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The Investigation of Peptide–Oligodeoxythymidylic Acid Interactions Using Template Chromatography†

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ABSTRACT: Poly(vinyl alcohol) has been substituted with oligodeoxythymidylic acid and the resulting polyanion irreversibly attached to DEAE-cellulose via ionic bonding. Peptide–oligonucleotide interactions have been studied using a column chromatography technique with the PV(pT)$_n$-DEAE-cellulose as stationary phase. Of all the naturally occurring amino acids, only tryptophan and to a lesser extent tyrosine interact significantly with the immobilized oligodeoxythymidylic acid residues under the conditions for base pairing. The homopolymers of tryptophan and tyrosine undergo greater retardation than the monomers, such that the effect is not additive but multiplicative. Thus Tyr-Tyr-Tyr shows an eightfold and Trp-Trp-Trp an approximately 30-fold larger retardation than tyrosine and tryptophan, respectively. The peptide–oligonucleotide interaction decreases considerably when nonaromatic amino acids are present in the peptide. Consequently, naturally occurring peptides and proteins which contain relatively small amounts of tryptophan and tyrosine compared with the nonaromatic amino acids undergo at the most only slight retardation on the PV(pT)$_n$-DEAE-cellulose. The retention of oligonucleotides and peptides containing these aromatic amino acids is due in both cases mainly to base stacking (roughly 67% of the total interaction) but involves different mechanisms. Thus, the peptides interact preferably with the cellulose matrix whereas the oligonucleotides with the immobilized oligonucleotides. Interaction via hydrogen-bond formation makes up the remaining 33% of the total interaction. The oligonucleotides and peptides of the mobile phase interact with each other also via this mechanism. The strength of the d(pA-A-A) interaction is roughly that of Trp-Trp whereas d(pA-A-A-A) is weaker than Trp-Trp-Trp.

The study of peptide–nucleotide interactions has until now been carried out mainly using physical-chemical methods which only enable indirect conclusions to be drawn (Montenay-Garestier and Hélène, 1971; Diminicoli and Hélène, 1974; Wagner and Arfmann, 1974; Arfmann et al., 1974; Morita, 1974; Pinkston and Li, 1974). In order to eliminate these drawbacks, we have developed a column chromatographic technique which we call template chromatography with which the peptide–nucleotide interactions can be studied and determined directly (Eckstein et al., 1975). A great advantage of template chromatography lies in the fact that very complex, multicomponent systems can be investigated which would otherwise be extremely difficult using other techniques. The matrix used in template chromatography is DEAE-cellulose to which polymer-bound oligonucleotides of known sequence are irreversibly attached (Schott, 1974a). The internucleotide phosphate groups of the covalently attached oligonucleotide are bound as anions to the DEAE-cellulose. The oligonucleotide attached covalently to poly(vinyl alcohol) and ionically to DEAE-cellulose leads to a very stable stationary phase. The oligonucleotide therefore remains bound under the elution conditions, whereas a non-poly(vinyl alcohol)-bound oligonucleotide does not interact with DEAE-cellulose. Since only the terminal phosphate group and the internucleotide phosphate groups are used to form the stationary phase, there are still enough portions of the oligonucleotide available for the components of the mobile phase. The degree of interaction of the components of the mobile phase with the stationary phase can be directly obtained from their retardation values. Thus mixtures of oligonucleotides can be separated on PV(pT)$_n$-DEAE-cellulose into complementary and noncomplementary nucleotides according to the principle of base pairing (Schott, 1974b). The template chromatography of amino acids on PV(pT)$_n$-DEAE-cellulose showed that only the aromatic amino acids undergo significant retardation (Eckstein et al., 1975). It was thus concluded that only those peptides which contain aromatic amino acids play an important role in peptide–nucleotide interactions.

A very important aspect of the gene regulation mechanism and especially for the codon–anticodon interaction is whether small peptides or specific segments of proteins

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compete with the complementary oligonucleotides in the base-pairing step for the common binding site. In order to gain more information on this fundamentally important mechanism we have studied the interaction of oligonucleotides ($PV(pT)_n$-DEAE-cellulose) and peptides containing aromatic amino acids using the template chromatography technique.

Materials and Methods[1]

Chemicals. The Na_2HPO_4 (LAB) and NaCl (p.a.) were obtained from E. Merck, Darmstadt, W. Germany, DEAE-cellulose (DE 23) was from Whatman, Maidstone, Kent, England, poly(vinyl alcohol) from Roth, Karlsruhe, W. Germany (mol wt 70000) and TPS (technical grade) from EGA-Chemie, Steinheim, W. Germany. The methanol (technical grade) and triethylamine (technical grade) were distilled once. The other solvents were distilled thus: pyridine (technical grade) over KOH, diethyl ether (technical grade) over sodium. HMPT was stored over molecular sieves (3 Å, E. Merck, Darmstadt, W. Germany).

Amino Acids and Peptides. These were obtained from the following sources: amino acids (E. Merck, Darmstadt, W. Germany), Tyr-Tyr, Tyr-Tyr-Tyr, Trp-Tyr, Trp-Trp, and Phe-Gly-Phe-Gly (Sigma, St. Louis, Mo.), Phe-Phe-Phe p.a. (Serva, Heidelberg, W. Germany), Leu-Trp-Leu (Mann Res. Lab., New York, N.Y.). All other peptides were synthesized in our laboratories.

Antibiotics and Enzymes. Gramicidin and α-lactalbumin (National Biochemical Corp., Cleveland, Ohio), tyrocidin hydrochloride (pharm.), lysozyme from chicken egg-white p.a. (3 × crystallized), and bovine chymotrypsinogen p.a. (Serva, Heidelberg, W. Germany).

Nucleotides. 2-Deoxythymidine 5′-monophosphate, disodium salt, was obtained from Waldhof, Mannheim, W. Germany; the nucleotides d(pT-T-T-T), d(pA-A-A), and d(pA-A-A-A) were synthesized in our laboratories.

Synthesis of $PV(pT)_n$-DEAE-Cellulose. The synthesis of the $PV(pT)_n$-DEAE-cellulose is achieved in three steps. Commercial deoxythymidine 5′-phosphate was condensed according to the modified Khorana method (Agarwal et al., 1972), using TPS, yielding oligothymidine 5′-phosphate with maximal 12 monomer units. The low molecular weight oligonucleotide fractions were separated from the high molecular weight fractions by chromatography on DEAE-cellulose. The high molecular weight oligonucleotides were condensed with the free hydroxyl group of PVOH using TPS in HMPT–pyridine. An aqueous solution of $PV(pT)_n$ was treated with DEAE-cellulose and filled into a chromatography column and impurities and weakly bound oligomers were washed out before use.

Synthesis of $Poly(pT)_n$. Twenty millimoles (176000 A_{260} units) of pT (pyridinium salt) was suspended in dry pyridine and thoroughly dried by repeated evaporation of the pyridine at the oil pump. The residue was dissolved in 40 ml of dry pyridine and condensed using 20 mmol (6 g) of TPS during 10 hr at room temperature under anhydrous conditions with shaking. The condensation reaction was interrupted through the addition of 40 ml of water and the reaction mixture left to stand overnight at room temperature. The reaction mixture was diluted with water to make a 0.04 *M* solution which was then applied to a 1000-ml DEAE-cellulose column. The column was washed with water until free of pyridine. Low molecular weight oligomers $poly(pT)_{<5}$, ca. 80% of the condensation product, were eluted using 0.3 *M* TEAB whereas the higher molecular weight oligomers ($poly(pT)_{>5}$, ca. 20%) were eluted with 1 *M* TEAB. The low molecular weight nucleotide fractions were combined, dissolved in absolute pyridine, and again polycondensed. The yield from four condensation reactions involving 80 mmol of pT was approximately 64 mmol of $poly(pT)_{<5}$ and 16 mmol of $poly(pT)_{>5}$. The high molecular weight nucleotides were combined (140000 A_{260} units), dissolved in absolute pyridine, treated with acetic anhydride in order to cleave pyrophosphate derivatives, and finally purified by chromatography on 1000 ml of DEAE-cellulose. The column was washed with 0.3 *M* TEAB to remove low molecular weight compounds and then eluted using a linear gradient (5 l. of 0.3 *M* TEAB in the mixing vessel, 5 l. of 1.0 *M* TEAB in the reservoir). $poly(pT)_{>7}$ (37000 A_{260} units) fractions were combined and the TEAB was removed, taken up in absolute pyridine and acetylated in the 3′ position using acetic anhydride, and finally precipitated from ether. One obtains after drying, 1.5 g of $poly(pT(Ac))_{>7}$ which was condensed on to PVOH.

Synthesis of $PV(pT)_n$. Powdered, dried PVOH (300 mg) was added to 10 ml of dry HMPT under stirring and dissolved upon warming and then 10 ml of pyridine was added. Dry $poly(pT(Ac))_{>7}$ (1.4 g) was suspended in 10 ml of dry pyridine, 600 mg of TPS (2 mmol) was added, and the solution was slowly added under anhydrous conditions to the warm polymer solution during 30 min.

After shaking for 3 hr at room temperature, the highly viscous reaction mixture was diluted with a mixture of 8 ml of HMPT and 10 ml of pyridine, allowed to condense for a further 12 hr, and finally precipitated from ether. The centrifuged precipitate was dissolved in lukewarm water and the acetate protecting group removed through the addition of 2 ml of concentrated NH_4OH. After 12 hr the reaction mixture was neutralized with carbon dioxide.

Loading and Preparation of DEAE-Cellulose with $PV(pT)_n$. Preswelled DEAE-cellulose (100 ml) was slowly added to the neutral $PV(pT)_n$ solution under stirring at room temperature. The suspension was stirred for a further 4 hr, poured into a chromatography column, and then washed free from pyridine with 0.04 *M* TEAB–20% methanol. The low molecular weight compounds were eluted using a linear TEAB gradient (2 l. of 0.04 *M* TEAB in the mixing vessel, 2 l. of 0.7 *M* TEAB in the reservoir). The column was washed with water to remove TEAB and then with 0.5 *M* NaCl buffer at 60°C until the extinction of the eluate decreased below 0.2 A_{260} unit. Approximately 8000 A_{260} units of $PV(pT)_n$ of the original ca. 30000 units of $poly(pT)_{>7}$ used in the condensation reaction with PVOH remained irreversibly bound.

Preparation of the DEAE-Cellulose Column. The commercial DEAE-cellulose was treated according to the manufacturer's instructions and after filling in the chromatography column was equilibrated with 0.5 *M* NaCl–0.01 *M* Na_2HPO_4 buffer (pH 6.8).

Chromatography. Glass columns (30 × 2 cm) containing

[1] The system of abbreviations is principally as has been suggested by the IUPAC-IUB commission (1970). In this paper the prefix d (for deoxy) refers in all cases to the entire nucleoside residues of the oligonucleotide chains described, for clarity, parentheses and hyphens usually following the prefix d have been omitted. PVOH, poly(vinyl alcohol); TPS, 2,4,6-triisopropylbenzenesulfonyl chloride; HMPT, hexamethylphosphorous triamide; TEAB, triethylammonium hydrogen carbonate buffer. One A_{260} unit is defined as the amount of nucleotide giving an absorbance of 1 at 260 nm when dissolved in 1 ml of solvent and measured in a 1-cm light-path quartz cell.

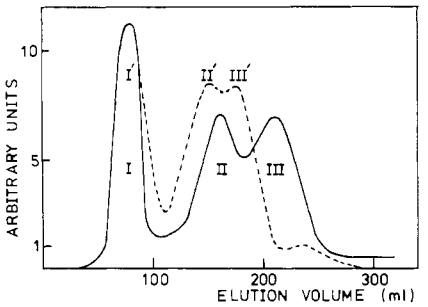

FIGURE 1: Template chromatography of a mixture of Trp, Trp-Trp, Leu-Trp-Leu, Ala-Trp-Trp, Trp-Trp-Ala, and Ala-Trp-Trp-Leu-Ala-Gln-Ser(*O*-*t*-Bu) in 0.5 *M* NaCl-0.01 *M* Na_2HPO_4 buffer on PV(pT)$_n$-DEAE-cellulose at 0°C (- - -, 25°C). Column size: 23 × 2 cm; fractions of 20 ml/hr were collected. Both elution profiles were automatically recorded at 280 nm using a LKB-Uvicord II unit. The characterization of the peak fractions is summarized in Table I.

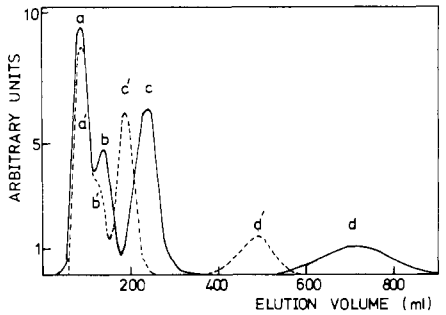

FIGURE 2: Template chromatography of a mixture of Trp, Trp-Trp, Trp-Trp-Trp, Tyr, Tyr-Tyr, and Tyr-Tyr-Tyr in 0.5 *M* NaCl-0.01 *M* Na_2HPO_4 buffer on PV(pT)$_n$-DEAE-cellulose at 0°C (- - -, 25°C). Column size: 23 × 2 cm; fractions of 20 ml/hr were collected. Both elution profiles were automatically recorded at 280 nm using a LKB-Uvicord II unit. The characterization of the peak fractions is summarized in Table I.

a sintered glass disc (D 1) and having a cooling jacket were used for the column chromatography. Between 8 and 120 μmol of each substance was dissolved in 1–10 ml of the elution buffer (0.5 *M* NaCl-0.01 *M* Na_2HPO_4 (pH 6.8)) and applied to the column. Occassionally methanol was added to dissolve the substance. The water in the jacket surrounding the column was kept constant at the predetermined temperature (Haake thermostat) during the elution procedure (hydrostatic pressure).

The elution profiles were measured automatically at 280 nm (LKB Uvicord II) and plotted simultaneously (Figures 1–3). The components within the various peaks were identified via their characteristic extinction values 250/260, 280/260, and 290/260 (Zeiss, PMQ II photometer) or by using paper chromatography. The composition of the various peaks is summarized in Table I.

Results and Discussion

Both the polysaccharide matrix and the immobilized thymidylic acid residues play a part in the retention of the compounds when chromatographed on oligonucleotide-DEAE-cellulose. Ion-exchange action can be neglected because the active sites are completely blocked by the polymer-bound oligonucleotides and furthermore the high salt concentration of the elution buffer completely nullifies the ion-exchange properties of the DEAE-cellulose. The substances and mixtures were chromatographed under identical conditions on both DEAE-cellulose and PV(pT)$_n$-DEAE-cellulose in order to determine the magnitudes of

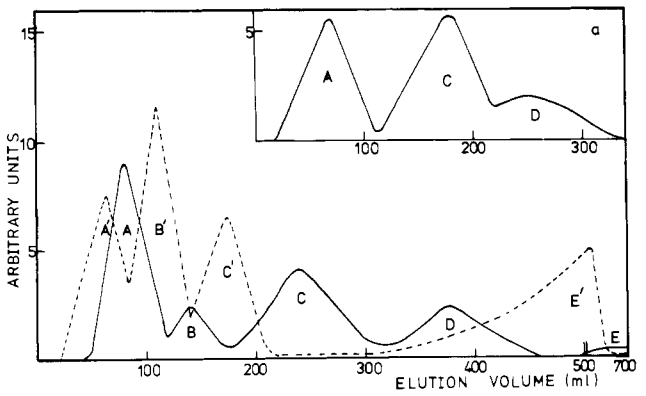

FIGURE 3: Template chromatography of a mixture of d(pT-T-T-T), d(pA-A-A), d(pA-A-A), Tyr-Tyr-Tyr, Trp-Trp, and Trp-Trp-Trp in 0.5 *M* NaCl-0.01 *M* Na_2HPO_4 buffer on PV(pT)$_n$-DEAE-cellulose at 0°C. The dotted line shows the elution profile obtained when the same mixture is chromatographed under the same conditions on DEAE-cellulose. Insert: Elution profile obtained upon rechromatography of the fractions comprising peak A′ in Figure 3, on PV(pT)$_n$-DEAE-cellulose at 37°C and otherwise identical conditions. Column size: 23 × 2 cm; fractions of 20 ml/hr were collected. All elution profiles were atuomatically recorded at 280 nm using a LKB-Uvicord II unit. The characterization of the peak fractions is summarized in Table I.

Table I: Characterization of the Peak Fractions in Figures 1–3. .

Figure	Peak	Compd
1	I, I′	Trp, Leu-Trp-Leu
	II, II′	Trp-Trp-Ala, Ala-Trp-Trp, Ala-Trp-Trp-Leu-Ala-Gln-Ser(*O*-*t*-Bu)
	III, III′	Trp-Trp
2	a, a′	Trp, Tyr-Tyr, Tyr
	b, b′	Tyr-Tyr-Tyr
	c, c′	Trp-Trp
	d, d′	Trp-Trp-Trp
3	A	d(pT-T-T-T)
	A′	d(pT-T-T-T), d(pA-A-A), d(pA-A-A-A)
	B, B′	Tyr-Tyr-Tyr
	C	Trp-Trp, d(pA-A-A)
	C′	Trp-Trp
	D	d(pA-A-A-A)
	E, E′	Trp-Trp-Trp
Insert to Figure 3	A	d(pT-T-T-T)
	C	d(pA-A-A)
	D	d(pA-A-A-A)

the peptide-oligonucleotide and peptide-polysaccharide interactions, respectively. If the retention on the oligonucleotide-DEAE-cellulose is larger than on the DEAE-cellulose, then assuming additivity, the difference between these two values is a quantitative measurement of the peptide-nucleotide interaction. Alanine undergoes no measurable retention on the column and, therefore, in order to eliminate additional column parameters, all elution volumes are expressed in terms of the elution volume of alanine. The relative elution volume (V_r) is obtained from the ratio between the elution volume found for a particular substance and that of alanine, viz., $V_r = V_{obsd}/V_{Ala}$. The peptide-oligonucleotide interaction is given by the difference in the relative elution volumes (ΔV_r) obtained through chromatography on both columns. The chromatographic data obtained for various amino acids and synthetic peptides are summarized in Table II, whereby the V_r values show a maximum error of ±2%.

Alanine and all nonaromatic amino acids do not measurably interact with the stationary phase and thus have the

Table II: Chromatographic Data of Amino Acids and Synthetic Peptides on PV(pT)$_n$-DEAE- and DEAE-Cellulose Columns.[a]

Components of the Mobile Phase	Elution Temp (°C)	Elution Volume: DEAE-Cellulose ml	V_r	PV(pT)$_n$-DEAE-Cellulose ml	V_r	ΔV_r
Ala and all non-aromatic amino acids	0	56	1.00	60	1.00	0.0
	20	58	1.00	60	1.00	0.0
Phe	0	60	1.07	67	1.12	0.05 ± 0.04
Tyr	0	67	1.20	75	1.25	0.05 ± 0.04
	20	65	1.12	70	1.17	0.05 ± 0.04
Trp	0	80	1.43	92	1.53	0.10 ± 0.06
	20	75	1.29	85	1.42	0.13 ± 0.05
Trp-Ala	0	77	1.37	90	1.50	0.13 ± 0.05
	20	80	1.38	87	1.45	0.07 ± 0.05
Trp-Tyr	0	106	1.89	120	2.00	0.11 ± 0.07
Trp-Leu-Ala-Gln-Ser (*O-t*-Bu)	0	74	1.32	80	1.33	0.01 ± 0.03
Leu-Trp-Leu	0	77	1.38	87	1.45	0.07 ± 0.05
Tyr-Tyr	0	78	1.39	102	1.70	0.31 ± 0.05
Trp-Trp	0	163	2.91	212	3.53	0.62 ± 0.13
	20	149	2.57	180	3.00	0.43 ± 0.09
	37	144	2.48	175	2.92	0.44 ± 0.10
Trp-Trp-Ala	0	140	2.50	180	3.00	0.50 ± 0.11
Ala-Trp-Trp	0	143	2.55	185	3.08	0.53 ± 0.09
pGlu-Trp-Trp-NH_2	0	174	3.10	201	3.35	0.25 ± 0.14
Ala-Trp-Trp-Leu-Ala-Gln-Ser(*O-t*-Bu)	0	110	1.97	136	2.27	0.30 ± 0.08
Phe-Gly-Phe-Gly	0	65	1.16	75	1.24	0.08 ± 0.07
Phe-Phe-Phe	0	63	1.12	70	1.17	0.05 ± 0.04
Tyr-Tyr-Tyr	0	110	1.96	142	2.37	0.41 ± 0.09
	37	99	1.70	120	2.00	0.30 ± 0.06
Trp-Trp-Trp	0	480	8.58	715	11.92	3.35 ± 0.48
	37	382	6.59	500	8.33	1.74 ± 0.28

[a] Column size in both cases: 23 × 2 cm.

values V_r = 1.0 and ΔV_r = 0. However, all the aromatic amino acids and the synthetic peptides containing aromatic amino acid residues have V_r values significantly larger than 1.0. Thus these compounds interact with the stationary phase to a greater or lesser extent. The retention of the amino acids phenylalanine and tyrosine is almost totally due to interaction with the polysaccharide portion of the stationary phase because the corresponding ΔV_r values are not significantly larger than zero.

Tryptophan undergoes the greatest retardation of all the naturally occurring amino acids. This retardation is mainly the result of a very strong interaction with the cellulose matrix (V_r = 1.43) which is only slightly, but significantly increased by the tryptophan–oligonucleotide interaction, ΔV_r = 0.1. This weak peptide–oligonucleotide interaction can also be observed for the following peptides which all contain one tryptophan residue: Trp-Tyr, Trp-Ala, and Leu-Trp-Leu. The peptide–nucleotide interaction of trpytophan peptides decreases and rapidly approaches zero as the number of nonaromatic amino acid residues in the molecule increases. Thus, the pentapeptide Trp-Leu-Ala-Gln-Ser(*O-t*-Bu) which contains four nonaromatic amino acid residues shows no measurable affinity for oligonucleotides. Furthermore, the position of the tryptophan within the peptide does not influence the retention in any way. It may thus be concluded that interaction with oligonucleotides can also take place when the tryptophan is in the middle of the peptide chain.

If the number of tryptophan or tyrosine residues in a particular peptide increases so does the retention. On the other hand, an increase in the number of phenylalanine residues in the peptide causes no increase in the retention. Thus the ΔV_r values for phenylalanine and Phe-Phe-Phe are identical, namely 0.05 ± 0.04. On the other hand, the ΔV_r value for the homopolymers of tyrosine increases considerably in going from the monomer to the trimer thus, 0.05 ± 0.04 (Tyr), 0.31 ± 0.05 (Tyr-Tyr), and 0.41 ± 0.09 (Tyr-Tyr-Tyr). As can be seen, the interaction does not increase linearly for each new tyrosine residue. The tryptophan oligomers undergo an even greater interaction with the stationary phase than the corresponding tyrosine oligomers. The V_r value for tryptophan increases from 1.53 to 3.53 in going to Trp-Trp and reaches the unexpectedly high value of 11.92 for Trp-Trp-Trp. The extremely large retention shown by Trp-Trp-Trp on PV(pT)$_n$-DEAE-cellulose is mainly (ca. 2/3, V_r = 8.58) due to interaction with the polysaccharide portion of the stationary phase. The interaction with the immobilized oligodeoxythymidylic acid amounts to approximately one-third of the total interaction (ΔV_r = 3.35). The ΔV_r values increase from 0.10 ± 0.06 for Trp to 0.62 ± 0.13 for Trp-Trp and reach the value of 3.35 ± 0.48 for Trp-Trp-Trp. Thus one can say that each new tryptophan residue causes an approximate sixfold increase in the ΔV_r values (0.10:0.62:3.35). This multiplicative increase is indicative of a cooperative effect which is known to occur in the base pairing of complementary oligonucleotides, but is up till now unknown for peptide–nucleotide interactions.

The ΔV_r values of those peptides which contain nonaromatic amino acid residues in addition to the Trp-Trp sequence are smaller than the value for the homopolymer itself. Thus, the ΔV_r value decreases from 0.62 to 0.50 in going from Trp-Trp to Trp-Trp-Ala. Similarly pGlu-Trp-Trp-NH_2 has a ΔV_r value some 50% smaller than that of Trp-Trp. This tryptophan peptide has also only one nonaromatic amino acid but due to the missing ionic charge at both ends, is similar to a Trp-Trp segment in a peptide chain. A similar environment for the tryptophan residues is present in the model peptide Ala-Trp-Trp-Leu-Ala-Gln-Ser(*O-t*-Bu) and the ΔV_r value of 0.30 corresponds closely with that of pGlu-Trp-Trp-NH_2. The interaction of all the amino acids and peptides investigated decreases as expected upon raising the temperature. This is due to the fact that the hydrogen bonding and stacking effect which bring about the interaction become weaker with increasing temperature. This temperature effect is especially noticeable with the homopolymers of tryptophan whereas it lies within the tolerance limits in the case of tyrosine and tryptophan peptides.

The strong interaction of tryptophan is drastically weakened through the presence of nonaromatic amino acid residues in the peptide. Thus, peptides with statistically distributed tryptophan and tyrosine residues should not be expected to interact with oligonucleotides. This assumption is based on the results of the template chromatographical investigations on tryptophan-containing, naturally occurring proteins on PV(pT)$_n$-DEAE-cellulose. The chromatographic data are summarized in Table III.

The V_r values for the water-soluble enzymes lysozyme and chymotrypsinogen, both of which contain a relatively high percentage of tryptophan, are smaller than 1.0, thus indicating that these substances undergo partial exclusion from the matrix. It can be tentatively concluded from the ΔV_r values that lysozyme undergoes slight interaction with the immobilized oligonucleotides whereas chymotrypsinogen is not retarded at all. The antibiotic tyrocidin and gramicidin and also the protein α-lactalbumin show a poor

Table III: Chromatographic Data of Amino Acids, Synthetic Peptides, and Naturally Occurring Antibiotics and Enzymes on PV(pT)$_n$-DEAE- and DEAE-Cellulose Columns.[a]

Substance mg (ml)	Amount of Tyr and Trp in Peptide[b] (%)	Elution Volumes: DEAE-Cellulose Buffer ml	DEAE-Cellulose Buffer V_r	DEAE-Cellulose Methanol–Buffer ml	DEAE-Cellulose Methanol–Buffer V_r	PV(pT)$_n$-DEAE-Cellulose Buffer ml	PV(pT)$_n$-DEAE-Cellulose Buffer V_r	PV(pT)$_n$-DEAE-Cellulose Methanol–Buffer ml	PV(pT)$_n$-DEAE-Cellulose Methanol–Buffer V_r	ΔV_r Buffer	ΔV_r Methanol–Buffer
Ala 20 (1)		70	1.00	71	1.00	58	1.00	59	1.00	0.00	0.00
Trp 3 (2)	100	96	1.37	90	1.27	82	1.41	82	1.39	+0.04	+0.12
Trp-Trp 1 (2)	100	217	3.10	136	1.91	209	3.60	157	2.66	+0.50 ± 0.13	+0.75
Lysozyme 20 (1)	7	61	0.87			55	0.95			+0.08 ± 0.04	
Chymotrypsinogen 10 (1)	5	46	0.66			37	0.64			−0.02 ± 0.02	
Tyrocidin[c] 7 (2)	~10–20			70	1.00			64	1.08		+0.08
Gramicidin[c] 6 (2)	~20–25			76	1.07			62	1.05		−0.02
α-Lactalbumin 20 (2)	6.5			67	0.94			63	1.07		+0.13

[a] Column size in both cases: 23 × 2 cm. [b] = [(ΣTyr + Trp)/Σ amino acid residues] 100. [c] Mixtures.

solubility in water and therefore they were chromatographed using a buffer solution containing 50% methanol. The V_r values for tryptophan and Trp-Trp in the methanol–buffer decreased by ca. 7% for Trp (DEAE-cellulose) and for Trp-Trp by 38% (DEAE-cellulose) and 26% ((PV(pT)$_n$)-DEAE-cellulose).

However, the ΔV_r values underwent a considerable increase in the methanol–buffer mixture when compared with aqueous buffer. This effect can be explained by assuming that the influence of the stacking effect which occurs predominantly on the cellulose matrix is reduced in the methanol–buffer (Cantor et al., 1969) whereas hydrogen-bond formation with the immobilized oligonucleotides increases. The weak retention found for the naturally occurring peptides and proteins investigated corresponds roughly to that of tryptophan and thus the differing tryptophan contents (5–25%) of these compounds play no significant role. These results are important when considering the peptide–nucleotide interaction of naturally occurring proteins. The large majority of all the proteins sequenced to date (Dayhof and Eck, 1968) contain considerably less statistically distributed tryptophan and tyrosine residues than the peptides and proteins which were chromatographed here. Furthermore such tryptophan and tyrosine sequences, which as we have shown interact extremely strongly with oligodeoxythymidylic acid, are very rare in naturally occurring peptides and proteins. Thus it is probable that several adjacent tryptophan and tyrosine residues are necessary to produce a significant interaction between such peptides and oligonucleotides. It is conceivable that tryptophan and tyrosine residues which show a statistical distribution in the primary structure can nevertheless be close together as a result of the protein's tertiary structure.

Most aromatic amino acids tend to be located in the tertiary structures of proteins. However there is good evidence that besides these buried tryptophan residues there are also exposed tryptophans present in proteins (Williams et al., 1965). Furthermore it is possible that tryptophan residues could be exposed by nucleotide sequences according to a "induced fit" mechanism. Thus, the tertiary structure of the protein possibly plays a decisive role in the interaction with oligonucleotides.

Table IV: Chromatographic Data of a Solution of (a) and (b) in 10 ml of 0.5 *M* NaCl–0.01 *M* Na_2HPO_4 Buffer on the PV(pT)$_n$-DEAE-Cellulose Column at 0 and 25°C.[a]

Component mg (μmol)	Elution Volume on PV(pT)$_n$-DEAE-Cellulose ml 0°C	ml 25°C	V_r 0°C	V_r 25°C
	Mixture a			
Trp 24 (120)	82	83	1.37	1.38
Leu-Trp-Leu 52 (120)	82	88	1.37	1.38
Trp-Trp-Ala 14 (30)	164	158	2.73	2.63
Ala-Trp-Trp 14 (30)	164	158	2.73	2.63
Trp-Trp 24 (60)	220	178	3.67	2.97
Ala-Trp-Trp-Leu-Ala-Gln-Ser(*O*-*t*-Bu) 15 (16)	Not identified			
	Mixture b			
Tyr 13.7 (75)	65	60	1.08	1.00
Tyr-Tyr 14.2 (39)	92	92	1.53	1.53
Tyr-Tyr-Tyr 14.7 (27)	140	130	2.33	2.17
Trp-Trp-Trp 9.4 (15)	726	496	12.10	8.27
Trp 8.3 (40)	92	90	1.53	1.50
Trp-Trp 8.3 (20)	240	200	4.00	3.33

[a] Column size: 23 × 2 cm.

These results do not give any information on the possible mutual interaction between peptides containing aromatic amino acid residues, in a mixture. In order to investigate this problem which is important for the understanding of natural systems, we chromatographed mixtures of peptides, the components of which contained nonaromatic amino acids and tryptophan residues. Figure 1 shows the elution profile obtained for the separation of a mixture of Trp, Trp-Trp, Leu-Trp-Leu, Ala-Trp-Trp, Trp-Trp-Ala, and Ala-Trp-Trp-Leu-Ala-Gln-Ser(*O*-*t*-Bu) on PV(pT)$_n$-DEAE-cellulose at 0 and 25°C. The chromatographic data are also summarized in Table IV. The good peak resolution

Table V: Chromatographic Data of a Solution of d(pT-T-T-T), d(pA-A-A), d(pA-A-A-A), Tyr-Tyr-Tyr, Trp-Trp, and Trp-Trp-Trp in 10 ml of 0.5 *M* NaCl–0.01 *M* Na_2HPO_4 Buffer (pH 6.8) on PV(pT)$_n$-DEAE- and DEAE-Cellulose Columns.[a]

Component in Solution mg (μmol)	Elution Volumes: DEAE-Cellulose 0°C ml	V_rI	PV(pT)$_n$-DEAE-Cellulose 0°C ml	V_rII	37°C ml	V_rIII	V_r(II-I)[c] ΔV_r(II-I)100[d]/V_rII	V_r(II-III)[e] ΔV_r(II-III)100[d]/V_rII
d(pT-T-T-T)	65	1.16	80	1.33	80	1.33	0.17 (12.8)	0 (0)
15 (12)	65	1.16	75[b]	1.25	75[b]	1.25	0.09 (7.2)	0 (0)
d(pA-A-A)	65	1.16	240	4.00	180	3.00	2.84 (71.0)	1.00 (25)
6 (6)	65[b]	1.16	220[b]	3.67	190[b]	3.17	2.50 (68.1)	0.50 (13.6)
d(pA-A-A-A)	65	1.16	375	6.25	256	4.27	5.09 (81.4)	1.98 (31.7)
8 (6)	65[b]	1.16	330[b]	5.50	265[b]	4.42	4.34 (78.9)	1.08 (19.6)
Tyr-Tyr-Tyr	110	1.96	140	2.33	130	2.17	0.37 (15.9)	0.16 (6.9)
7 (14)	110	1.96	142[b]	2.37	120[b]	2.00	0.41 (17.3)	0.37 (16.0)
Trp-Trp	175	3.13	240	4.00	180	3.00	0.87 (21.8)	1.00 (25)
4 (10)	163[b]	2.91	212[b]	3.53	175[b]	2.92	0.62 (17.5)	0.61 (17.3)
Trp-Trp-Trp	520	9.29	650	10.83	430	7.17	1.54 (14.2)	3.65 (33.8)
18 (30)	480[b]	8.58	715[b]	11.92	500[b]	8.33	3.35 (28.1)	3.59 (30.1)

[a] Column size: 23 × 2 cm. [b] Value for pure material. [c] Contribution of immobilized oligodeoxythymidylic acid toward the total interaction with the PV(pT)$_n$-DEAE-cellulose matrix. [d] As in *c* in %. [e] Amount of possible hydrogen bonds involved in the interaction with the immobilized oligonucleotides.

indicates that the components of the mobile phase interact to differing extents with the stationary phase. As expected, the degree of interaction increases as the number of tryptophan residues in the peptide increases whereas an increase in the elution temperature causes a decrease in the interaction (Figure 1). The interaction between the stationary phase and the components in the mixture is in almost all cases approximately 10% weaker than when the individual components are chromatographed alone. The only exception is Trp-Trp which undergoes identical retardation in the mixture as when chromatographed alone. A distinct mutual interaction between the components of the mixture which would lead to a more striking difference in the chromatographic behavior could not be detected even in the following example. Thus, a mixture of the homopolymers of tryptophan and tyrosine was chromatographed on PV(pT)$_n$-DEAE-cellulose. These compounds show the strongest interaction with the stationary phase of all the peptides investigated and it is therefore possible that they can associate with each other in solution. Figure 2 shows the elution profile obtained for the separation of the mixture containing Trp, Trp-Trp, Trp-Trp-Trp, Tyr, Tyr-Tyr, and Tyr-Tyr-Tyr on PV(pT)$_n$-DEAE-cellulose at 0 and 25°C. The chromatographic data are summarized in Table IV. As in the first example, the good peak resolution shows that the components of the mobile phase interact to differing extents with the stationary phase. Furthermore the interaction shows a significant temperature dependency as can be seen from the elution profiles at 0 and 25°C. This temperature effect is especially noticeable with Trp-Trp and Trp-Trp-Trp for which the elution volume in both cases is some 30% smaller at 25°C than that at 0°C. Tyrosine and Tyr-Tyr undergo an approximate 10% weaker retardation in the mixture than when chromatographed as single components. The V_r values obtained for tryptophan, Trp-Trp-Trp, and Tyr-Tyr-Tyr in the mixture correspond to those obtained when chromatographed alone. On the other hand, Trp-Trp is retarded more strongly (ca. 14%, V_r = 4.00) than in the corresponding single component system (V_r = 3.53). This increase in the retention is possible due to aggregation formation between Trp-Trp units although this still has to be unequivocably proved. The results obtained from the multicomponent systems are in good agreement with those for individual components and show that mutual interaction between aromatic amino acids is if at all, then considerably smaller, than the peptide–oligonucleotide interaction.

As we have shown, peptides which contain tyrosine and tryptophan residues can interact via these amino acids with oligodeoxythymidylic acid. The degree and extent of the interaction depend upon the number and the spacial arrangement of these residues within the peptide. Thus, one observes especially large interactions when these aromatic amino acids occur blockwise and are not statistically distributed within the peptide. The interaction between the immobilized oligodeoxythymidylic acid and Tyr-Tyr-Tyr and Trp-Trp-Trp clearly show that the monomer units in the homopolymers participate in the interaction in a multiplicate and not an additive manner, analogous to the interaction between complementary oligonucleotides. At this stage the question arose as to whether the strengths of interaction of small peptides and base-paired, complementary oligonucleotides are comparable. This comparison is very important for the understanding of the peptide–nucleotide interaction and we therefore chromatographed a mixture of tryptophan-containing peptides and oligodeoxyadenylic acid on PV(pT)$_n$-DEAE-cellulose in an attempt to clarify the situation.

Figure 3 shows the elution profiles obtained for the chromatography of a mixture of d(pT-T-T-T), d(pA-A-A), d(pA-A-A-A), Tyr-Tyr-Tyr, Trp-Trp, and Trp-Trp-Trp on PV(pT)$_n$-DEAE- and DEAE-cellulose at 0°C. The chromatographic data are summarized in Table V. The good peak resolution obtained indicates once more that the components of the mobile phase interact to a greater extent with the stationary phase than with themselves. In addition, the resolution obtained on the PV(pT)$_n$-DEAE-cellulose is considerably better than that on the DEAE-cellulose, thereby confirming that the immobilized oligodeoxythymidylic acid residues play an important role in the interaction. By comparing the V_r values of the mixture with those of the pure substances it must be concluded that the homologues of tryptophan and tyrosine probably interact with each other and also with the additional components of the mixture. The V_r values of the oligonucleotides and of Tyr-Tyr-Tyr on

DEAE-cellulose remain unaltered in the mixture compared with the corresponding single component values. However, the V_r values for the tryptophan oligomers are some 7% larger in the multicomponent system than the values for the pure substances. These variations are even more noticeable on the PV(pT)$_n$-DEAE-cellulose. Thus the V_r values of all the oligonucleotides in the multicomponent system at 0°C are between 7–13% higher than the single component values. On the other hand, the V_r value for Trp-Trp-Trp is some 9% smaller.

These significant differences in the elution volumes can be explained by postulating that the components of the mobile phase form some type of aggregate species via hydrogen bonding and/or stacking effects. This postulate is supported by the results obtained by performing the chromatographic separation at 37°C. Thus, the V_r values of the individual components of the mixture apart from Trp-Trp-Trp, are only slightly different (max. 5%) than the values obtained by chromatographing the pure substances separately. Furthermore the components d(pA-A-A) and d(pA-A-A-A) are eluted earlier in the mixture than they are when chromatographed alone. This shows that the secondary forces are partially destroyed through warming to 37°C.

The increased retention of the tryptophan oligomers on the DEAE-cellulose can thus be explained through the formation of aggregates in solution. Such aggregates should show a greater tendency to interact with the cellulose matrix so that Trp-Trp and also Trp-Trp-Trp undergo stronger retardation in the mixture than the individual substances alone. Presumably peptide–oligonucleotide complexes are also formed in solution which will interact with the immobilized oligodeoxythymidylic acid to differing extents. Thereby, the weaker binding components of the peptide–nucleotide complexes (oligodeoxyadenylic acid and Trp-Trp) are eluted later and the strongly binding component (Trp-Trp-Trp) earlier than in the single component systems. Tyr-Tyr-Tyr apparently plays no part in this interaction since its V_r values does not differ significantly from the value in the single component system.

The strength of the peptide–oligonucleotide and the oligonucleotide–oligonucleotide interactions can be estimated in a semiquantitative manner from the V_r values in Table V. The V_r values at 0°C on PV(pT)$_n$-DEAE-cellulose are directly proportional to the strength of the interaction. All the secondary forces such as base stacking and hydrogen bonding which occur between the components of the mobile phase and both the cellulose matrix and the immobilized oligonucleotide are included in the V_rII value. That portion of the total interaction, due to the immobilized oligonucleotides is approximately equivalent to the difference between V_rII and V_rI. The relative retention of a compound on DEAE-cellulose (V_rI) increases on the PV(pT)$_n$-DEAE-cellulose (V_rII) by that amount due to the contribution of the immobilized oligodeoxythymidylic acid residues.

A comparison of the ΔV_r(II–I) values in percent shows that peptides and oligonucleotides have completely different affinities toward the stationary phase. Thus, 70–80% of the total interaction of the oligodeoxyadenylic acid occurs via the immobilized, complementary oligodeoxythymidylic acid, whereas only 14–28% of the interaction of the noncomplementary oligodeoxythymidylic acid and peptides occurs via the immobilized nucleotides. The aromatic peptides and the noncomplementary nucleotides exhibit a much greater affinity for the cellulose matrix than for the immobilized nucleotides.

Complementary tri- and tetranucleotides do not form intermolecular hydrogen bonds at 37°C and consequently the retention suffered by these compounds at 37°C (V_r III) is presumably due to base stacking. Thus the difference V_rII − V_rIII corresponds approximately to that portion of the total interaction due to hydrogen-bond formation.

The ΔV(II–III) values show that all the components form hydrogen bonds to a grater or lesser extent with the PV(pT)$_n$-DEAE-cellulose, apart from oligodeoxythymidylic acid which forms none.

That portion of the total interaction due to hydrogen bond formation is approximately 25% in the case of d(pA-A-A) and increases to 32% for d(pA-A-A-A).

The values obtained from the multicomponent systems indicate that peptide–nucleotide interaction occurs in the mobile phase. Thus the interaction of oligodeoxyadenylic acid and of Trp-Trp is twice as large in the mixture than in the single component systems whereas the value for Tyr-Tyr-Tyr is only half as large.

Trp-Trp-Trp shows the greatest tendency of all to form hydrogen bonds and this seems unaffected by the presence of other components in the mobile phase. Thus this tendency shown by Trp-Trp-Trp is some 3.5 times greater than that for d(pA-A-A) and 1.80 times greater than d(pA-A-A-A). The contribution in percent toward the total interaction due to hydrogen bonds is roughly the same for d(pA-A-A) and Trp-Trp; furthermore the values for d(pA-A-A-A) and Trp-Trp-Trp are very similar. However, for all the compounds investigated here, the hydrogen-bond formation accounts for maximally 30% of the total interaction. Consequently, base stacking plays the most important role in the retention on PV(pT)$_n$-DEAE-cellulose.

The results of the template chromatography show that at low temperatures and also under physiological conditions (37°C, salt buffer), tryptophan peptides and low molecular weight oligonucleotides interact with each other via hydrogen-bond formation and with other compounds via base stacking. Thus, due to base stacking, tryptophan peptides have an extremely high affinity for the cellulose matrix. Oligonucleotides also interact via base stacking but do so preferably with the immobilized oligonucleotides and not with the cellulose matrix. As a result of the different mechanism involved, it is conceivable that tryptophan peptides induce interactions between oligonucleotides and other substances which normally, in the absence of such peptides, are not possible. Such a mechanism could possibly regulate molecular biological processes.

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Endonuclease II of *Escherichia coli:* DNA Reacted with 7-Bromomethyl-12-methylbenz[*a*]anthracene as a Substrate†

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ABSTRACT: An endonuclease II preparation from *Escherichia coli* makes single-strand breaks in DNA which has been treated with the carcinogen 7-bromomethyl-12-methylbenz[*a*]anthracene. In addition, the enzyme preparation excises N^6-(12-methylbenz[*a*]anthracenyl-7-methyl)adenine and N^2-(12-methylbenz[*a*]anthracenyl-7-methyl)guanine residues from the DNA. These are released as the modified purine bases, not as purine nucleoside derivatives. The rate of release of the adenine derivative is three to four times that of the guanine derivative.

Endonuclease II is an enzyme from *Escherichia coli* which hydrolyzes phosphodiester bonds in DNA treated with alkylating agents such as methyl methanesulfonate (Friedberg and Goldthwait, 1968; Friedberg et al., 1969), dimethyl sulfate, and *N*-methyl-*N*-nitrosourea (Kirtikar and Goldthwait, 1974), as well as in DNA exposed to γ irradiation (Kirtikar et al., 1975). This activity has been purified 1600-fold (Hadi et al., 1973). In this partially purified preparation of endonuclease II, there is an activity which recognizes depurinated and depurinated reduced DNA (Hadi and Goldthwait, 1971) and this activity was originally considered to be the same enzyme as that active on alkylated DNA. Since then the activities have been separated as will be noted in the Results and Discussion sections.

This 1600-fold purified enzyme preparation is also active on DNA treated with the polycyclic aromatic carcinogen 7-bromomethyl-12-methylbenz[*a*]anthracene. This carcinogen reacts with the amino groups of the bases in DNA both in vitro and in vivo (Rayman and Dipple, 1973a,b). It is a potent carcinogen in several animal test systems (Dipple and Slade, 1970, 1971; Roe et al., 1972), it exhibits mutagenic and cytotoxic effects in mammalian cells (Huberman et al., 1971; Duncan and Brookes, 1973) and bacterial systems (Maher et al., 1974), and it is toxic to bacteriophage (Dipple and Shooter, 1974). The enzyme preparation makes phosphodiester breaks in DNA treated with this carcinogen and also releases derivatives of the purine bases.

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Materials and Methods

Radioactive Materials. 7-Bromo[^{14}C]methyl-12-methylbenz[*a*]anthracene (specific radioactivity 5.6 Ci/mol) and [^{3}H]-7-bromomethyl-12-methylbenz[*a*]anthracene (specific radioactivity 725 Ci/mol) were prepared as previously described (Rayman and Dipple, 1973a). [^{3}H]Thymidine- and [^{3}H]purine-labeled T_7 DNA were prepared as described previously (Kirtikar et al., 1975). [^{3}H]Thymidine-labeled T_4 DNA was prepared according to procedures of Melgar and Goldthwait (1968).

Modified DNA Samples. Treatment of various DNA samples with hydrocarbon was carried out under the reaction conditions described by Rayman and Dipple (1973a). Samples of both T_7 and commercial salmon sperm DNAs, reacted with [^{3}H]-7-bromomethyl-12-methylbenz[*a*]anthracene [0.44 mmol of hydrocarbon per mol of DNA-P for T_7 DNA and 1.2 mmol of hydrocarbon per mol of DNA-P for salmon sperm DNA] were prepared. Salmon sperm DNA was also reacted with 7-bromo[^{14}C]methyl-12-methylbenz[*a*]anthracene to give DNA with specific activity of 0.23 μCi/mmol of DNA nucleotide. [^{3}H]Purine-labeled T_7 DNA (specific activity 1960 cpm/nmol of DNA nucleotide) was reacted with unlabeled 7-bromomethyl-12-methylbenz[*a*]anthracene at a hydrocarbon to DNA nucleotide ratio of 1:10. Other DNA samples used as substrates were prepared according to published procedures. [^{3}H]Purine-labeled T_7 DNA was treated with methyl methanesulfonate (Friedberg and Goldthwait, 1968) at a drug to DNA nucleotide ratio of 10:1 and the same labeled DNA was used to prepare depurinated reduced DNA by the procedures described by Hadi and Goldthwait (1971). [^{3}H]Purine-labeled T_7 DNA was exposed to 22.5 krad of γ irradiation from a ^{60}Co source (Kirtikar et al., 1975). [^{3}H]Thymidine-labeled T_4 DNA was irradiated with approximately 200 ergs/mm^2, a dose which should introduce approximately 60 dimers into each DNA molecule (P. Kuebler and D. Schlaes, unpublished results).