Studies on Some Interactions and Reactions of Oligonucleotides in Aqueous Solution*

R. Naylor and P. T. Gilham

ABSTRACT: The interactions between thymidine and deoxyadenosine oligonucleotides of various chain lengths as measured by percentage hypochromicity have been studied in salt solutions at 0°. The amount of complexing was found to be dependent on the chain lengths of the two interacting species especially with those with chain lengths of less than eight nucleotides. However, no interaction could be detected with thymidine and deoxyadenosine oligonucleotides of chain lengths less than five and four nucleotides, respectively. The amount and types of interaction between thymidine penta- and hexanucleotides with polyadenylic acid have been investigated with a view to using the complexes formed to direct the synthesis of internucleotide linkages in aqueous solution. Water-soluble carbodiimides have been studied as reagents for the activation of terminal phosphate groups of oligonucleotides in aqueous solution. In model experiments these reagents were shown to be capable of effecting the rapid cyclization of nucleoside 2′(3′)-phosphates at pH 6 and to be capable of converting adenosine 5′-phosphate to its ethyl ester and to adenylyl-(5′→5′)-adenosine on reaction with the appropriate hydroxylic component. By using the complexes formed with polyadenylic acid to achieve favorable orientations of the oligonucleotides and by employing a water-soluble carbodiimide as an activating agent, thymidine penta- and hexanucleotides have been converted in aqueous solution to thymidine deca- and dodecanucleotides in yields of 3 and 5%, respectively.

The study of the possible interactions between short-chain polynucleotides in solution is of importance in the understanding of a number of facets of nucleic acid structure. For example, the structures of ribosomal and transfer ribonucleic acids appear to be stabilized by the interactions of relatively short sections of their chains while, in sequence analysis studies, the properties and characteristics of mixtures of oligonucleotides obtained by the degradation of nucleic acids are likely to be dependent on such interactions. Also, the capacity of oligonucleotides to form complexes with themselves requires some consideration in the development of methods for their fractionation. Zachau (1965) has found that such complex formation interferes with the chromatographic separation on Sephadex of oligonucleotide mixtures obtained by enzymic degradation of transfer ribonucleic acids, whereas the interaction between ribonuclease digests of ribonucleic acid with columns of thymidine oligonucleotides covalently linked to cellulose has been used to fractionate the components of such digests (Gilham, 1964; Gilham and Robinson, 1964).

A number of studies have been reported in which the interactions between oligonucleotides and long-chain polynucleotides have been measured. Lipsett *et al.* (1961) have investigated the interaction of adenosine oligonucleotides with polyuridylic acid and have shown that adenosine polymers as small as the dinucleotide were capable of complexing. The complexes formed between thymidine oligonucleotides and polyadenylic acid have been studied by Rich (1960) and those between guanosine oligonucleotides and polycytidylic acid by Lipsett (1964). While these studies have given some information on the complexing capacity of oligonucleotides it seemed likely that these complexes derived some of their stability by virtue of the fact that one of the interacting species was a long-chain polynucleotide. It is to be expected that a lower thermal stability would be exhibited by complexes formed by interactions between short-chain polymers only.

In a preliminary report (Gilham, 1962b) thymidine dodecanucleotide has been shown to form a complex with deoxyadenosine hexanucleotide. The melting curve (Figure 1), derived from the measurement of the optical density of the mixture at various temperatures, shows that the complex has a melting point, T_m, of 16° and that the complete formation of the complex results in 15% hypochromicity. The interaction is essentially instantaneous and the melting curve is reproducible, and independent of whether it is measured by increasing or decreasing the temperature. This complex appears to be composed of one molecule of the dodecanucleotide and two molecules of the hexanucleotide since the percentage hypochromicity did not increase following the addition of further quantities of either component. However, with a mixture of thymidine and deoxyadenosine hexanucleotides, essentially no complex could be detected above 0° by this

* From the Department of Biological Sciences, Purdue University, Lafayette, Indiana. *Received April 4, 1966.* Supported by grants from the National Science Foundation and the National Institutes of Health. Part of this work was carried out at the Institute for Enzyme Research, University of Wisconsin.

TABLE I: Percentage Hypochromicities of Mixtures of Thymidine and Deoxyadenosine Oligonucleotides.[a]

	pT(pT)$_2$pT	pT(pT)$_3$pT	pT(pT)$_4$pT	pT(pT)$_5$pT	pT(pT)$_6$pT	pT(pT)$_7$pT	pT(pT)$_8$pT
d-pApApA	0	0	0	0	0	0	0
d-pA(pA)$_2$pA	0	0	0	1	3	5	6
d-pA(pA)$_3$pA	0	0	0	5	11	13	12
d-pA(pA)$_4$pA	0	0	1	6	12	15	14
d-pA(pA)$_5$pA	0	0	4	10	13	15	15
d-pA(pA)$_6$pA	0	1	6	11	14	14	14
d-pA(pA)$_7$pA	0	2	8	12	13	14	14

[a] All mixtures contained equal molar concentrations of thymidine and deoxyadenosine bases with a total nucleoside concentration of 0.05–0.1 mM. The hypochromicity of each mixture was measured at 260 mμ at 0° in 1 M sodium chloride–0.01 M sodium phosphate, pH 6.3, and recorded as the percentage decrease, on mixing, of the sum of the optical densities of the two separate components at that temperature. Percentage hypochromicities at 257 and 266 mμ were similar to those at 260 mμ.

method. In order to study the dependence of complex formation on the size of polymer, the percentage hypochromicities were determined for mixtures of oligonucleotides of various chain lengths (Table I). The results show that the capacity to form complexes is dependent on the chain lengths of both interacting species. The amount of interaction is constant when the chain lengths of both species are greater than seven, while no interaction can be detected for mixtures containing thymidine or deoxyadenosine polymers with chain lengths less than five and four nucleotides, respectively.

For purposes of comparison the interactions between thymidine oligonucleotides and polyadenylic acid have also been measured. Table II lists the percentage hypochromicities observed in two different salt solutions and, here again, the amount of hypochromicity is constant for mixtures containing thymidine polymers

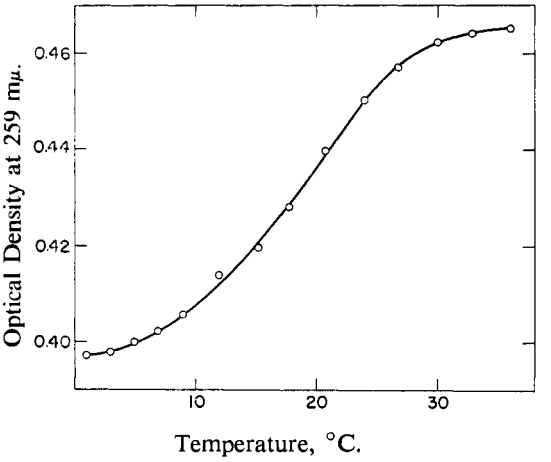


FIGURE 1: Temperature dependence of complex formation between thymidine dodecanucleotide and deoxyadenosine hexanucleotide. The mixture contained equal molar concentrations of thymidine and deoxyadenosine moieties dissolved in 1 M sodium chloride–0.01 M sodium phosphate, pH 7.

TABLE II: Hypochromicities of Mixtures of Thymidine Oligonucleotides and Polyadenylic Acid.[a]

	Chain Length of Thymidine Oligonucleotide						
	4	5	6	7	8	9	10
% hypochromicity[b]	0	4	16	18	19	19	18
% hypochromicity[c]	0	2	13	17	20	20	19

[a] All mixtures contained equal molar concentrations of thymidine and adenosine bases with a total nucleoside concentration of 0.05–0.1 mM. The hypochromicity of each mixture is recorded as the percentage decrease, on mixing, of the sum of the optical densities of the two separate components at the given temperature. [b] Measured at 260 mμ in 1 M sodium chloride–0.01 M sodium phosphate, pH 6.3, at 0°. [c] Measured at 262.5 mμ in 1 M lithium chloride–0.01 M cacodylate buffer, pH 6.9 at 0.5°.

of chain lengths greater than seven while for those with chain lengths less than five there is no observable interaction.

These results are in conflict with those obtained by Rich and Tinoco (1960) who reported that the smallest thymidine oligonucleotide to interact with polyadenylic acid under the conditions described in Table II (footnote *c*) was the heptanucleotide for which they calculated a hypochromicity value of 8%. Also, their hypochromicity values for the octa-, nona- and decanucleotides are lower than those reported here. The values listed in Table II do not fit the theoretical curve derived by Rich and Tinoco relating the hypochromism of a complex to the chain length of the oligonucleotide involved in that complex, and thus, in the molecular

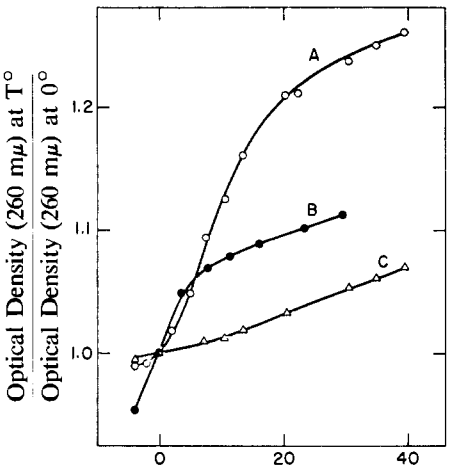


FIGURE 2: Temperature dependence of complex formation between polyadenylic acid and thymidine hexanucleotide (A) and between polyadenylic acid and thymidine pentanucleotide (B). The mixtures contained equal molar concentrations of the purine and pyrimidine moieties in 1 M sodium chloride–0.01 M sodium phosphate, pH 6.3, with a total base concentration of 0.07 mM. Curve C is derived from the addition of the curves obtained separately for polyadenylic acid and thymidine hexanucleotide in the same concentrations as those used for the respective polynucleotides in the above mixture.

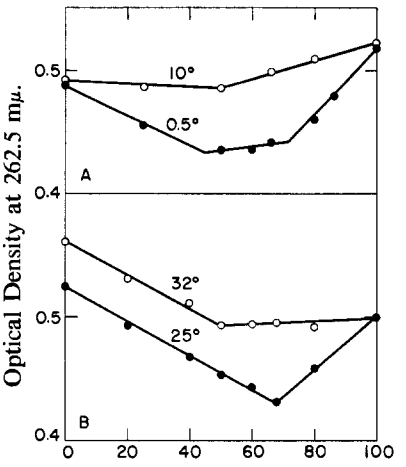


FIGURE 3: Optical density at 262.5 mμ of various mixtures of polyadenylic acid and thymidine hexanucleotide (A) and of polyadenylic acid and thymidine decanucleotide (B) in 1 M lithium chloride–0.01 M cacodylate buffer, pH 6.9, at different temperatures. The total base concentration (*ca.* 0.06 mM) remained the same for all points within each curve.

structure of the complexes formed between thymidine oligonucleotides and polyadenylic acid at 0.5°, their estimate of two to three unbound residues occurring at each end of each oligonucleotide may be too high. The thymidine oligonucleotides used in the study by Rich and Tinoco as well as the present one were obtained by the chemical polymerization of thymidine 5′-phosphate, and it is important that the fractions obtained by the DEAE-cellulose chromatography of the crude product be well characterized with respect to chain length and structure since some of the peaks appearing in the elution pattern of the chromatography represent products containing structures other than the normal linear polynucleotide arrangement.

The binding of the thymidine penta- and hexanucleotides with polyadenylic acid was studied further in order to make use of these complexes in attempts to effect condensation of oligonucleotides in aqueous solution. Figure 2 shows the temperature dependence of complex formation between these species. Curve C represents a relationship that would be obtained if there were no interaction between the two species (the rise in optical density with increase in temperature is due to the breakdown of the indigenous structure of polyadenylic acid). Curve A for the complexing of the hexanucleotide is parallel with curve C at 35–40° and thus this is the temperature at which the complex formation begins. From −2 to −4° there is no further decrease in the optical density and it is concluded that at these temperatures the complex formation is complete. By contrast, the interaction between the pentanucleotide and polyadenylic acid (curve B) begins at about 16° and is not yet complete at −4°.

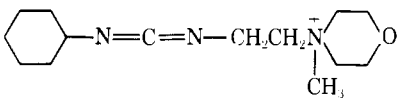
In order to obtain some information on the type of structures involved in these complexes a study was made of the decrease in optical density of mixtures containing different amounts of the interacting species (Figure 3). The mixing curves show that, at 10°, the thymidine hexanucleotide forms a 1:1 complex with polyadenylic acid while at 0.5° it appears that 1:1 or 2:1 complexes may be formed. For comparison the thymidine decanucleotide was shown to form a 1:1 complex at 32° and a 2:1 complex at 25°. There are a number of earlier observations reported on such 2:1 complexes involving oligonucleotides. Rich (1960) has observed that the complex between polythymidylic acid (chain length, 11–13) and polyadenylic acid at 24° changed from a 1:1 to a 2:1 complex with a change in salt concentration from 0.15 to 1.0 M, and Lipsett *et al.* (1961) have found that the addition of magnesium ions caused the formation of 2:1 complexes in the interaction of polyuridylic acid with adenosine oligonucleotides.

Condensation of Oligonucleotides in Aqueous Solution

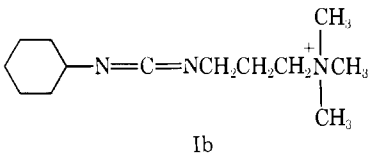
The synthesis of the internucleotide linkage in aqueous solution from a nucleoside phosphate and a nucleoside can be considered to require a succession of two reactions: the activation of the phosphate group followed by the condensation of this activated phosphate with the hydroxyl group of then ucleoside

moiety. It is to be expected that, in aqueous solution, the realization of the second step would present the most difficulty since the competition by water for the activated phosphate would preclude its condensation with the nucleosidic hydroxyl group. Thus the formation of a diester bond would be expected only if the phosphate and hydroxyl groups of the reacting species could be held in a close spatial arrangement.

In order to study the activation of phosphate groups in aqueous solution we have used the nucleoside 2′(3′)-phosphates where the sterically favorable position of the neighboring hydroxyl group permits the detection of the activation of the phosphate group by the formation of an internal phosphodiester linkage to yield the nucleoside cyclic phosphate. As activating agents we have investigated the use of diimides of the type, I, which by virtue of their quaternary ammonium groups are water soluble.



Ia

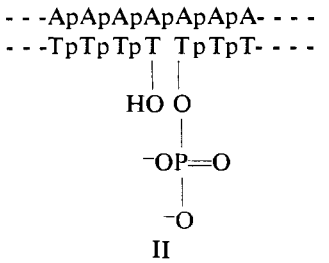


Ib

Sheehan and Hlavka (1956) have used reagent Ia for the activation of amino acids and the synthesis of peptide linkages, and the *N*-diethyl analog of Ib has been used in a study of the synthesis of nucleoside triphosphates in aqueous solution (Smith and Khorana, 1958). Reagent Ia has also been used to modify the action of enzymes used in the degradation of ribonucleic acid since, in solutions of pH values 8–9, it has been found to add to the bases of those nucleotides which have p*K* values in the vicinity of 9 (Gilham, 1962a; Lee *et al.*, 1965; Naylor *et al.*, 1965). The addition reaction is dependent on the presence of the anion of the nucleotide base and it is now shown that, in solutions of lower pH values, this reaction is inhibited and the reagent becomes capable of the selective activation of nucleotide phosphate groups. In the presence of an excess of Ia or b the four common nucleoside 2′(3′)-phosphates are converted at pH 6 to the corresponding cyclic phosphates in less than 1 hr. The course of the reaction can be followed by chromatography or by the amount of added hydrochloric acid required to maintain the pH at 6. The use of these reagents thus constitutes a simple method for the cyclization of 2′(3′)-phosphates of nucleosides or polynucleotides.

The rate of cyclization was studied at various pH values. The reaction was very slow at pH 8 and it increased markedly on lowering the pH, reaching a constant rate at about pH 5. These findings are consistent with a mechanism which involves the attack of the phosphate anion on the protonated diimide followed by a displacement at the phosphate adduct by the neighboring hydroxyl group. When equimolar amounts of the nucleotide and the reagent were used, yields greater than 75% of the cyclic phosphate were obtained, indicating that the neighboring hydroxyl group competes quite effectively with water in the attack on the activated phosphate group. The stability of the reagents in water at pH 6 was studied and the half-life of each reagent was found to be about 6 hr. When nucleoside 5′-phosphates were treated with Ia or b in aqueous solution no evidence of the synthesis of phosphodiester bonds could be detected by paper chromatography although the reaction of a mixture of adenosine and adenosine 5′-phosphate with the reagents gave a small yield of adenylyladenosine. This dinucleoside phosphate was shown to be the 5′→5′ isomer by virtue of its hydrolysis by snake venom phosphodiesterase to adenosine and adenosine 5′-phosphate and its resistance to hydrolysis by spleen phosphodiesterase or by alkali. Higher yields of phosphodiesters can be achieved by increasing the concentration of the hydroxylic component. For example, with a large excess of ethanol, adenosine 5′-phosphate is converted to its ethyl ester the yield of which depends on the ethanol concentration.

With these considerations it is obvious that, in order to effect the synthesis of the internucleotide linkage in aqueous solution, a method is required by which the hydroxyl group of one molecule can be held in close proximity to the phosphate group of another during the activation of the latter. It was expected that this arrangement could be realized in the molecular complexes described above, since, with the assumption of a head-to-tail "Watson–Crick" arrangement of the thymidine oligonucleotides along the polyadenylic acid strand (II), the 3′-hydroxyl



II

group of one thymidine polymer should lie in a favorable steric relationship to the terminal 5′-phosphate group of the adjacent polymer. Thus, the reaction of a mixture of thymidine hexanucleotide and polyadenylic acid with reagent Ib at −3° in 1 M sodium chloride gave thymidine dodecanucleotide in 5% yield. The product was identified by comparison with an authentic sample of the dodecanucleotide obtained by the polymerization of thymidine 5′-phosphate in anhydrous solution. The reaction appeared to be catalyzed by the complex formed by the two polynucleotides and appeared also to be a consequence of the activation of the terminal phosphate of the hexanucleotide since no dodecanucleotide was produced when either the polyadenylic acid or the reagent was omitted from the reaction mixture. Under the same reaction conditions

as above thymidine pentanucleotide, polyadenylic acid, and the activating reagent gave a small yield of thymidine decanucleotide.

The observation that oligonucleotides can be condensed under these conditions is consistent with the assumed head-to-tail arrangement of the molecules in the complexes discussed above; the small yield may be due to the lack of steric rigidity of the hydroxyl and phosphate groups resulting from the expected looser binding of the terminal nucleotide moieties. Alternatively, there may be spaces between some of the oligonucleotide molecules where the adenosine moieties of the polyadenylic acid remain unbound to the thymidine components, an arrangement which would be expected to prevent the condensation of the hydroxyl and phosphate groups. However, this approach of using polynucleotide interactions to effect specific condensations of oligonucleotides in aqueous solution may offer some incentive to the work aimed at the synthesis of larger polynucleotides and nucleic acids, and the use of the reagents described above may be of value in attempts to connect up segments of single chains or to repair single chain breaks in those natural polymers, such as deoxyribonucleic acid, which possess fairly rigid secondary structures.

Experimental Section

Materials. 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulfonate (reagent Ia *p*-toluenesulfonate) was purchased from Aldrich Chemical Co., Milwaukee, Wis. Reagent Ib was prepared by the general methods for preparing substituted carbodiimides described by Schmidt *et al.* (1958). 3-Dimethylaminopropylamine (28 g) dissolved in light petroleum ether (bp 40–60°) (28 ml) was added dropwise over a period of 2 hr to a stirred solution of cyclohexyl isothiocyanate (40 g) in light petroleum ether (28 ml). The mixture was then stirred at room temperature for a few hours and filtered. The solid product was washed with cold petroleum and dried over solid sodium hydroxide. The *N*-(3-dimethylaminopropyl)-*N'*-cyclohexylthiourea (63.7 g, 96%) had mp 63° after recrystallization from benzene–light petroleum ether mixture. The thiourea (70 g) was dissolved in dichloromethane (300 ml) and stirred at 0° while an alkaline sodium hypochlorite solution (2 M, 600 ml) was added dropwise during 1 hr. The mixture was allowed to come to room temperature and stirred for a further 3 hr. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic solutions were washed with a small amount of water and dried over sodium carbonate. The solvent was removed and the product was distilled in a short-path still at 50–55° (0.01 mm) to yield the carbodiimide as a yellow oil (39 g, 64%). A portion of this product (10 g) was dissolved in cold dry ether (10 ml), a solution of methyl *p*-toluenesulfonate (8.9 g) in dry ether (10 ml) was added, and the mixture was placed in an ice bath. The precipitated 1-cyclohexyl-3-(3-dimethylaminopropyl)carbodiimide metho-*p*-toluenesulfonate (Ib *p*-toluenesulfonate) was removed by filtration, washed with ether, and dried (16 g, 85%). *Anal.* Calcd for $C_{20}H_{33}N_3O_3S$: C, 60.75; H, 8.42; N, 10.62. Found: C, 60.88; H, 8.48; N, 10.45.

Polyadenylic acid (sedimentation constant: 8.6–11.6 in 0.1 M NaCl–0.05 M phosphate buffer, pH 7.5) was purchased from Miles Chemical Co., Elkhart, Ind. Thymidine oligonucleotides were prepared according to the method of Khorana and Vizsolyi (1961). Deoxyadenosine oligonucleotides were prepared by a modification (Gilham, 1964) of the method described by Ralph and Khorana (1961). For the determination of the molar extinction value per thymidine base, solutions of the thymidine oligonucleotides (1.2–1.6 ODU, at 266 mμ) in 0.1 M Tris–acetate buffer, pH 8.0 (1 ml), were treated with snake venom phosphodiesterase (0.02 mg, Worthington Biochemical Corp., Freehold, N. J.) at 25° in a spectrophotometer cell. In each case the reaction was followed by the increase in absorption at 266 mμ (measured against a blank containing the same solution without the oligonucleotide) until there was no further increase. The product, thymidine 5'-phosphate, was taken to have a molar extinction of 9550 at 266 mμ and the molar extinctions per thymidine moiety in the oligonucleotides were calculated from the percentage increase in optical density on hydrolysis; $\epsilon_{266/\text{thymidine}}$: pTpT, 9200; pT(pT)$_2$pT, 9100; pT(pT)$_4$pT, 9000; pT(pT)$_8$pT, 8900.

Optical density measurements were made with the Unicam SP 500 spectrophotometer using a 1-cm light path cell in a water-jacketed cell carriage. For the determination of percentage hypochromicities the various mixtures of oligonucleotides were kept at 0° for 12 hr before measurement of optical density and, for the determination of melting curves, the solutions were allowed to stand at each plotted temperature for 20 min before measurement. For the calculation of thymidine base concentration an extinction coefficient of 9000 at 266 mμ was used and the concentrations of deoxyadenosine residues were determined using an equivalent value (11,000 at 257 mμ) to that determined for the adenosine residues in adenosine oligonucleotides by Singer *et al.* (1962).

Chromatography. Paper chromatography was carried out by the descending method in the solvent systems: A, *n*-propyl alcohol (55 ml)–concentrated ammonia (10 ml)–water (35 ml); B, isopropyl alcohol (70 ml)–concentrated ammonia (10 ml)–water (20 ml); C, ethyl alcohol (70 ml)–1 M ammonium acetate, pH 7.5 (30 ml); D, *n*-propyl alcohol (90 ml)–concentrated ammonia (20 ml)–water (70 ml).

Reaction of Nucleoside 2'(3')-Phosphates with Water-Soluble Carbodiimides. Disodium uridine 2'(3')-phosphate (38.6 mg, 0.1 mmole) was dissolved in water (3 ml) and the pH was adjusted to 5.8. Reagent Ia or Ib (0.5 mmole) was added to the solution at 25° and the pH was kept at 5.8 by the addition of 0.1 N hydrochloric acid. No further addition of acid was necessary after 1 hr. The mixture was allowed to stand for 4 hr and applied to Whatman 3MM paper (16 in.) and chromatographed in solvent A or B. The yield of the

cyclic phosphate was quantitative and the single band of product was cut out and eluted. The isolation of the product was more facile if, before chromatography, the reaction mixture was passed through a column (12 × 2 cm) of Dowex 50 (NH_4^+) ion-exchange resin to remove the carbodiimide and its corresponding urea product. Relevant R_F values in solvent A were: uridine 2′(3′)-phosphate, 0.42; uridine 2′,3′-cyclic phosphate, 0.56; *p*-toluenesulfonate, 0.80. The product was completely converted to uridine 3′-phosphate by the action of pancreatic ribonuclease. Similar results were obtained for the cyclization of the other three nucleoside 2′(3′)-phosphates. In similar reactions at different pH values the yields of cyclic phosphate in the first 2 min were: 3% at pH 7.5; 8% at pH 7; 41% at pH 6; 63% at pH 5; and 68% at pH 4. With a restricted amount of either carbodiimide (0.1 mmole), each nucleoside phosphate (0.1 mmole) in water (2 ml, maintained at pH 6.5) gave 75–80% yields of the corresponding cyclic phosphate. The yields were quantitative, however, when 2 equiv of either reagent were used.

Reaction of Adenosine 5′-Phosphate with Ethanol. A solution of the sodium salt of adenosine 5′-phosphate (25 μmoles) and ethanol (25 μl, 0.43 mmole) in water (0.275 ml) was treated with reagent Ib (50 mg, 0.125 mmole) at pH 6.0 and 25°. After 24 hr the mixture was chromatographed on Whatman No. 1 paper with solvent C, the two components being adenosine 5′-phosphate (80%, R_F 0.29) and adenosine 5′-ethyl phosphate (20%, R_F 0.66). For identification, the ethyl ester was shown to be unchanged after treatment with alkaline phosphatase and to be degraded to adenosine 5′-phosphate by snake venom phosphodiesterase. The product had the expected electrophoretic mobilities relative to adenosine 5′-phosphate of 1.0 at pH 3.2 and 0.45 at pH 7.0. Under similar reaction conditions with ethanol (50 μl, 0.86 mmole)–water (0.25 ml) the yield of the ethyl ester was 36% and with ethanol (75 μl, 1.29 mmole)–water (0.225 ml) the yield was 53%.

Reaction of Adenosine 5′-Phosphate with Adenosine. A solution of adenosine 5′-phosphate (0.1 mmole) and adenosine (27 mg, 0.1 mmole) in water (1 ml) was treated with reagent Ib (200 mg, 0.5 mmole) at pH 6.0 and 25°. After 24 hr the mixture was chromatographed on Whatman 3MM paper in solvent B. The yield of adenylyl-(5′→5′)-adenosine (R_F 0.2) was 25 ODU at 260 mμ (1%). On degradation with snake venom phosphodiesterase the product gave equal quantities of adenosine 5′-phosphate and adenosine but was unaffected by spleen phosphodiesterase or by treatment with 0.2 M sodium hydroxide.

Stability of Water-Soluble Carbodiimides in Aqueous Solution. A solution of reagent Ib (80 mg, 0.2 mmole) in water (2 ml) was adjusted to pH 6.0 and kept at this pH and at 25° for 24 hr. At various times an aliquot (0.2 ml) was withdrawn and added to a solution of sodium cytidine 2′(3′)-phosphate (0.02 mmole) in 0.2 ml of water at pH 6. This mixture was then kept at pH 6–7 for 12 hr and then analyzed by chromatography on Whatman No. 1 paper in solvent C to determine the percentage conversion of the cytidine phosphate (R_F 0.38) to the cyclic phosphate (R_F 0.64). Time of sample (hr): 0, 1, 3, 6.25, 10.5, and 24; % cyclic phosphate formed: 74, 63, 50, 36, 22, and 8. Thus, the half-life of reagent Ib in water at pH 6.0 was about 6 hr. Similar results were obtained for the stability of reagent Ia.

Condensation of Thymidine Oligonucleotides in Aqueous Solution. Thymidine hexanucleotide sodium salt (100 ODU at 266 mμ) and polyadenylic acid sodium salt (100 ODU at 257 mμ) were dissolved in 1 M sodium chloride (1 ml). The solution was cooled to −3° and its pH was adjusted to 6.0 with dilute hydrochloric acid. Reagent Ib (50 mg) was added and the solution kept at −3° and pH 6.0 for 2 days. A further 20 mg of the reagent was added and after an additional 2 days under the same conditions the solution was diluted with water (3 ml) and passed through a column (10 × 0.8 cm) of Dowex 50 (Na^+) ion-exchange resin. The effluent was dialyzed against water for 6 hr and concentrated. Chromatography of the mixture on Whatman 3MM paper (4 in.) with solvent D gave four bands. The R_F values of the bands relative to the R_F value of thymidine 5′-phosphate were A, 0; B, 0.38; C, 0.68; and D, 0.89 (trace). The bands A, B, and C corresponded in their R_F values to polyadenylic acid, thymidine dodecanucleotide, and thymidine hexanucleotide, respectively. The band B (5 ODU at 266 mμ) had λ_{max} 266 mμ. A portion of this product was dephosphorylated with alkaline phosphatase and the product compared with that obtained from the dephosphorylation of authentic thymidine dodecanucleotide by chromatography on Whatman No. 4 paper with solvent A (Table III). For terminal phosphate determination a small amount of band B was applied to a small area of Whatman No. 1 paper and chromatographed for 2 days with solvent A. The spot was cut out and eluted and 1.22 ODU (266 mμ) was treated with alkaline phosphatase (0.04 mg) in 0.02 M Tris–chloride buffer, pH 8 (0.2 ml), for 2 hr at 25°. The released phosphate, determined by the method of Chen *et al.* (1956), was 0.1 μmole/ODU (266 mμ). An authentic sample of $pT(pT)_{10}pT$, under the same conditions, produced 0.095 μmole/ODU (266 mμ).

TABLE III: Chromatography Data.

	Distance Moved (cm)
Band B	22.0
Band B dephosphorylated	26.5
$pT(pT)_{10}pT$	21.3
$T(pT)_{10}pT$	26.5
$pT(pT)_4pT$	29.3

In two control experiments, in which either the polyadenylic acid or the reagent was omitted, none of the band B could be detected. Under similar reaction conditions thymidine pentanucleotide gave a product (3 ODU at 266 mμ, R_F 0.16 on Whatman No. 4 paper in solvent A) which was identical with an authentic sample of thymidine decanucleotide. The polymer, on reaction with alkaline phosphatase as above, gave the dephosphorylated product, R_F 0.29 (Whatman No. 4 paper, solvent A) identical with the R_F value of $T(pT)_8pT$. This product was treated with snake venom phosphodiesterase (25 μg) in 0.05 M Tris–chloride buffer, pH 8.0 (0.2 ml), for 18 hr at 25°. The products of the enzyme digestion were separated by chromatography in solvent A and a value of 7.1 was obtained for the ratio of thymidine 5′-phosphate to thymidine produced. The enzyme digestion of authentic $T(pT)_8pT$ gave a corresponding value of 7.6.

References

Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28*, 1756.

Gilham, P. T. (1962a), *J. Am. Chem. Soc. 84*, 687.

Gilham, P. T. (1962b), *J. Am. Chem. Soc. 84*, 1311.

Gilham, P. T. (1964), *J. Am. Chem. Soc. 86*, 4982.

Gilham, P. T., and Robinson, W. E. (1964), *J. Am. Chem. Soc. 86*, 4985.

Khorana, H. G., and Vizsolyi, J. P. (1961), *J. Am. Chem. Soc. 83*, 675.

Lee, J. C., Ho, N. W. Y., and Gilham, P. T. (1965), *Biochim. Biophys. Acta 95*, 503.

Lipsett, M. N. (1964), *J. Biol. Chem. 239*, 1256.

Lipsett, M. N., Heppel, L. A., and Bradley, D. F. (1961), *J. Biol. Chem. 236*, 857.

Naylor, R., Ho, N. W. Y., and Gilham, P. T. (1965), *J. Am. Chem. Soc. 87*, 4209.

Ralph, R. K., and Khorana, H. G. (1961), *J. Am. Chem. Soc. 83*, 2926.

Rich, A. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1044.

Rich, A., and Tinoco, I. (1960), *J. Am. Chem. Soc. 82*, 6409.

Schmidt, E., Ross, D., Kittl, J., and Düsel, H. H. von (1958), *Ann. 612*, 11.

Sheehan, J. C., and Hlavka, J. J. (1956), *J. Org. Chem. 21*, 439.

Singer, M. F., Heppel, L. A., Rushizky, G. W., and Sober, H. A. (1962), *Biochim. Biophys. Acta 61*, 474.

Smith, M., and Khorana, H. G. (1958), *J. Am. Chem. Soc. 80*, 1141.

Zachau, H. G. (1965), *Biochim. Biophys. Acta 108*, 355.