Use of phosphorus oxychloride in synthesizing nucleotides and oligonucleotides

W.S. Mungall, G.L. Greene, P.S. Miller, and R.L. Letsinger

Department of Chemistry, Northwestern University, Evanston, Illinois 60201, USA

Received 27 February 1974

ABSTRACT

Procedures are described for phosphorylating protected nucleotides, oligonucleotides and phosphoramidate oligonucleotide derivatives at the 3'-hydroxyl group. The conditions (phosphorylation with phosphorus oxychloride and pyridine in dioxane followed by hydrolysis with aqueous pyridine) are sufficiently mild that base labile (trifluoroacetyl amino; β -cyanoethyl phosphotriester) and acid labile (O-monomethoxytrityl; phosphoramidate) functions are retained intact. Application of the technique is illustrated by the synthesis of dpT, dTp, d(CF₃CONH)Tp, dTpNTP, and dTpNTP_NTP. In addition, the utilization of phosphorus oxychloride in joining thymidine derivatives and dinucleoside phosphotriester blocks via phosphodiester links is described.

INTRODUCTION

Phosphorus oxychloride has been used for phosphorylating hydroxyl groups for many years². In pyridine, which has commonly been employed as a solvent, it is quite active; however, it generally gives undesirable mixtures of products in reactions with nucleosides. The degree of discrimination between 5' (primary) and 3' (secondary) hydroxyl groups is low³, phosphodiesters may be formed in addition to phosphomonoesters⁴, and pyrophosphates may be produced when the intermediate phosphorodichloridate is hydrolyzed⁵. For these reasons phosphorus oxychloride has been largely replaced in applications in the nucleotide field by derivatives of the type ROP(O)Cl₂ and (RO)₂POCl, where RO- is a group that can be selectively converted to HO- subsequent to the phosphorylation reaction⁶.

Interest in phosphorus oxychloride as a phosphorylating reagent was recently revived by the observation that in trimethyl or triethyl phosphate as a solvent it preferentially attacks the 5'-OH of nucleosides, affording a high

yield of the nucleoside 5'-phosphate on hydrolysis⁷. We report in the present paper another set of conditions under which phosphorus oxychloride can be utilized to advantage in the preparation of nucleotides, namely, in an unreactive solvent (dioxane) in the presence of a limited amount of pyridine or lutidine at room temperature⁸. This work was stimulated by a need for a convenient method to introduce a phosphoryl group at the 3'-oxygen terminus of an oligonucleotide or a nucleoside that possessed protecting groups sensitive to basic and acidic reagents. Agents such as β -cyanoethyl phosphate⁹ and bis-(p-nitrophenyl)phosphorochloridate¹⁰ were precluded because of the alkaline conditions needed to remove the β -cyanoethyl and phenyl protecting groups.

RESULTS

Experiments with thymidine and 5'-O-tritylthymidine showed that the reaction of phosphorus oxychloride can be satisfactorily controlled. On treatment with three equivalents each of phosphorus oxychloride and pyridine in dioxane for half an hour at room temperature followed by addition of aqueous pyridine, thymidine afforded thymidine 5'-phosphate (>90%). No 3'-phosphate was detected by paper chromatography or by use of 5'-nucleotidase, an enzyme that hydrolyzes 5' but not 3'-phosphates. As in the phosphorylation in trialkylphosphates⁷ and in several other reactions of nucleosides¹¹, the hydroxyl group at the 5' position proved to be much more reactive than that at the 3'-position. An experiment with 5'-O-tritylthymidine showed, however, that the 3'-hydroxyl group can be phosphorylated successfully (81% of dTp obtained) with the same reagent by extending the reaction time.

This phosphorylation technique was used to advantage in the conversion of 5'-trifluoroacetyl-amino-5'-deoxythymidine (1) to the corresponding 3'-phosphate (2), an intermediate desired in connection with some synthetic studies of oligonucleotide analogs. The N-trifluoroacetyl group is quite sensitive to alkali; however, it held up satisfactorily during the phosphorylation reaction and the subsequent hydrolysis with aqueous pyridine. Compound 2 was purified by elution from DEAE-Sephadex with aqueous ammonium bicarbonate. Some loss of the trifluoroacetyl group occurred during this step;

even so, 2 was obtained as the pyridinium salt in 50% yield.

Phosphorus oxychloride also served well for phosphorylating the 3'-hydroxyl group of the protected phosphoramidate analogs, 3 and 4. Following hydrolysis of the intermediate phosphorodichloridate and cleavage of the protecting groups, the corresponding dinucleotide and trinucleotide phosphoramidate analogs, 5 and 6, were obtained in good yields (>74%)

An example of the utility of phosphorus oxychloride in joining protected oligonucleotide blocks is provided by the synthesis of dTpTpTpT (9) from compounds 7 and 8. For this, 7 was treated successively with phosphorus oxychloride and water to give the corresponding 3'-phosphate, which in turn was coupled to 8 (1.5 equivalents) by activation with triisopropylbenzene-sulfonyl chloride. The relatively good yield, 74%, of the tetranucleoside triphosphate (9) obtained after removing the protecting groups shows that a phosphodiester condensation can be employed effectively in joining oligonucleotide blocks synthesized via the phosphotriester approach.

In connection with this synthesis it was of interest to see if blocks such as 7 and 8 could be joined directly with phosphorus oxychloride without hydrolysis of the intermediate phosphorodichloridate and subsequent reactivation with an arenesulfonyl chloride. Results from a model study in which thymidine units were joined were encouraging. The steps were: (1) phosphorylation of 5'-O-(p-methoxytrityl)thymidine with excess phosphorus oxychloride and pyridine in dioxane, (2) removal of excess phosphorus oxychloride by distillation with dioxane, (3) condensation of the intermediate ROPOCl_2 with a 3'-O-protected thymidine (3'-O- β -benzoylpropionylthymidine)

and (4) hydrolysis with aqueous pyridine. dTpT was obtained in 78% yield. The method is rapid; however, care must be used to remove all the excess phosphorus oxychloride, which can lead to formation of 5'-5' phosphodiester derivatives when the 3'-O-protected nucleoside is added. When applied to the coupling of 7 and 8, however, this procedure was much less satisfactory than that proceeding via the intermediate 3'-phosphate. The yield was lower (34% of dTpTpTpT from a 4 hr. coupling reaction), and the product contained some material sensitive to snake venom phosphodiesterase but resistant to spleen phosphodiesterase (probably an isomer resulting from reaction of residual phosphorus oxychloride with 8).

EXPERIMENTAL SECTION

Nucleotidic reactants were dried by evaporation of several portions of pyridine at less than 30° at reduced pressure. Air readmitted to the reaction vessel was first passed through a column (50 x 1 cm) of anhydrous magnesium perchlorate. Reactions were run at room temperature in septum sealed flasks, and solvents and liquid reagents were transferred by the use of dry syringes.

Whatman 3MM paper was used for paper chromatography. The solvent systems were prepared on a volume basis: solvent A, i -C₃H₇OH-NH₄OH-H₂O (7:1:2); solvent C, 1M NH₄OAc-C₂H₅OH (3:7, adjusted to pH 7.5); solvent D, n -C₃H₇OH-2M HCl(3:1); solvent E, C₂H₅OH-0.5M NH₄OAc (7:3, pH 3.5); solvent F, n -C₃H₇OH-NH₄OH-H₂O (55:10:35). R_f^F refers to R_f values obtained with solvent F. Paper electrophoresis was performed using Whatman 3MM paper in a Savant flat plate electrophoretic chamber and a Savant Model HV 2,000 volt power source operated at 2,000 volts. Mobility relative to thymidine 5'-phosphate is expressed as R_m^{dpT} values. Thin layer chromatography (tlc) was conducted on Eastman silica gel 6060 sheets with fluorescent indicator. Preparative TLC was carried out using ChromAR 1000 sheets (20 x 20 cm) purchased from Mallinckrodt Chemical Co. Chromatographic grade silica gel (No. 3405 Baker Chemical Co.) was used for column chromatography. Elemental analyses were performed by the Micro-Tech Laboratories, Skokie, Illinois.

For quantitative determination of products isolated by paper chromatography, the bands were cut out and eluted with water. Blanks correspond-

ing to each band were used to correct for any uv absorbing material eluted from the paper. The term A_{267} unit refers to the extinction of 1 ml of a nucleotidic solution using a quartz cell with a 1 cm light path at 267 nm wavelength. For the purpose of calculating yields, the extinction coefficient of products containing 1, 2, 3, and 4 thymidine units are taken to be 9600, 18500, 25800, and 34000, respectively¹². Assays of the natural oligonucleotides with spleen and snake venom phosphodiesterase were carried out as previously described¹³; for the phosphoramidate analogs a modified procedure was used¹⁴.

5'-Trifluoroacetyl-amino-5'-deoxythymidine (1). 5'-Amino-5'-deoxythymidine (2.0 g; 8.3 mmol) was dissolved in 20 ml of anhydrous pyridine and treated with triethylamine (2.0 g; 10 mmol) and trifluoroacetic anhydride (2.1 g; 10 mmol) for 60 min at room temperature. The solvent was removed in vacuo to give a light yellow solid. Trituration of the solid with two 10-ml portions of water afforded 2.4 g (86%) of cream-colored crystals, mp 252-254°. The product was homogeneous on tlc in tetrahydrofuran (R_f 0.53) and was used without further purification. An analytical sample, mp 253-255°, was obtained by crystallization from hot ethanol; $\lambda_{\max}(\text{EtOH})$ 267 nm (ϵ 9500), $\lambda_{\min}$ 234 nm; principal ir bands at 2.9, 5.8, 5.9, 6.0, 6.4, 6.7, 7.7 and 7.8 μ .

Anal. Calcd for $\text{C}_{12}\text{H}_{14}\text{F}_3\text{N}_3\text{O}_5$:C, 42.74; H, 4.18; N, 12.46. Found: C, 42.59; H, 4.27; N, 12.48.

Thymidine 5'-phosphate, dpT. - Phosphorus oxychloride (0.028 ml, 0.30 mmol) was added to a solution of 24.2 mg (0.10 mmol) of thymidine and 0.024 ml (0.30 mmol) of pyridine in 7 ml of dioxane. The solution was stirred for 35 min; then 2 ml of 50% aqueous pyridine was added. The mixture was stirred overnight, concentrated to less than 1 ml, and diluted with H_2O to 10 ml. Chromatography of an 0.1 ml aliquot on paper with solvent F revealed 4 bands of nucleotidic material: R_f 0.34 (trace), R_f 0.49 (dark), R_f 0.65 (trace), and R_f 0.76 (trace). The thymidylic acid at R_f 0.49 amounted to 9.04 A_{267} units (94%). It was homogeneous on paper chromatography in solvent D (R_f 0.75), which separates 3'- and 5'-thymidylic

acids (controls run on the same paper gave R_f 0.75 for dpT and R_f 0.83 for dTp). That phosphorylation occurred at the 5'-oxygen was confirmed by hydrolysis of the product with 5'-nucleotidase (Sigma Chemical Co., 18 units/mg protein). The procedure was that of Shaller *et al*^{11d}. This enzyme converted the product quantitatively to thymidine (R_f^A 0.66). In a control reaction, it was shown that dTp was not attacked under the same conditions as judged by the paper chromatographic assay.

Thymidine 3'-phosphate, dTp. Dry 5'-O-tritylthymidine (48.4 mg, 0.10 mmol) in 5 ml of dioxane was similarly treated with pyridine (0.024 ml, 0.30 mmol) and phosphorus oxychloride (0.028 ml, 0.30 mmol) for 21 hr at room temperature. Following addition of 2 ml of 50% aqueous pyridine, the solution was stirred for 6 hr and then concentrated at reduced pressure to a gum. The residue was treated with 5 ml of acetic acid-water (4:1) at 100° for 25 min, the acetic acid was evaporated, 5 ml of water was added, the mixture was extracted with two 10 ml portions of ether, and the aqueous phase was diluted to 10 ml. On chromatography of a 0.10 ml aliquot in solvent F two uv absorbing bands centered at R_f 0.52 (dTp; strong band) and R_f 0.76 (dT; faint) were observed. The dTp isolated amounted to 7.8 A_{267} units (81% yield). It was homogeneous on paper chromatography in Solvent D (R_f 0.83), and it ran the same as a standard sample of dTp and differently from a standard sample of dpT (R_f 0.75) on the same paper. The product was also completely resistant to the 5'-nucleotidase enzyme under the standard conditions.

5'-Trifluoroacetyl-amino-5'-deoxythymidine 3'-monophosphate (2). 5'-Trifluoroacetyl-amino-5'-deoxythymidine (1.23 g; 3.6 mmol) was dried by evaporation from several portions of anhydrous pyridine. The residue was suspended in 40 ml of dry dioxane and treated with phosphorus oxychloride (1.0 ml; 11 mmol) and pyridine (0.90 ml; 11 mmol) for 90 min at room temperature. After concentration of the reaction mixture to a gum under reduced pressure, benzene was added and the mixture was concentrated again to remove residual phosphorus oxychloride. The residue was treated with 20 ml of cold, 50% aqueous pyridine for 45 min. Thin-layer chromatography in tetrahydrofuran showed that the phosphorylation was complete, as judged by

the disappearance of the starting nucleoside (R_f 0.53) and the appearance of a new uv-absorbing material at the origin.

The aqueous pyridine solution was concentrated to a gum and the residue was evaporated from two portions of water to remove pyridine. The resulting gum was then dissolved in 25 ml of water and the pH of the solution was adjusted to 7.2 with concentrated ammonium hydroxide. Aqueous barium acetate (8 ml of a 2 M solution) was added and the mixture was stirred for several hr at room temperature. The barium phosphate was removed by centrifugation and washed with three 10-ml portions of water. After adjusting the pH of the combined aqueous portions to 7.5 with concentrated ammonium hydroxide, one-half of the solution was applied to a DEAE Sephadex column (4.3 x 50 cm). The column was eluted first with water (1.4 l) and then with a linear gradient of water (2 l) and 0.40 M ammonium bicarbonate (2 l; pH 7.8). Fractions (22 ml each) were collected at a rate of ten per hr. Several uv-absorbing bands were eluted; fractions 197-216 contained the desired product, 2. It was isolated as the ammonium salt by repeated lyophilization. An aqueous solution of the product was then passed through a column of Dowex 50 resin (acid form; 2.2 x 38 cm) to convert the salt to the free acid and remove a trace amount of $d(NH_2)Tp$. The intensity of the uv spectrum (λ_{max} 267 nm; λ_{min} 234 nm) indicated a total yield of 8600 A_{267} units (50% yield) of 2. Removal of the solvent in vacuo and repeated concentration from pyridine afforded white, hygroscopic crystals of the pyridinium salt of the product. The product was homogeneous by paper chromatography (solvent A; R_f^A 0.40; some streaking occurred due to hydrolysis of the trifluoroacetyl group) and by paper electrophoresis at pH 7.2 (R_m^{dpT} 0.90). Analysis for phosphorus by the method of Chen and Toribara¹⁵ gave a phosphorus:thymine ratio of 1.0:1.0. The nmr spectrum at 60 MHz in D_2O was very similar to that of thymidine, but with two extra groups of bands attributable to the pyridinium ion downfield from 7.8 δ . Integration of the resonances for the pyridinium ions and the methyl group of thymine (2.0 δ) showed 1.6 pyridinium ions per methyl, indicating that some pyridine was removed from the nucleotide salt during isolation (e.g., during drying in vacuo). For analysis the nucleotide was converted to the barium salt by

reaction with barium acetate in water followed by precipitation with acetone, filtration, and drying in vacuo at room temperature.

Anal. Calcd for $\text{BaC}_{12}\text{F}_3\text{H}_{13}\text{N}_3\text{O}_5\text{P}$: C, 26.08; H, 2.37; N, 7.60. Found: C, 26.25; H, 2.63; N, 7.60.

For further characterization, 6.5 A_{267} units of 2 (pyridinium salt) was allowed to stand in a small volume of concentrated ammonium hydroxide for 1 hr at room temperature. The solution was then concentrated and chromatographed in solvent A. One uv-absorbing band was observed, R_f^A 0.09 (ninhydrin positive). The band was eluted with water to yield 5'-amino-5'-deoxythymidine-3'-phosphate; λ_{max} 266 nm; 5.5 A_{266} units (87% recovery); homogeneous on paper electrophoresis, R_m^{dpT} 0.45.

Thymidylyl(3'-5')-5'-amino-5'-deoxythymidine 3'-phosphate ($dT_{\text{pN}}T_{\text{p}}$)
(5) 5'-O-Isobutyloxycarbonylthymidylyl-(3'-5')-5'-amino-5'-deoxythymidine phenyl ester¹⁴ (3) (216 mg, 0.30 mmol), dried by the evaporation of three 1 ml portions of pyridine, was dissolved in dioxane (8 ml) and treated with 2, 6-lutidine (0.10 ml, 0.9 mmol) and phosphorus oxychloride (0.08 ml, 0.9 mmol). After 15 hr at room temperature the reaction flask was cooled in an ice bath and 10 ml of ice cold 50% aqueous pyridine was added. After an additional 3 hours 2.4 ml of cold 2 M sodium hydroxide was added to neutralize the acid present and the solution was concentrated under reduced pressure. Pyridine was periodically added during the concentration process. The resulting syrup was treated with 0.1 M sodium hydroxide (25 ml) in 50% aqueous dioxane. Precipitated salt was removed by filtration and an additional 25 ml portion of base was added. After 7 hr the orange solution was neutralized with a minimum of pyridinium Dowex-50 resin (3 ml). The resin was removed by filtration and washed. The combined filtrate and washings were concentrated under vacuum. The concentrated solution was chromatographed on a DEAE-cellulose column¹⁶ (2.5 x 34 cm) by elution first with 10^{-3} M ammonium bicarbonate (600 ml) and then with a 4 l linear gradient of ammonium bicarbonate ranging from 10^{-3} M to 0.2 M in ethanol-water (1:9) at pH 8. Fractions of 13 ml volume were collected after the gradient was started. Fractions 13-34 contained non-nucleotidic

uv absorbing material. The desired product was eluted in fractions 48-115; R_f^F 0.39 (homogeneous). The pooled fractions were concentrated under reduced pressure and lyophilized. The resulting white to pale-yellow powders were dissolved in water and lyophilized again, and this process was repeated several times until a constant weight was attained. A total of 4100 A_{267} units (74% yield) of dT_{pNT_p} was obtained. In agreement with this structure, the product was converted quantitatively to dT_{pNT}^F (R_f^F 0.58) on treatment with alkaline phosphatase under the standard conditions. In addition, on hydrolysis with 80% acetic acid-water, it yielded equimolar amounts of dT_p (R_f^A 0.11, R_m^{dpT} 1.00) and $d(NH_2)T_p$ (R_f^A 0.05; R_m^{dpT} 0.45).

Thymidylyl-(3'-5')-5'-amino-5'-deoxythymidylyl-(3'-5')-5'-amino-deoxythymidine 3'-phosphate ($dT_{pNT_pNT_p}$) (6). Compound 4 (203 mg, 0.18 mmol) was phosphorylated at 15° with 0.945 ml (0.5 mmol) of phosphorus oxychloride by the procedure described for the preparation of 5. For isolation of the tetranucleotide analog, 4/5 of the product mixture was applied to a DEAE-cellulose column (2.5 x 35 cm) and eluted with an ammonium bicarbonate buffer, as described for dT_{pNT_p} . Fractions of 19 ml were collected. $dT_{pNT_pNT_p}$, 3700 A_{267} units (99%), was obtained in fractions 175-191. It was homogeneous on paper chromatography in Solvent F (R_f^F 0.31). Hydrolysis with 80% aqueous acetic acid for 20 minutes at ~90° yielded dT_p and $d(NH_2)T_p$ in a 1:2 ratio. On treatment with alkaline phosphomonoesterase (Worthington Biochemicals, BAPSF) it was converted cleanly to a single nucleotide product, which behaved correctly chromatographically for $dT_{pNT_pNT}^F$ (R_f^F 0.44).

Thymidylyl-(3'-5')-thymidylyl-(3'-5')-thymidylyl-(3'-5')-thymidine, $dT_pT_pT_pT$ (9). $dMTr_p(CE)T$ (7)¹⁷ (17.4 mg 20 μ mol), dried by evaporation of pyridine (3 x 1 ml), was dissolved in 0.7 ml of dioxane and treated with 5 μ l of dry pyridine and 5.6 μ l (60 μ mol) of phosphorus oxychloride for two hours. The phosphorochloridate was hydrolyzed by addition of 1 ml of 50% aqueous pyridine, and after 2 hours the solvent was evaporated and the residue was taken up in 1 ml of saturated sodium chloride. On extraction with chloroform (2 x 1 ml) and evaporation of the chloroform layer, the 3'-O-

phosphoryl derivative of 7 was obtained as the pyridinium salt. As evidence that phosphorylation had occurred, the protecting groups were removed from a sample by treatment with an equivolume mixture of pyridine and ammonium hydroxide for 2 hours, followed by evaporation of the solvent and treatment with 80% aqueous acetic acid at 44° for 2 hours. Paper chromatography in Solvent F afforded dT_pT_p (5) (R_f 0.46, 19.7 A₂₆₇ units, 84%) along with some dT_pT (R_f^F 0.66; 3.64 A₂₄₇ units) and a trace of material at R_f 0.30.

An aliquot containing 2 μmol of dMTrT_p(CE)T_p along with 2.9 μmol of dT_p(CE)T_βB (8)¹⁷ was dried by repeated evaporation with pyridine, and the resulting gum was mixed with 4 μmol of triisopropylbenzenesulfonyl chloride in 0.1 ml of dry pyridine. After 8 hr the solution was mixed with 0.5 ml of ammonium hydroxide and 0.5 ml of pyridine. This mixture was allowed to stand 12 hr to remove the base labile groups; then it was concentrated and treated with 80% aqueous acetic acid at 44° for 1 hr to cleave the methoxytrityl group. The mixture was then concentrated and taken up in 0.5 ml of 50% aqueous ethanol. Chromatography on paper in solvent E yielded four bands: R_f^E 0.10 (dT_pT_pT_pT); R_f^E 0.30 (dT_pT_p); R_f^E 0.56 (dT_pT) and R_f^E 0.92 (protecting group residues). Elution of the band at R_f 0.10 gave dT_pT_pT_pT (74%). For enzymatic analysis the product was freed of trace impurities by paper electrophoresis at pH 7.0 and by chromatography in solvent C. Digestion with snake venom phosphodiesterase afforded dT (R_f^B 0.68; 1.84 A₂₆₇ units) and d_pT (R_f^B 0.36; 5.05 A₂₆₇ units) in a 1.0/2.74 ratio. Similarly, digestion with spleen phosphodiesterase gave only two products, corresponding to dT (R_f^B 0.68; 2.63 A₂₆₇ units) and dT_p (R_f^B 0.37; 8.70 A₂₆₇ units), in a 1.0/3.3 ratio.

Thymidylyl-(3'-5')-thymidine, d-TpT. - Dry 5'-O-Tritylthymidine (48.4 mg, 0.10 mmol) in 3 ml of dioxane was phosphorylated with phosphorus oxychloride (0.028 ml, 0.30 mmol) and pyridine (0.024 ml, 0.30 mmol) at room temperature (4.5 hr reaction). The mixture was concentrated to 0.5 ml at reduced pressure and two 1 ml portions of dioxane were evaporated at reduced pressure from this residue to remove excess phosphoryl chloride.

The resulting gum was treated with anhydrous 3'-O-mono-p-methoxytrityl-thymidine^{11c} (103 mg, 0.20 mmol) and 0.024 ml of pyridine in 2.5 ml of dioxane. The mixture was stirred for 5 hours, treated with 0.5 ml of aqueous pyridine and concentrated to a syrup. The protecting groups were removed by treatment with 2 ml of 80% acetic acid at 100° for 10 minutes. The acetic acid was evaporated and the residue was mixed with 10 ml of water. After extraction with two 3 ml portions of ether, the aqueous phase was diluted to 25 ml with water. A 1 ml aliquot of this solution chromatographed on paper in solvent F revealed three components: Rf 0.54 (2.09 A₂₆₀ units), Rf 0.66 (57.8 A₂₆₀ units; dTpT) and Rf 0.76 (3.04 A₂₆₀ units, dT). The dTpT represents a 78% yield based on 5'-O-tritylthymidine. It was completely degraded by both snake venom and spleen phosphodiesterase (nucleoside:nucleotide = 0.93:1.00 from reaction of the venom enzyme and 0.95:1.00 from reaction of the spleen enzyme).

ACKNOWLEDGEMENT

This research was supported by the National Institute of General Medical Sciences of the National Institutes of Health (Grant GM10265).

REFERENCES

1. Part XIX in series on Nucleotide Chemistry.
Part XVIII: Wilkes, J. S., Hapke, B. and Letsinger, R. L. (1973), *Biochem. Biophys. Research Comm.*, 53, 917.
2. Fischer, E., (1914), *Chem. Ber.*, 47, 3193.
3. Barker, G. R. and Foll, G. E. (1957), *J. Chem. Soc.*, 3798.
4. Muramatsu, N. and Takenishi, T. (1965), *J. Org. Chem.*, 30, 3211.
5. Chambers, R. N., Moffatt, J. G. and Khorana, H. G. (1957), *J. Amer. Chem. Soc.*, 79, 3747.
6. (a) Khorana, H. G. (1961), "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., Chapter 2, summarizes the older literature. (b) Eckstein, F. (1968) 101, 2998. (c) Hata, T., Mushika, T. and Mukaiyama, T. (1969), *J. Amer. Chem. Soc.*, 91, 4532. (d) Khwaja, T. A., Reese, C. B. and Stewart, J. C. M. (1970), *J. Chem. Soc. (C)*, 2092.

7. Yoshikawa, M., Kato, T. and Takenishi, T. (1967), *Tetrahedron Letters*, 50, 5065; (1969) *Bull. Chem. Soc. Japan*, 42, 3505.
8. Rose, W. G. (1947), *J. Amer. Chem. Soc.*, 69, 1384, and subsequent workers employed similar conditions (phosphorus oxychloride and pyridine in chloroform) in the preparation of phospholipids.
9. Tener, G. (1969), *J. Amer. Chem. Soc.*, 83, 159.
10. Ukita, T. and Hayatsu, H. (1962), *J. Amer. Chem. Soc.*, 84, 1879.
11. (a) Imai, K., Fujii, S., Takanohashi, K., Furukawa, Y., Nasudo, T. and Honjo, M. (1969), *J. Amer. Chem. Soc.*, 34, 1547. (b) Franke, A., Scheit, K. and Eckstein, F. (1968), *Chem. Ber.*, 101, 2998. (c) Ogilvie, K. K. and Letsinger, R. L. (1967), *J. Org. Chem.*, 32, 2365. (d) Schaller, H., Weimann, G., Lerch, B. and Khorana, H. G. (1963), *J. Amer. Chem. Soc.*, 85, 3821.
12. Jacob, T. M. and Khorana, H. G. (1965), *J. Amer. Chem. Soc.*, 87, 368.
13. Letsinger, R. L. and Ogilvie, K. K. (1969), *J. Amer. Chem. Soc.*, 91, 3350.
14. Letsinger, R. L. and Mungall, W. S. (1970), *J. Org. Chem.*, 35, 3800.
15. Chen, P. S., Toribara, T. Y. and Warner, H. (1956), *Anal. Chem.*, 28, 1756.
16. Khorana, H. G. and Connors, W. J. (1966), *Biochem. Preparations*, 11, 113.
17. Letsinger, R. L., Ogilvie, K. K. and Miller, P. S. (1969), *J. Amer. Chem. Soc.*, 91, 3360.