cognate enzyme (figure 3). TR-bound MTrUp has some resemblance to agarose-NH-Ø-pUp developed by Wilchek (30) and marketed for removal of RNases. Since the binding capacity of the MTrUp column is close to that of an equivalently derivatized Wilchek-column, using the -NH-Ø-P- grouping as spacer arm, it is likely that the putative stacking interactions between the bulky trityl groups of the resin and ligands, constitute an effective spacer or extension arm. As with the commercial resin, inclusion of Up (2',3') in the binding buffer prevents RNase binding, or, elutes off prebound RNase, presumably by a simple displacement process at the active site. Changing the pH from 5.2 to 9.2, however, gave a more efficient elution of RNase. It is known (31) that this causes a large drop in the association constant between substrate and RNase. As above with nucleic acids (table 2, footnotes 2 + 5), MTrUp could be preassociated with pRNase in free solution. The resulting complex will bind to the tritylated column as efficiently as in figure 3B. Experiments on the binding of lysozyme to 6'-trityl N-acetyl glucosamine, the latter being prebound to TS, qualitatively resemble those described in figure 3 (21). However the intrinsic binding constant between enzyme and monomeric sugar is too low (59) to give a useful retention of enzyme.

Depending upon the concentration and type (in a Hofmeister sense (33)) of salt added, proteins can be induced to bind to TRs in the absence of any tritylated ligands. Medium salt concentrations (e.g. ~ 50 mM) cause most enzymes to bind tightly to the TRs (table 3). Table 3 indicates a fairly good correlation exists between the degree of binding of a particular protein to TS columns and its aromatic amino acid content. The only apparent exception is pRNase which fails to bind to medium or even high TRs, despite its 7.5% aromatic amino acid content. Strong 3-dimensional homologies have been noted between pRNase and lysozyme.
Figure 3. Binding (and release) of pancreatic RNase to trityl cellulose columns using hydrophobically anchored MTrUp (3') as the affinity ligand. (B) 60 A_{260}^\text{u} of MTrUp (3' isomer) was pre-bound to a TC column (4 ml bed vol.; ~140\mu MTr/ml, equilibrated with 0.2 M NaOAc pH 5.2). Subsequently the following solutions were added and fractions collected: #1, 4 ml 0.1 M NaOAc (pH 5.2)/0.2 M NaCl (referred to hereafter as SA/SC); #2, 300 \mu g RNase/2ml SA/SC followed by a 4 ml wash-in with SA/SC; #3 and 4, 8 ml washes with SA/SC respectively; #5, 2.8 ml 0.1M NH_4HCO_3 pH 9.2/0.8M NaCl; #6, 7 and 8, 3 ml, 3 ml and 6 ml washes respectively with the preceding pH 9.2 solution; done at room temperature.

Note: maintaining NaCl at 0.2M and increasing the pH to 9.2 causes traces of MTrUp to leak off column—perhaps because the ring N of MTrUp, with a \( pK_a \approx 9 \), is ionizing, rendering the molecule more hydrophilic/less hydrophobic. Increasing the NaCl to 0.8M prevents this leakout. (A) Subsequently having removed the MTrUp with 60% ethanol, and washed with 4M urea; the column was re-equilibrated with SA/SC solution and an identical sequence of fractions collected, as just described for part B. Essentially no activity was bound to the column in the absence of the MTrUp ligand. Recovery of enzyme activity in both cases was ca 80%; activity was measured with a Kunitz assay (32). The temperature dependance of MTrUp(A_{260}^\text{u}) binding to medium TS at HS is: 4° (100%); 12° (90%); 24° (40%); 37° (30%).

(58); this overall similarity not withstanding, one striking difference between the 2 proteins is the fact that pRNase has no tryptophan while lysozyme has 6. Since most proteins have an aromatic amino acid content around 10%, most of them should bind to TRs. In view of a recent glass fiber filter binding assay (60)
Table 3. Binding of various proteins to trityl sepharose.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Aromatic Amino Acids</th>
<th>% Bound to TS</th>
<th>Protein</th>
<th>% Aromatic Amino Acids</th>
<th>% Bound to TS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Protamine</td>
<td>0</td>
<td>0</td>
<td>8. Fdp'ase</td>
<td>7.9</td>
<td>60</td>
</tr>
<tr>
<td>2. Histone H1</td>
<td>0.5</td>
<td>0</td>
<td>9. Lysozyme</td>
<td>9.3</td>
<td>100</td>
</tr>
<tr>
<td>3. Histone H2a</td>
<td>2.3</td>
<td>0</td>
<td>10. Vpd'ase</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>4. Histone H4</td>
<td>3.9</td>
<td>0</td>
<td>11. Pdnase</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>5. Prnase</td>
<td>7.5</td>
<td>0</td>
<td>12. Albumin</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>6. Histone H2b</td>
<td>5.6</td>
<td>24</td>
<td>13. Insulin</td>
<td>13.7</td>
<td>100</td>
</tr>
<tr>
<td>7. Histone H3</td>
<td>5.2</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Proteins (1-5 mg) dissolved in 5 ml 0.1M Na2HPO4 pH 7, unless noted otherwise; before application solutions centrifuged to remove any turbidity. Binding determined by A280 of eluant or biuret.
2. Tryptophan, phenylalanine and tryosine.
3. Medium TS column (~ 4 ml) used.
4. Dissolved in 0.1M NaaoAC pH 5.2/0.2M NaCl.
5. Dissolved in 25 mM Na2HPO4 pH 7/0.2M NaCl.
6. Dissolved in 30 mM Tris pH 8.8, 30 mM Mg(OAC)2.
7. Dissolved in 50 mM Tris pH 7.5, 5 mM MgCl2.

for viral proteins attached covalently to DNA; it is expected that these TRs could be employed similarly. Analogies to NO2-cellulose filter assays (61) should also be noted.

These hydrophobically retained enzymes mimic commercially available, solid phase-attached (covalently) enzymes in that they display moderate amounts of activity and retain it over a number of days at room temperature and above (table 4). In particular calf alkaline phosphatase (CAP) and venom phosphodiesterase (vpd'ase) retain good activity for better than a week, the latter enzyme being kept at 37-45° for 3 days. Particularly impressive in this regard is the tenacity of ~10% of the initially bound CAP (immobilized presumably through hydrophobic bonds alone) to resist elution with 1% SDS, or 40% ethanol (footnote 6, table 4). In general, E. coli bacterial alkaline phosphatase (BAP) gave results similar to CAP in preliminary experiments (21).

In view of: 1) the well established membrane-association of eucaryotic phosphatases, including CAP (63), 2) the possible membrane-association of (E. coli) periplasmic BAP (62), 3) the hydrophobic nature of membranes, the surprisingly efficient binding of phosphatases to TRs and their retention of activity may
Table 4. Activity of enzymes bound hydrophobically to trityl resins.

<table>
<thead>
<tr>
<th></th>
<th>vpd'ase³</th>
<th></th>
<th>CAP⁴</th>
<th></th>
<th>PNP'ase⁷</th>
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<tr>
<td></td>
<td>time²</td>
<td>temp.³</td>
<td>activity</td>
<td>time²</td>
<td>activity</td>
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<td>(days)</td>
<td></td>
<td>(A₄₄₀ u/5 hrs)</td>
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<tr>
<td>1. 0</td>
<td>2005</td>
<td>-</td>
<td>2.8</td>
<td>4. 6</td>
<td>18</td>
</tr>
<tr>
<td>2. 2</td>
<td>21</td>
<td>50</td>
<td>3.0</td>
<td>5. 8</td>
<td>20</td>
</tr>
<tr>
<td>3. 3</td>
<td>17</td>
<td>220</td>
<td>2.9</td>
<td>6. 9</td>
<td>12</td>
</tr>
</tbody>
</table>

1. Vpd'ase assay solution: 30 mM Tris pH 8.8, 30 mM Mg (oAc)₂, 0.3 mM Bis-pNP₂ phenyl PO₄; all assays done at room temp and in duplicate. After applying vpd'ase (200 µg, 5 units) to medium TS column (3 ml) in assay buffer minus substrate and washing with same solution (10-20 ml) to check if any activity washes out, the substrate was added to assay solution (6 ml) and it passed directly through the column (-5'). This was immediately followed by a wash (assay solution minus substrate) -15 ml. The total A₄₄₀ of effluent was then read and recorded as "activity". Control incubations (in free solution) were done with 200 µg vpd'ase in 6 ml assay solution for 5' at room temp. TR-immobilized vpd'ase activity was 60-85% of control incubations.

2. Days elapsed since zero time.

3. Temperature-time correlation refers to time elapsed, since prior assay, at indicated temperature; e.g. 37° in line 4 means that during the time interval between days 5 and 7, the column was kept at 37° prior to being assayed (@ room temp.) on day #7.

4. CAP assay solution: 50 mM NH₄HCO₃ pH 9.2, 1.9 M NaCl, 1 mM pNO₂ phenyl PO₄; all assays done at room temp. In general column assays were done as described in footnote 1 for vpd'ase, except that: 1.5 mg CAP applied; high TC column used; incubation time was 5 hours, during which the eluent was continuously recirculated through the column.

5. TR-immobilized CAP activity was 83% of control incubation.

6. After doing assay on line 1, the column was flushed with 1% SDS, 40% ethanol and finally H₂O (-20 ml each) after which it was left at room temp. for 2 days and assayed. Unlike part A above, column was left entirely at room temp. (-22°).

7. PNP'ase was isolated as a crude (NH₄)₂SO₄ cut (F₁) from M. luteus and assayed according to established methods (16). The enzyme (2.5 units) in 0.4 M glycine pH 9.3 and 20 mM Mg(oAc)₂ was applied to each of 2 TS columns (medium and high TS) and washed in with the same. Next the assay solution, including ADP, was applied and the incubation continued for 30'. The columns were then washed with HS to remove the assay solution and then with LS to elute the poly A product. TR-immobilized PNP'ase activity was -60% control incubations. Similar results obtained on both TRs.
not be coincidental. In a broad sense, little is, in fact, known as to the exact intracellular locus of most enzymes, i.e. whether they are "free" within a given organelle in the cyto- or nucleoplasm or are membrane-associated. Perhaps the generally strong binding and retention of enzyme activity by these hydrophobic polysaccharide-immobilized enzymes merely reflects their prepurification membrane-associated natural state.

DISCUSSION

Table 1 and figure 1 indicate that the base specificities and binding capacities for nucleic acids by dT cellulose and TRs are very close indeed. Likewise the similar mutual dependance of poly A binding on HS conditions and poly A release on LS is to be noted. These similarities notwithstanding, the nature of the chemical derivatization of the respective columns (oligo dT- or trityl-), their differing behavior regarding poly A binding in 0.1% SDS (footnote 5, table 1) and other results (21) suggest that the mechanisms whereby poly A is bound by these respective columns is very different. In the case of TRs the suggested binding mode is hydrophobic (diagram 1, bottom). The conventional explanation for the salt dependance of poly A binding to dT cellulose is: H bonds are maximized at HS because the excess Na\(^+\) counterions minimize the (phosphate) charge repulsion of the juxtaposed poly A/oligo dT polyanions. At LS this charge minimization is inadequate and poly A is eluted. In the case of TRs, according to the "internal pressure-solvent electrostriction" postulate (33): HS conditions so modify solvent (H\(_2\)O) structure that hydrophobic bonds are encouraged. At LS hydrophobic bonding is reduced as a result of the reduced internal pressure or cohesiveness of the solvent.

Some clues as to why poly A and denatured DNA bind so strongly to TRs at HS (and poly I and poly U less so) may be found in the structures of the homopolymers of A, C, G, U, I and X and single stranded DNA respectively. In general, there appears to be a tight correlation between the degree of openness or unstacking of a polymer's bases and its binding to TRs. Poly A maintains its open, single stranded, partially unstacked form under a wide variety of conditions (27,29,38). The open, unstacked structure of denatured DNA is well established (36). On the other hand,
poly I (39) and poly U (25,26), with increasing salt, change from a single stranded, open form, to a more ordered H-bonded form (3 or 4 stranded with I (40), hairpin duplex with U (41)). Poly C (29), poly G (29) and poly X (42,43) have more or less permanently compact, tightly base stacked structures, the latter two forming multistranded H-bonded aggregates. Particularly convincing in this regard are the reported effects of ethanol on the structures of poly A (23,24) and poly U (25,26; and probably poly I) and how this, in turn, effects their respective binding to TRs. Denaturants, including ethanol (23,24), convert poly A into a "denatured" form, wherein the bases lose all internal stacking and, instead, face outward. In contrast, ethanol (10-20%) has a structure-promoting effect on poly U (25,26) as reflected in higher $T_m$s. This implies decreased availability of its bases for intermolecular (e.g. with trityl) contacts. Under these conditions, poly A has its highest binding capacity to TRs, while poly U (and poly I fail to bind (21).

Almost as helpful in understanding why poly A binds to TRs is to consider both the relative stacking forces between the monomeric bases themselves: A>G>I>C>U (44) and also, between them and hydrophobic groups on columns. Elution patterns of nucleosides/tides from polystyrene-derivatized columns indicate that A-containing monomers bind more tenaciously than can be accounted for by ionic forces alone (45-48). Furthermore, inclusion of ethanol in the eluant causes them to elute sooner, in a position consistent with ionic forces alone (47). Even columns composed of only moderately hydrophobic polyacrylamide-derivatives show this predisposition of A-containing monomers to interact hydrophobically (49). In retrospect the tendency of poly A toward hydrophobic associations was, in fact, almost predictable (table 1, ref. 14), wherein, the then bothersome tendency of protected dinucleotides of A to stick to TC was noted. In summary, the distinct tendency of poly A to bind to TRs is probably due both to the fact that it has a more open, unstacked structure under a variety of conditions than the other polynucleotides, and that A monomers themselves have a higher stacking potential than other bases.

This apparently bizarre immobilization of poly A by hydrophobic resins, may have its physiological counterpart in the obser-
vation by Penman that poly A containing mRNA is often associated with hydrophobic membrane fractions (50), as well as in reports (51,52) on the tight binding between the poly A tails of mRNA and their cognate 78,000 M.W. protein, the salt dependence of which suggests hydrophobic type bonding (52). Studies on oligopeptide-polynucleotide recognition specificity (53) indicate that U- and A-enriched regions bind most strongly to aromatic amino acids, especially tryptophan. Likewise interferon binds (54) not only to the affinity ligand Cibacron blue (which itself binds to TRs; 21), but also binds to poly I, A and U, all of which bind to (high) TRs. Brawerman has noted (55) the tendency of hydrophobic regions of phenol denatured proteins to bind strongly to the poly A tails of mRNA at higher salt and pull them into the phenol phase. Reports such as these suggest that these little-noticed hydrophobic prop-
erties of nucleic acids are not without in vivo significance.

We feel that this trityl-group-supported method of nucleic acid affinity chromatography (diagram 1, top; figure 2 and table 2) is useful for 2 reasons: 1) it is a simple approach, because it takes advantage of the pre-positioned trityl group already on the synthetic ligand, which, in turn, affords a matrix-attachment yield of absolutely 100%. In fact, natural enzyme-produced polynucleotides may also be tritylated and hence incorporated into this scheme (table 2). Furthermore the hydrophobic handle or sticky end attached to any ligand, be it a nucleic acid, sugar or amino acid, need not be limited to trityl with its limited speci-
ficity (unhindered 1° -OH groups), but can be extended to other trityl-related groups: p(triphenylmethyl) aniline and naphtyliso-
cyanate, which have a much wider specificity range and also bind to TRs (14). 2) More experimental flexibility with regard to anneal-
ing complementary sequences should be possible, since it can be done either in free solution (under a variety of conditions as circumstances require) or directly on the column. It is antici-
pated that annealing in free solution will give better discrimina-
tion and yield (56) relative to columns involving conventional covalently anchored ligands.

These TRs show a striking resemblance to chromatographic materials currently in wide use: dT-cellulose and NO₂-cellulose. The functional homology with dT-cellulose is obvious (table 1),
their vastly different binding mechanisms not withstanding. The functional homology with NO₂-cellulose is equally, if not more striking, in that it includes not only the moderate poly A specificity of NO₂ cellulose, but also the binding of denatured DNA as well as the binding of most proteins at HS. It appears difficult to account for these functional homologies between such apparently dissimilar materials as the hydrophilic NO₂-cellulose and the hydrophobic TRs. However, as has been noted (57), the NO₂-cellulose designation is not very appropriate, since in addition to the NO₂ groups, the cellulose is also acetylated, which renders it sufficiently hydrophobic so as to require the addition of some detergent(s) (undesignated) to make it wetable and hence permeable to aqueous solutions. This more comprehensive description of the chemical makeup of NO₂-cellulose clearly makes it easier to explain the functional similarity between it and TRs.

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ABBREVIATIONS
TR, trityl resin; TC, trityl cellulose; TS, trityl sepharose; HS, high salt, 0.5M NaCl, 0.05 M Tris pH 7.5; LS, low salt, 0.05 M Tris, pH 7.5; Tr, trityl, (0)3-; MTr, mono p-methoxy trityl; DMTr, di p-methoxy trityl; SDS, Na dodecyl sulfate; FmRNA, fibroin (silk) mRNA; A260, absorbance units at 260 nm in 1 cm path; vpd'ase, venom phosphodiesterase; pDNase, pancreatic deoxyribonuclease; BAP, bacterial alkaline phosphatase; CAP, calf (intestinal) alkaline phosphatase; PNP'ase, polynucleotide phosphorylase; Fdp'ase, Fructose 1,6-diphosphatase; DEPC, diethyl pyrocarbonate.

REFERENCES
36. pp. 375-392 in ref. 33.
44. pp. 537-562, ref. 28.
61. References #26, 30 and 37 in ref. #60.