Acknowledgments

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Use of Phosphate-Blocking Groups in Ligase Joining of Oligodeoxyribonucleotides†

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ABSTRACT: The polynucleotide ligase from bacteriophage T_4 is able to join oligomers in which those terminal phosphate groups not directly involved in the formation of the new phosphodiester bond are in the form of alkyl phosphorothioates. These latter may be helpful in preventing alternate wrong joinings and serving as a handle for subsequent fragment modification.

One current strategy for the construction of deoxyribonucleotide duplexes ("genes") large enough and of the proper primary sequence to contain information that can, in principle, be transcribed into biologically nontrivial RNA involves the chemical synthesis of oligomers of sufficient size to be joined into larger arrays enzymatically (Agarwal *et al.*, 1970.)[1] This operation, catalyzed by polynucleotide ligase, requires that two segments to be joined must be held in adjacent positions by separately associating, *via* conventional antiparallel Watson–Crick bonding, with a third fragment (the "splint") of appropriate complementary sequence so that the 3′-hydroxyl group of one (the "acceptor") is brought into close juxtaposition to the 5′-terminal phosphate of the other (the "donor"). The splint thus provides specific template guidance for the ligation proper.

There have been a few observations that *in vitro* joining may deviate from this scheme. Thus, it was found (Tsiapalis and Narang, 1970) that the fidelity of the joining is not perfect; the ultimate base on the oligomer acceptor does not have to be complementary to the corresponding counterbase on the splint. Furthermore, certain types of duplex "end-to-end" joining or terminal cross-linking were found to be complicating

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[1] Other approaches consist of the isolation of operons by genetic and physicochemical means (Shapiro *et al.*, 1969) or by "reverse" transcription of purified messengers (Ross *et al.*, 1972; Verma *et al.*, 1972; Kacian *et al.*, 1972).

TABLE I: Oligodeoxyribonucleotides Used in Joining Reaction.[a]

Oligomer	Designation	Reference
d-EtS-p(T-G-C-T-A-A-A-T-T-T-G-A)	Protected fragment[2] 5	Heimer *et al.*, 1972a,b
d-p(A-A-G-A-C-A-G-C-A-T-A-T)	Fragment 2	Poonian *et al.*, 1972
d-EtS-p(T-G-T-C-T-T-T-C-A-A-A-T)	Protected fragment 3	This paper
d-EtS-p(T-T-A-A-T-C-C-A-T-A-T-G-C)	Protected fragment 1	Cook *et al.*, 1972
d-p(T-G-T-C-T-T-T-C-A-A-A-T)	Fragment 3	This paper
d-EtS-p(A-A-G-A-C-A-G-C-A-T-A-T)	Protected fragment 2	Poonian *et al.*, 1972

[a] See Discussion, Figure 2.

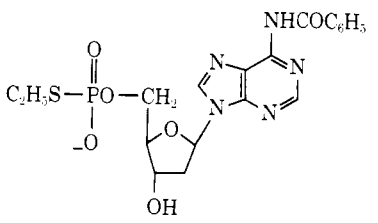
events (Sgaramella *et al.*, 1970; Weiss, 1970). It was reasoned that in order to minimize "wrong" joinings it would be useful to devise means whereby those terminal phosphates of the ternary nucleotide complex that could potentially result in an undesired phosphodiester linkage were prevented from doing so. We now find that it is possible in a ligase reaction to use both splint and acceptor molecules that have their 5′-terminal phosphates blocked with alkylthio groups—substituents that can serve both as protecting groups during the chemical oligonucleotide synthesis, and activating groups for subsequent modification (Cook *et al.*, 1969). It is hoped that this extension of the substrate specificity of the enzyme will prove useful in the recovery of the valuable oligomer fragments not participating in the joining reaction.

Experimental Section

Enzymes. Polynucleotide kinase and polynucleotide ligase from bacteriophage T_4 infected bacterial cells were the same as used previously (Harvey and Wright, 1972). Calf intestinal mucosa alkaline phosphatase (type VII) was obtained from Sigma Chemical. The phosphatase was dialyzed against 0.01 M Tris-HCl buffer (pH 8.0) containing 1 mM $MgCl_2$. The solution was stored at a concentration of 1 mg/ml at −20°. No phosphodiesterase activity was found under the following conditions: 10 nmol of the heptamer d-p(T-G-T-C-T-T-T)[2] was incubated 1 hr at 37° with 20 μg of calf alkaline phosphatase. The dephosphorylated heptamer was labeled with ^{32}P using polynucleotide kinase and [γ-^{32}P]ATP as described above. The labeled heptamer was separated by DEAE-cellulose chromatography (Harvey *et al.*, 1971). The enzyme-treated heptamer was found to elute at the same position as the marker heptamer. This shows that no nucleotides were excised by contaminating diesterases.

Bacterial alkaline phosphatase was obtained from Worthington Biochemical and dialyzed against 0.01 M Tris-HCl buffer (pH 8.0). Pancreatic DNase (1 × crystallized), snake venom phosphodiesterase, spleen phosphodiesterase, and micrococcal nuclease were all purchased from Worthington Biochemical and used without further purification.

[2] Nomenclature as specified in *Biochemistry* 9, 4022 (1970), and amplified in collaboration with Dr. W. E. Cohn. EtS preceding 5′-terminal phosphate symbol p denotes *S*-ethyl phosphorothioate. Thus, d-EtS-p(bzA) is



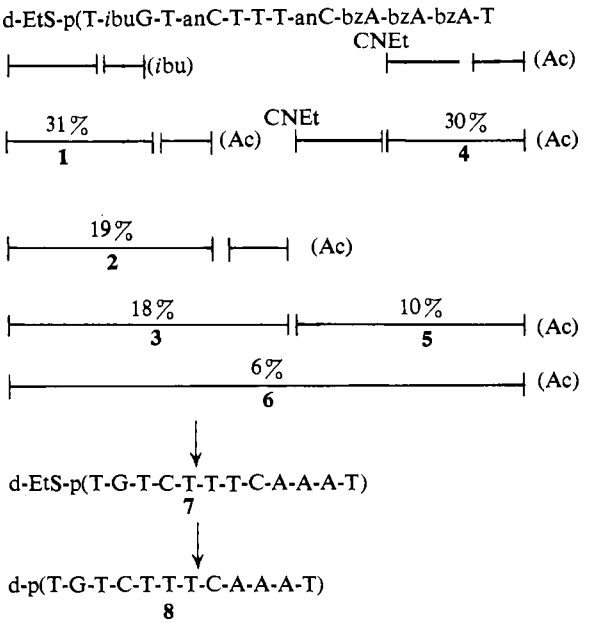


FIGURE 1: Chemical synthesis of fragment 3 (**8**) and its 5′-protected derivative **7**.[3] Yields are given above the bars.

Oligonucleotides. The several oligodeoxyribonucleotides used in this study are summarized in Table I. In this paper we describe the synthesis of fragment 3 (Figure 1) and its 5′-protected derivative. General methods for oligonucleotide synthesis, including the procedure for condensation reactions, chromatographic techniques, analytical methods, and extinction values employed have been described in earlier papers. Figure 1 summarizes the synthetic approach[3]: the method of fragment condensation is employed. The outcome of individual steps is summarized under Results. Experimental details are given as legends to the figures. The 5′ terminus of the growing chain is carried as phosphorothioate ethyl ester throughout; its removal by mild oxidative hydrolysis constitutes the final chemical manipulation.

The dodecamer **7** was obtained by treatment of **6** with concentrated ammonium hydroxide overnight, followed by evaporation and chromatography on a Sephadex G-15 gel column (1 × 100 cm) which was eluted with 0.5 M triethylammonium

[3] The method of representation is patterned after peptide schemes; see, for instance, Rittel *et al.*, 1957.

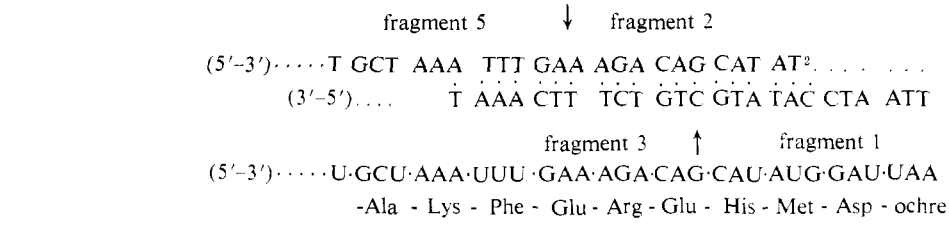


FIGURE 2: Relationship of DNA fragments to corresponding RNA and peptide sequence.

bicarbonate (pH 7.5). Unprotected dodecamer **8** was obtained by treatment of **6** (30 OD_{280} units) in phosphate buffer (pH 7), 0.4 M (0.25 ml), and water (0.5 ml) with a solution of iodine (0.2 M) in aqueous potassium iodide (0.4 M, 0.25 ml) for 18 hr. The product was diluted to 5 ml with water, and extracted with ether (three 5-ml portions). The aqueous portion was evaporated, treated with concentrated ammonium hydroxide (5 ml) overnight and centrifuged. The supernatant was evaporated and applied to an agarose gel column (Bio-Gel A, 0.5 m, 200–400 mesh, 0.85 × 115 cm) which was eluted with 0.5 M $Et_3NH_2CO_3$; fraction size, 4 ml, flow rate, 8 ml/hr; **8** (21 OD_{260} units, 75%) was located in fractions 17–20.

Preparation of 5′-^{32}P Labeled Fragment 2 and 5′-^{35}P Labeled Fragment 3. The 5′-phosphate was removed from fragment 2 in a reaction mixture containing 20 μmol of Tris-HCl buffer (pH 7.6), 5–8 nmol of fragment 2, and 5 μg of calf alkaline phosphatase in a total volume of 0.1 ml. The reaction was incubated at 37° for 1 hr and stopped by heating at 100° for 3 min. This completely inactivated the alkaline phosphatase from calf intestine. The reaction mixture was then brought up to 0.3 ml by addition of 3 μmol of $MgCl_2$, 2 μmol of dithiothreitol, 10 nmol of [γ-^{32}P]ATP (10 × 10^6 cpm), and 10 units of polynucleotide kinase. After incubation for 1 hr at 37°, the reaction mixture was separated on a Sephadex G-25 gel column (0.9 × 43 cm) by development with 0.5 M triethylammonium bicarbonate buffer (pH 7.6) at a flow rate of 6 ml/hr, 0.45-ml fractions being taken. The ^{32}P-labeled fragment 2 was found at the void volume, well separated from the peak of [γ-^{32}P]ATP which followed. Fractions containing the desired 5′-^{32}P labeled fragment 2 were concentrated *in vacuo* to dryness and dissolved in 1 ml of H_2O.

5′-^{35}P labeling of fragment 3 was carried out by the same procedure as described for fragment 2. [γ-^{35}P]ATP was made by the procedure of Weiss *et al.* (1968) except the ATP was purified on a DEAE-cellulose column (0.9 × 15 cm) eluted with a linear gradient of 0–0.5 M triethylammonium bicarbonate buffer (pH 7.6) in a total volume of 400 ml.

Joining of Fragments and Separation of Products. The reaction mixture contained 66 mM Tris-HCl buffer (pH 7.6), 6.6 mM $MgCl_2$, 6.6 mM dithiothreitol, 5 nmol of ATP, and 1 nmol of each strand (see Discussion and Figure 2) in a total volume of 0.06 ml. The reaction mixture was incubated at 0° with 5 units of T_4 ligase. At 1-hr intervals, 1-μl samples were removed and the ^{32}P label assayed for resistance to bacterial alkaline phosphatase. The ^{32}P resistant to phosphatase reached a maximum (40–60% in diester linkage) after 2 hr of incubation. The entire reaction mixture was layered on an Agarose 0.5-m column (0.9 × 62 cm). The column was developed at 6 ml/hr with 0.5 M triethylammonium bicarbonate and 0.45-ml fractions were taken.

Assays for Phosphodiester Bond Formation. The assay used for phosphodiester bond formation measured change of ^{32}P label from phosphatase labile to resistant. This was accomplished by incubation of the sample with 0.1 ml of 0.1 M Tris-HCl buffer (pH 8.0) and 5 μg of bacterial alkaline phos-

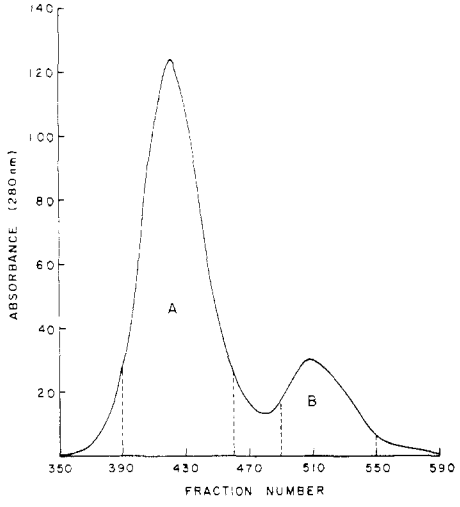


FIGURE 3: Preparation of **1**. Condensation, 20 mmol of d-EtSpT (Cook *et al.*, 1972), 20 mmol of d-*p*ibuG(*i*Bu), 45 mmol of $MesSO_2$-Cl (mesitylenesulfonyl chloride), 75 ml of pyridine, 3 hr. Work-up, 25 ml of water; after storage overnight at 0°, dilute to 200 ml with pyridine, treat with 200 ml of 2 N sodium hydroxide for 5 min at 10°, neutralize with pyridinium Dowex 50, subject to preliminary DEAE-cellulose chromatography (not shown) with linear gradient of 0.3 M 20% ethanolic $Et_3NH_2CO_3$ buffer (pH 7.5) into 0.15 M 20% ethanolic $Et_3NH_2CO_3$ buffer. The material emerging with buffer molarity of 0.17–0.21 was concentrated and reapplied to a DEAE column (9.2 × 75 cm). Gradient, convex, 0.3 M 20% ethanolic $Et_3NH_2CO_3$ buffer pH 7.5 into 9 l. of 0.1 M 20% ethanolic buffer; flow rate, 2.5 ml/min; fraction size, 20 ml.

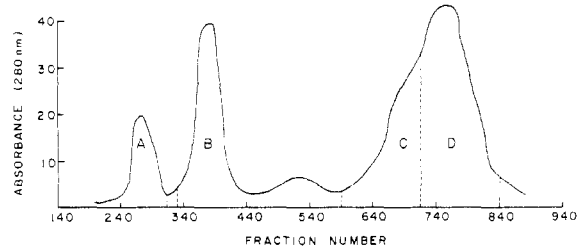


FIGURE 4: Preparation of **2**. Condensation, 6.1 mmol of **1**, 5 mmol of d-p(T-anC(Ac)) (Kumar and Khorana, 1969), 19 mmol of $MesSO_2$-Cl, 40 ml of pyridine, 3 hr. Work-up, 40 ml of water; after storage overnight at 5° adjust to 75 ml with pyridine and treat with 75 ml of 2 N NaOH for 20 min at 25°, neutralize with pyridinium Dowex 50. Chromatography, DEAE-cellulose (6.6 × 90 cm), convex gradient 0.275 M $Et_3NH_2CO_3$ pH 7.5 into 9 l. of 0.05 M buffer; fraction size, 20 ml; flow rate, 2.5 ml/min. Fractions 720–840 were concentrated and d-p(T-anC) was removed by gel permeation chromatography (not shown) on Sephadex G-25, superfine. Fractions 205–250 contained pure 2. Column size, 5 × 100 cm; flow rate, 1 ml/min of buffer 0.2 M $Et_3NH_2CO_3$ pH 7.5; fraction size, 4 ml.

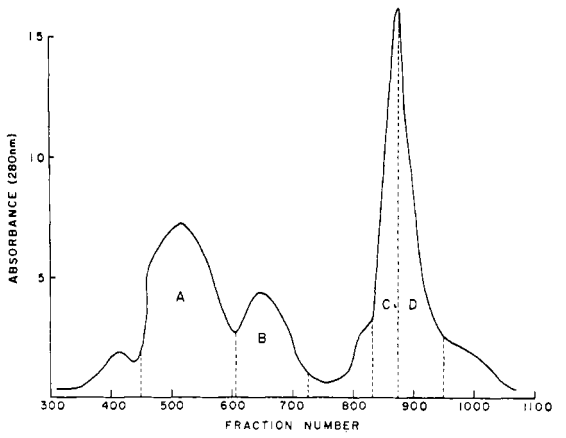


FIGURE 5: Preparation of **3**. Condensation, 0.85 mmol of **2**, 1.7 mmol of d-p(T-T-T(Ac)) (Narang *et al.*, 1969), 7.4 mmol of $MesSO_2Cl$, 30 ml of pyridine, 3 hr. Work-up, 30 ml of water; after storage overnight at 5° adjust to 100 ml with pyridine and treat with 100 ml of sodium hydroxide (2 N) at 0° for 10 min and neutralize with pyridinium Dowex 50. Chromatography, DEAE-cellulose (4.4 × 98 cm) bicarbonate form; gradient, linear, 12 l. of 0.45 M $Et_3NH_2CO_3$ pH 7.5 into 12 l. of 0.05 M buffer; flow rate, 2.5 ml/min; fraction size, 20 ml.

phatase for 30 min. After cooling, 0.2 ml of a solution containing 2 mM sodium pyrophosphate, 25 mM potassium phosphate buffer (pH 7.0), and 5 mg/ml of bovine albumin, followed by 0.2 ml of a 20% Norit suspension (packed volume) were added. The suspension was filtered through a 2.5-cm diameter glass fiber disk (Schleicher and Schuell). The residue was washed three times with cold 0.01 N HCl. The wet filter disk with washed Norit was placed in a vial with 10 ml of toluene-based scintillation fluid and ^{32}P determined in Packard scintillation spectrometer. Samples containing ^{33}P in place of ^{32}P as label were handled the same except the supernatant was counted and substracted from total radioactivity to determine the Norit adsorbable (or phosphatase resistant) label. This was necessary because of the lower energy of ^{33}P, which is counted in the ^{14}C channel.

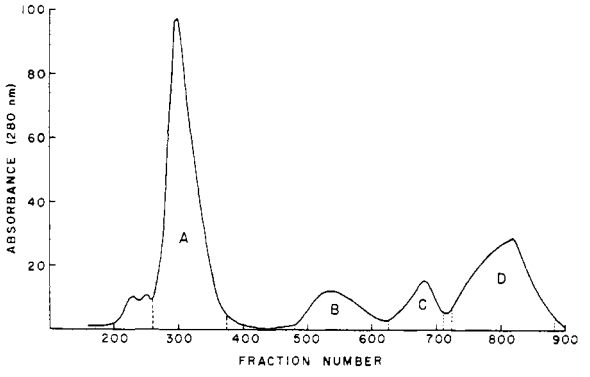


FIGURE 6: Preparation of **4**. Condensation, 5 mmol of d-CNEt-p-(bzA-bzA) (Narang *et al.*, 1967), 20 mmol of d-pT (Ac), 35 mmol of $MesSO_2Cl$, 100 ml of pyridine, 3 hr. Work-up, 50 ml of water, 70 ml of $iPro_2EtN$; after storage overnight at 5°, dilute to 150 ml with pyridine and treat with 150 ml of 2 N sodium hydroxide at 0° for 20 min; neutralization with pyridinium Dowex 50. Chromatography, DEAE-cellulose, bicarbonate, (6.6 × 90 cm). Gradient, convex, 0.25 M $Et_3NH_2CO_3$ pH 7.5 into 0.05 M, 9 l.; flow rate, 2.5 ml/min; fraction size, 20 ml. Peak D was evaporated and acetylated using 25 ml of acetic anhydride in 40 ml of pyridine for 18 hr. After water addition (25 ml) the solution was evaporated and isolated by precipitation in the usual way.

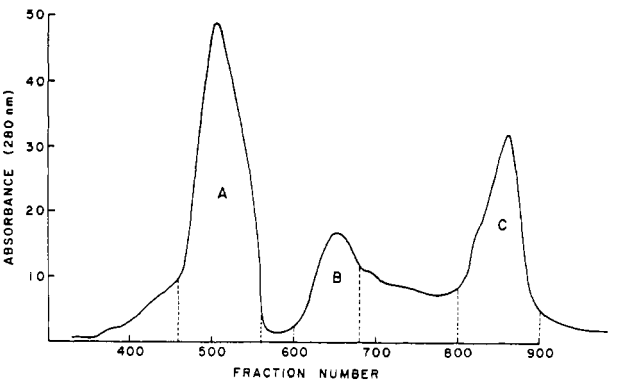


FIGURE 7: Preparation of **5**. Condensation, 3.68 mmol of d-CNEt-p-(anC-bzA (Kumar and Khorana, 1969), 1.44 mmol of d-p(bzA-bzA-T(Ac), 9.15 mmol of $MesSO_2Cl$, 25 ml of pyridine, 2.5 hr. Work-up, 20 ml of water, 18 ml of $iPro_2EtN$, and storage overnight at 5°. After dilution to 50 ml with pyridine, treat with 50 ml of 2 N sodium hydroxide at 0° for 20 min and neutralize with pyridinium Dowex 50. Chromatography, DEAE-cellulose, bicarbonate (4.4 × 78 cm). Gradient, linear, 12 l. of 0.45 M $Et_3NH_2CO_3$ pH 7.5 into 12 l., 0.05 M; fraction size, 20 ml; flow rate, 2.5 ml/min. Peak C was evaporated and applied to a Sephadex G-50 (superfine) gel column (5 × 100 cm) (not shown) and eluted with 0.2 M $Et_3NH_2CO_3$; fraction size, 5 ml; flow rate, 1 ml/min. Pure d-p(anC-bzA-bzA-bzA-T) was obtained in fractions 234–250. The product was acetylated as described for **4**.

Analysis for "Nearest Neighbor." The degradation of ^{32}P- or ^{33}P-labeled joined strands for "nearest neighbor" analysis (Josse *et al.*, 1969) was done as follows. The reaction mixture (0.2 ml) contained 4 OD_{260} calf thymus DNA, 50 mM triethylammonium bicarbonate buffer (pH 8.7), 2 mM $CaCl_2$, and approximately 10,000 cpm of ^{32}P- or ^{33}P-labeled joined strand. The mixture was incubated 4 hr with 60 units of micrococcal nuclease. After incubation, the mixture was adjusted to pH 5.0 with 1 N acetic acid (≈ 5 μl) and 2 μl of 1 M

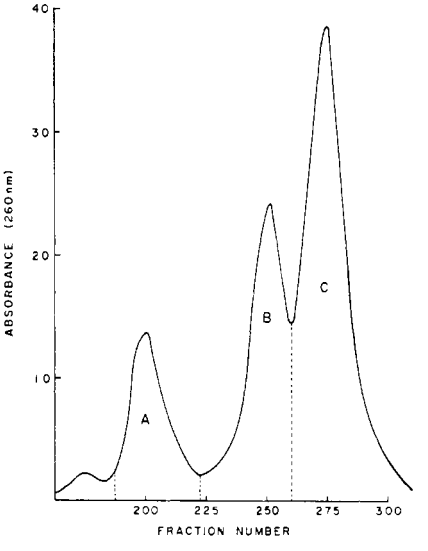


FIGURE 8: Preparation of **6**. Condensation, 0.044 mmol of **3**, 0.05 mmol of **5**, 0.55 mmol of $MesSO_2Cl$, 2 ml of pyridine, 1.5 hr. Work-up, 1.5 ml of water, 0.5 ml of $iPro_2EtN$; after storage overnight at 5°, apply to a Sephadex G-50 (superfine) column (5 × 100 cm), elute with 0.2 M $Et_3NH_2CO_3$; fraction size, 3.8 ml; flow rate, 1 ml/min. Peak A was rechromatographed on a DEAE-cellulose column (2.5 × 85 cm) (not shown); gradient, linear, 8 l. of 0.4 M $Et_3NH_2CO_3$ into 8 l. of 0.3 M; fraction size, 20 ml; flow rate, 1 ml/min. Fractions 340-420 contained pure **6**.

TABLE II: Identification of Chromatographic Peaks in Figures 3–8.

Figure	Peak A	Peak B	Peak C	Peak D
3	d-EtS-p(T-*i*buG)	d-p*i*buG		
4	$MesSO_3H$[a]	d-EtS-p(T-*i*buG)	d-p(T-anC)	d-EtS-p(T-*i*buG-T-anC) + d-p(T-anC)
5	d-p(T-T-T)	d-EtS-p(T-*i*buG-T-anC)	**3** + impurity	Pure **3**
6	dpT	Unknown	d-p(bzA-bzA)	d-p(bzA-bzA-T)
7	d-p(anC-bzA) + d-p(bzA-bzA-T)	Unknown	d-p(anC-bzA-bzA-bzA-T) + impurity	
8	**6** + impurity	**3**	Mostly **5**	

[a] $MesSO_3H$, mesitylenesulfonic acid.

TABLE III: Monomer Composition of Synthetic Intermediates of Fragment 3.

Intermediate	Mol % (Theory) A	C	G	T
d-EtS-p(T-G)			49.6 (50)	50.4 (50)
d-EtS-p(T-G-T-C)		25.2 (25)	24.4 (25)	50.4 (50)
d-EtS-p(T-G-T-C-T-T-T)		15.6 (14.3)	13.0 (14.3)	71.4 (71.4)
d-p(A-A-T)	63.8 (66.7)			36.2 (33.3)
d-p(C-A-A-A-T)	59.3 (60)	20.0 (20)		20.7 (20)
d-EtS-p(T-G-T-C-T-T-T-C-A-A-A-T)	25.1 (25)	17.0 (16.7)	6.5 (8.3)	51.4 (50)

potassium phosphate (pH 6.5) was added to inhibit phosphatases. The reaction was incubated with 1.65 units of spleen phosphodiesterase for 2 hr and stopped by heating to 100° for 2 min. The 3′-nucleotides were separated and examined for radioactivity.

Other Materials. The ATP used was obtained and purified as described earlier (Harvey and Wright, 1972). Crystalline bovine albumin was purchased from Schwarz–Mann. Agarose (Bio-Gel A, 0.5 m) was obtained from Bio-Rad Laboratories and columns prepared as recommended by the distributor.

Results and Discussion

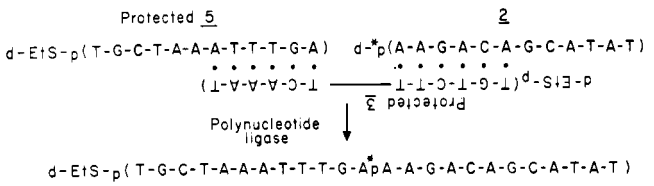
The studies here detailed were carried out in connection with a wider synthetic program of molecules containing information for defined peptide sequences. Specifically, the segments joined by ligase here constitute the "right" end of a "gene" coding for a modified *S*-peptide of ribonuclease A (Finn *et al.*, 1968). Figure 2 depicts the relationships between the chemically synthesized DNA fragments consisting of an (upper) nonsense and (lower) sense strand, the corresponding RNA sequence, and the cognate peptide chain. Chemical synthesis of deoxyribonucleotide oligomers is practical up to a point: when a size of 10–20 units is reached, enzymatic joining of such molecules becomes possible.

The synthesis of fragment 3 is shown in Figure 1. Figures 3–8 summarize the chromatographic purification of the reaction mixtures, and Table II identifies the peaks therein. The *S*-ethyl group was employed as the 5′-terminal blocking group; it was retained until completion of the chain and removed using iodine–water (see Cook *et al.*, 1972, for further details of this group). Oligonucleotides were analyzed (Table III) for their base content by ammonia hydrolysis followed by snake venom diesterase digestion and high-pressure liquid chromatography (Gabriel and Michalewsky, 1972). Paper chromatographic properties are summarized in Table IV.

TABLE IV: Paper Chromatography of Fragment 3 and Synthetic Intermediates.

Compound	Mobility (dpT = 1.0) for System[a] A	B	C
d-EtS-p(T-G)	0.98	1.18	1.20
d-EtS-p(T-G-T-C)	0.72	0.85	0.88
d-EtS-p(T-G-T-C-T-T-T)	0.36	0.52	0.65
d-p(A-A-T)	1.09	0.55	0.77
d-p(C-A-A-A-T)	0.96	0.30	0.52
d-EtS-p(T-G-T-C-T-T-T-C-A-A-A-T)	0.22		0.18
d-p(T-G-T-C-T-T-T-C-A-A-A-T)	0.18		0.09

[a] System A, isobutyric acid–concentrated ammonium hydroxide–water (57:4:39, v/v/v); B, ethanol–1 M ammonium acetate, pH 7 (1:1, v/v); C, 1-propanol–concentrated ammonium hydroxide–water (55:10:35, v/v/v).



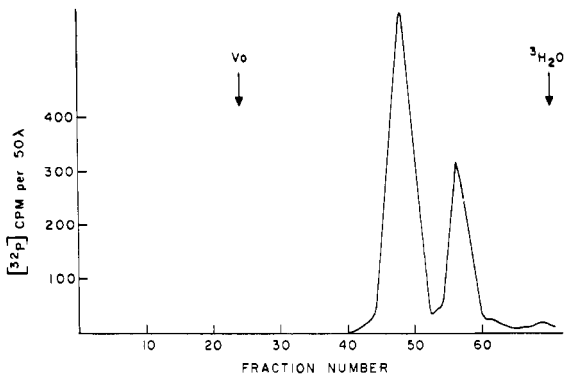
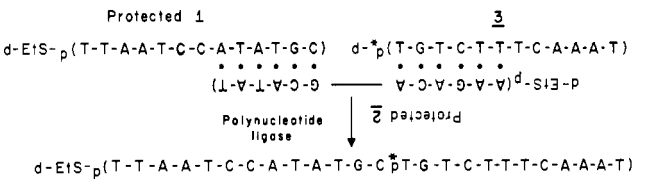


FIGURE 9: Separation of product after joining fragment 2 to protected fragment 5 in the presence of protected fragment 3. The ligase-joining reaction and details of separation by gel chromatography are given in the Experimental Section. The first peak to emerge is the joined material; the second represents the mixture of starting materials.



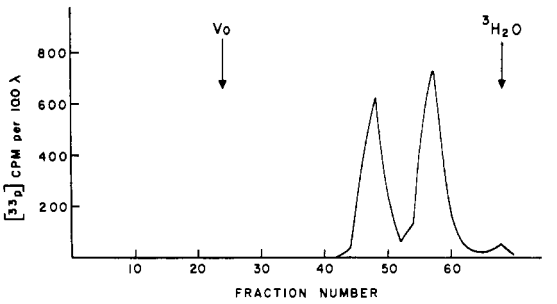


FIGURE 10: Separation of product after joining fragment 3 to protected fragment 1 in the presence of protected fragment 2. Details of joining reaction and gel chromatography are given in the Experimental Section. Peaks as in Figure 9.

Two joinings were carried out with polynucleotide ligase from bacteriophage T_4: fragment 2 (Figure 2) was joined to the protected fragment 5 under the template guidance of protected fragment 3, and fragment 3 was in turn joined to protected fragment 1 under similar control of protected fragment 2. The reactions were monitored by prior labeling of the donor components (fragments 2 and 3) with tracer phosphate at the 5′ terminus, and their incorporation into molecules of greater size was observed in gel chromatography (Figures 9 and 10). As expected, the peaks emerging in front of the input donor molecules had the phosphate label in phosphatase-resistant phosphodiester linkage.

Nearest neighbor analysis proved the specificity of the indicated joinings: the radioactive 3′-nucleotide produced from the micrococcal spleen digest of the joining of fragment 2 to protected fragment 5 was exclusively [^{32}P]dAp, whereas, in the case of joining fragment 3 to protected fragment 1, [^{33}P]dCp was obtained.

There are several problems connected with such biochemical condensations: in addition to the possible complications mentioned in the introductory statement, the fact that the three components of such joining reactions are of similar size makes their recovery difficult. Here the presence of the phosphorothioate termini may be helpful; aside from the fact that they modify the chromatographic behavior of oligomers by their lipophilicity, and that they inherently carry one charge less than the corresponding primary phosphates, their reaction toward a large variety of nucleophiles (Cook *et al.*, 1969) makes subsequent modification—including reaction with macromolecular species—possible and thus may also be helpful in recovery attempts.

In summary, the substrate specificity of polynucleotide ligase from bacteriophage T_4 has been extended to oligomers carrying phosphorothioate termini at those sites not involved in the generation of the new phosphodiester bond.

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Binding of Ethidium Bromide to Double-Stranded Ribonucleic Acid†

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ABSTRACT: The interaction of ethidium bromide with double-stranded RNA (*Penicillium chrysogenum*) has been investigated using spectroscopic, spectropolarimetric, hydrodynamic, and thermal melting techniques. The binding isotherms (Scatchard plots) are dependent on ionic strength. The apparent binding constants and number of binding sites are quite similar to those found for DNA under similar conditions (Waring, M. J. (1965a), *J. Mol. Biol. 13*, 269). Hydrodynamic studies of the dye–RNA complex show a 53% increase in its viscosity increment, a 13% decrease in its relative sedimentation coefficient, and a decrease in its buoyant density in Cs_2SO_4 as compared to RNA alone. Thermal melting studies show a marked increase in the T_m ($\Delta T_m = 26°$). Visible-region circular dichroic bands are induced when the dye is bound to RNA. These effects are also very similar to the results of studies on ethidium bromide–DNA complexes (Dalgleish, D. G., Peacoche, A. R., Fey, G., and Harvey, C. (1971), *Biopolymers 10*, 1853; LePecq, J. B., and Paoletti, C. (1967), *J. Mol. Biol. 27*, 87). Our data appear to indicate two modes of binding of the dye to RNA which are consistent with electrostatic and intercalative interactions.

Ethidium bromide is a dye which has been widely used in nucleic acid binding studies. As a drug, it has trypanocidal, antibacterial, and antiviral activities (Dickenson *et al.*, 1953; Newton, 1964). The dye inhibits DNA polymerase (Elliott, 1963) and DNA-dependent RNA polymerase (Waring, 1964). *In vitro* the dye binds to both RNA and DNA (Waring, 1965a).

Two main modes of binding to native DNA have been suggested based on the results of spectral and hydrodynamic studies. The primary and generally stronger mode of binding has been interpreted as "intercalation" where a part of the ethidium ion sandwiches between adjacent base pairs. Spectral shifts in the 480-mμ absorption band of the dye (Waring, 1965a) together with a decrease in sedimentation coefficient (LePecq and Paoletti, 1967) and an increase in viscosity (LePecq, 1965) with extent of binding occurs on formation of the complex. The hydrodynamic changes, indicative of lengthening of the DNA polymer, support the intercalation hypothesis. A decrease in buoyant density upon binding of the dye to DNA has also been observed (LePecq and Paoletti, 1967).

Hydrodynamic changes also occur in closed circular DNA in the presence of ethidium bromide. These changes can be related to changes in superhelical density due to intercalation (Crawford and Waring, 1967; Bauer and Vinograd, 1968). Recent electron microscopic studies show a 27% increase in molecular length for a linear DNA–ethidium bromide complex and a relief of superhelical twisting in closed circular DNA in the presence of ethidium bromide (Freifelder, 1971).

The second and generally weaker mode of binding is most evident at low salt and high ethidium bromide concentration. This mode is thought to be an electrostatic interaction between the phosphate groups in the double-stranded nucleic acid backbone and the dye molecules.

The same types of spectral effects have been observed when ethidium bromide binds to RNA. A number of RNAs of ill-defined secondary and tertiary structure have been studied including ribosomal (Waring, 1965a), "core" (Waring, 1965b) and tRNA (Bittman, 1969). LePecq and Paoletti (1967) postulated intercalative binding of ethidium bromide preferentially to helical regions in RNA. Waring (1965b) using spectral techniques studied binding of the dye to a group of synthetic polynucleotides. He was able to establish a relationship between the degree of secondary (helical) structure and the strength of primary binding. In these spectral studies on RNA and RNA-like polynucleotides, primary binding is considered synonymous with intercalation. However, this proposal must be viewed with some reservation since there is no supporting hydrodynamic evidence for these systems and the spectral effects in themselves are not sufficient to define a mode of binding.

In the present study the interaction of ethidium bromide with native double-stranded RNA (ds-RNA),[1] having secondary and tertiary structural characteristics and hydro-

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[1] Abbreviation used is: ds-RNA, double-stranded RNA.