When the purine nucleoside-3',5' cyclic phosphates were incubated for protracted periods or with excessive amounts of protein, traces of ultraviolet adsorbing products appeared with no electrophoretic mobility at pH 7.5. Presumably, these are nucleosides (or bases) resulting from the actions of contaminating traces of phosphatase (or other degradative enzymes). Analogous products were obtained in the experiments with pyrimidine deoxynucleoside-3',5' cyclic phosphates, which required large concentrations of enzyme and prolonged reaction before any degradation was evident.

Studies on Polynucleotides. XXX.[1] A Comparative Study of Reagents for the Synthesis of the C₃'–C₅' Internucleotidic Linkage[2]

BY T. M. JACOB AND H. G. KHORANA

The formation of thymidylyl(3'→5')thymidine by the condensation of (a) pyridinium 3'-O-acetylthymidine-5' phosphate with 5'-O-tritylthymidine and (b) pyridinium 5'-O-acetylthymidine-3' phosphate with 3'-O-acetylthymidine has been studied under identical conditions using the following reagents: dicyclohexylcarbodiimide, ethoxyacetylene, N-ethyl-5-phenylisoxazolium fluoroborate, the reagent prepared by the reaction of phosgene with dimethylformamide, ethyl metaphosphate, p-toluenesulfonyl chloride, and mesitylenesulfonyl chloride. Dicyclohexylcarbodiimide and the aromatic sulfonyl chlorides gave the highest (90% or better, using stoichiometric amounts of the protected nucleotide and the nucleoside) yields of the desired product. The rate of internucleotide bond synthesis using the aromatic sulfonyl chlorides was much higher than that obtained with dicyclohexylcarbodiimide. The mechanism of internucleotide bond synthesis using the aromatic sulfonyl chlorides is discussed in relation to the previous findings with dicyclohexylcarbodiimide.

A comparative study of several reagents for the purpose of polymerization of mononucleotides recently was reported.[3] Dicyclohexylcarbodiimide (I, DCC) gives the best results,[3] and this reagent has, in fact, been used in essentially all of the synthetic work in the polynucleotide field reported from this laboratory.[1,4,5] However, during the past few years a number of new reagents have been proposed for the activation of carboxylic and phosphoric acid groups. The reagents proposed include ethoxyacetylene[6] (II), substituted

$C_6H_{11}N\!=\!C\!=\!NC_6H_{11}$ $HC\!\equiv\!COC_2H_5$
I II
IV

III, R = ethyl
R' = phenyl
V
VI, R = H or CH₃
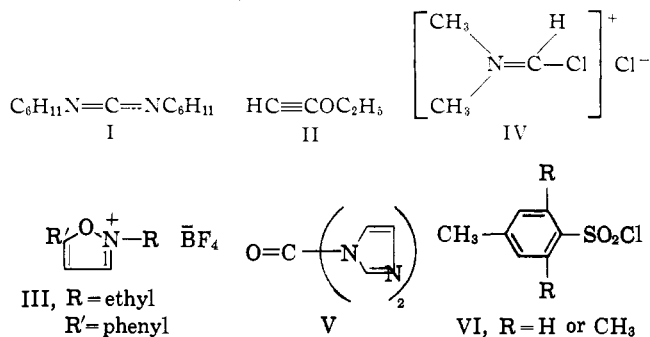
isoxazolium salts[7] (III), the product (IV) from the reaction of phosgene with dimethylformamide,[8] ethyl

metaphosphoric acid prepared by the reaction of ether with phosphorus pentoxide,[9] trichloroacetonitrile,[10] and carbonylbis(imidazole)[11] (V). The present paper reports on a comparative study of several of the reagents[12] for the synthesis of the C₃'–C₅' internucleotidic linkage using relatively simple nucleotide and nucleoside derivatives. The results show that DCC and the aromatic sulfonyl chlorides[3,13] (VI) are the most efficient reagents. A characteristic feature of the latter class of reagents, however, is the very much higher rate of reaction than that obtained using DCC. A brief report of part of these results has been made previously.[14]

Dicyclohexylcarbodiimide.—The formation of thymidylyl(3'→5')thymidine (VII) from (a) pyridinium 3'-O-acetylthymidine-5' phosphate (VIII) and 5'-O-tritylthymidine (IX, System A), and from (b) 5'-O-acetylthymidine-3' phosphate (X) and 3'-O-acetylthymidine (System B), using 0.1 M solutions in dry pyridine has been studied. Both of these condensations have been given close study in this laboratory using DCC as the reagent.[5,15,16] Two new observations were made using the latter reagent in the present work. When the duration of the aqueous pyridine treatment was reduced from 16 hr.[15] to about 1 hr., the presence of labile intermediates was noted. These

(1) Paper XXIX: Y. Lapidot and H. G. Khorana, J. Am. Chem. Soc., 85, 3857 (1963).

(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, the National Science Foundation, Washington, D. C., and The Life Insurance Medical Research Fund, New York, N. Y.

(3) H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, J. Am. Chem. Soc., 84, 414 (1962).

(4) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 5.

(5) (a) P. T. Gilham and H. G. Khorana, J. Am. Chem. Soc., 80, 6212 (1958); (b) H. Schaller and H. G. Khorana, ibid., 85, 3841 (1963), and previous papers cited therein.

(6) (a) J. F. Arens and H. C. Volger, Rec. trav. chim., 77, 1170 (1958), and earlier papers in this series; (b) H. H. Wasserman and P. S. Wharton, J. Am. Chem. Soc., 82, 661 (1960); (c) H. Wasserman and D. Cohen, ibid., 82, 4435 (1960).

(7) (a) R. B. Woodward and R. A. Olofson, ibid., 83, 1007 (1961); (b) R. B. Woodward, R. A. Olofson, and H. Mayer, ibid., 83, 1010 (1961).

(8) (a) M. Zaoral and Z. Arnold, Tetrahedron Letters, No. 14, 9 (1960); (b) see also a related study by H. H. Boshard, R. Mory, M. Schmid, and H. Zollinger, Helv. Chim. Acta, 42, 1653 (1959).

(9) (a) K. Langheld, Ber., 43, 1857 (1910); (b) G. Schramm, H. Grotsch, and W. Pollmann, Angew. Chem., 74, 53 (1962).

(10) F. Cramer and G. Weimann, Ber., 94, 996 (1961).

(11) H. Schaller, H. A. Staab, and F. Cramer, ibid., 94, 1621 (1961); A. F. Turner and H. G. Khorana, unpublished work (see ref. 4).

(12) An experiment using trichloroacetonitrile, carried out under conditions identical with those used in the present work, was reported previously [G. Weimann and H. G. Khorana, J. Am. Chem. Soc., 84, 419 (1962), and footnote 14]. Up to a period of 64 hr. at room temp., none of the desired product, thymidylyl(3'→5')thymidine, was formed. The use of the reagent carbonylbis(imidazole) in internucleotide bond synthesis was tested recently under identical conditions in this laboratory. None of the desired thymidylyl(3'→5')thymidine was formed (H. Schaller and H. G. Khorana, unpublished work).

(13) H. G. Khorana, G. M. Tener, J. G. Moffatt, and E. H. Pol, Chem. Ind. (London), 1523 (1956); G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, J. Am. Chem. Soc., 80, 6224 (1958).

(14) T. M. Jacob and H. G. Khorana, Chem. Ind. (London), 932 (1962).

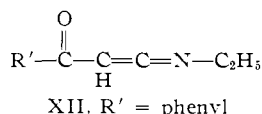(15) G. Weimann and H. G. Khorana, J. Am. Chem. Soc., 84, 4329 (1962).

(16) H. Schaller and H. G. Khorana, ibid., 85, 3828 (1963).
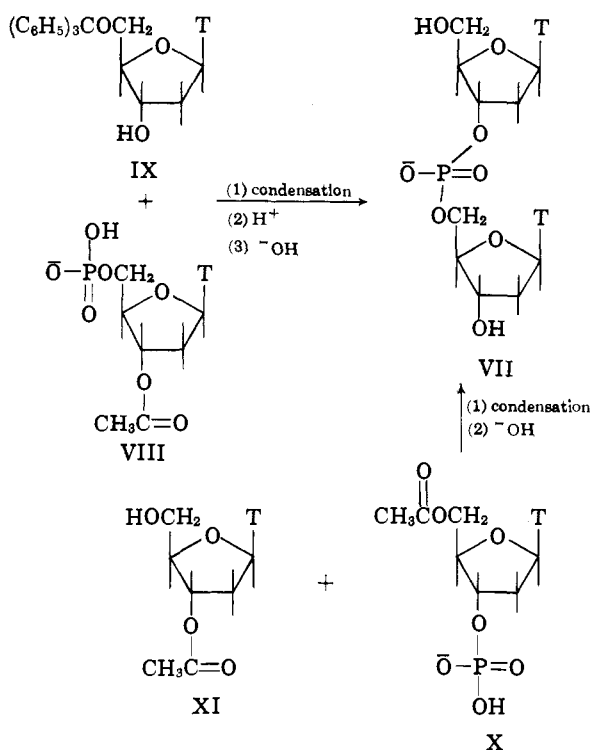
evidently comprise the adducts of DCC with the thymine ring (cf. ref. 16) and break down during prolonged aqueous pyridine treatment to the previously well-characterized products. The final yield (about 80%) of the desired VII using stoichiometric amounts of X and XI was identical with that reported previously.[15] The second observation was that in System A the yield of VII from stoichiometric amounts of VIII and IX could be increased from 70%[5a,16] to better than 90% when additional steps were taken to remove traces of the inhibitory strong amines present in the reagent grade, commercially available pyridine. This was accomplished by either distilling the commercial pyridine from chlorosulfonic acid or by including some dry pyridinium Dowex-50 ion-exchange resin[17] in the reaction mixtures.

N-Ethyl-5-phenylisoxazolium Fluoroborate (III).[18]— In both Systems A and B, the kinetics and the final yields of thymidylyl(3'→5')thymidine (VII) were very similar to those found with DCC. Thus, in System A, the yield of VII was about 50% in one day and 67% in three days, while in System B, the yield of VII was 60% in one day and 68% after two days. These results were not unexpected since Woodward and Olofson[7a] showed that the isoxazolium salts of the general type III are rapidly converted to the ketoketenimines of type XII and, therefore, the manner of activation of a phosphomonoester and the nature of

$$R'-\overset{\overset{O}{\|}}{C}-\underset{H}{C}=C=N-C_2H_5$$

XII, R' = phenyl

the actual phosphorylating agent involved would probably be similar to that established previously for the DCC mediated reactions.[15]

In experiments with the isoxazolium salts, the slow formation of two additional unidentified side products was observed. One of these evidently was derived from the nucleotide component alone, its properties being consistent with structure XIII, which could have formed by O→N migration[19] of the phosphoryl group in the initial adduct XIV.[20,21] The ultraviolet absorp-

tion characteristics of the second side product indicated a chromophore different from that of thymidine. This result could point to an addition reaction between the reagent and the thymine ring. Alternatively, it is possible that this ultraviolet absorbing material arose from the reagent alone.

Ethoxyacetylene (II).—Under the standard conditions, ethoxyacetylene caused only the formation of P^1,P^2-dithymidine pyrophosphate up to 96 hr. at room temperature. The same result was previously obtained with trichloroacetonitrile.[12] Thus, it is clear that whereas the activation of a phosphomonoester may occur with these reagents, the resulting pyrophosphate lacks under mild conditions the nucleophilicity to undergo further reaction with these reagents, a step which is essential for conversion of the initially formed symmetrical pyrophosphate to a phosphorylating agent.

Ethyl Metaphosphate.—The reagent prepared by the prolonged reaction of diethyl ether with phosphorus pentoxide was introduced by Langheld[9a] as a phosphorylating agent for alcohols. Recently, it has been claimed to be effective for the polymerization of mononucleotides.[9b] While adequate characterization of the polymeric products as yet has not been reported, we tested this reagent under our standard conditions for the formation of thymidylyl(3'→5')thymidine. A complex mixture of products was produced and, while a trace of the desired product might have been formed, its amount was too little to make the method of practical value in the stepwise synthesis of oligonucleotides. Furthermore, as might have been expected
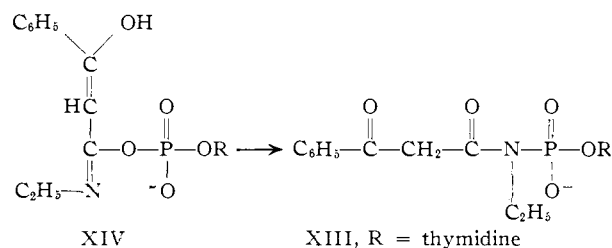
(17) The use of pyridinium Dowex-50 ion-exchange resin to ensure the removal of any strong amines in polynucleotide synthesis using DCC previously has been made whenever dimethylformamide is used as the solvent [ref. 16 and see also R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, J. Am. Chem. Soc., **85**, 1983 (1963)].

(18) We are grateful to Prof. R. B. Woodward and Dr. R. A. Olofson of Harvard University for generous gifts of several of the reagents which they have developed recently for peptide synthesis.[7]

(19) This migration could occur also during the reaction of P^1,P^2-di-(thymidine-5') pyrophosphate with XII, in which case the initial product would be i and this would break down during the subsequent aqueous
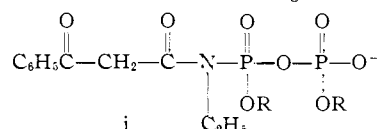
pyridine or alkaline treatment to XIII and the mononucleotide.

(20) This formulation is analogous to that proposed for carboxylic acids by Woodward and Olofson.[7a]

(21) The formation of N-phosphorylureas has been noted in DCC promoted polymerization reactions. Their formation was slow and the amount was extremely small [H. G. Khorana and J. P. Vizsolyi, J. Am. Chem. Soc., **83**, 675 (1961); H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, ibid., **83**, 686 (1961); G. M. Tener, ibid., **83**, 159 (1961)].

from the results of earlier workers,[4,22,23] direct phosphorylation of the nucleoside component by the reagent occurred, and the product, ethyl thymidine-3' phosphate, obtained after acidic removal of the trityl group was characterized (see Experimental).

Although our own experiments with the ethyl metaphosphate reagents are not exhaustive[24] in scope, it is our view that the direct phosphorylation of the hydroxyl groups by the reagent would be difficult to avoid. Furthermore, there is the extreme danger of glycosyl bond fission[24] and of the removal of the highly sensitive groups (such as dimethoxytrityl[25]) during treatment with this reagent.

Dimethylformamide and Phosgene.—The crystalline reagent (IV) prepared by the reaction of phosgene with dimethylformamide has recently been recommended for the activation of carboxyl groups with a view to the formation of peptide bonds.[8] As applied to the synthesis of thymidylyl(3'→5')thymidine using System A, although a rapid reaction occurred and the desired product was indeed formed, the yield never exceeded 30–40%. The use of a larger excess of the reagent caused the formation of side products. Because of the general probability of side reactions[26] with the heterocyclic rings, the reagent does not appear to us to be promising in the field of polynucleotide synthesis.

Arylsulfonyl Chlorides.—There were indications from early work[13] that the internucleotidic bond synthesis occurred at a faster rate with the arylsulfonyl chlorides than when DCC was used as the condensing agent. In the present work, when mesitylenesulfonyl chloride was the reagent in System A, the yield of thymidylyl-(3'→5')thymidine was 71% in 1 hr. and around 90% in 6 hr. at room temperature. There was no increase on prolonging the reaction time. The yield using p-toluenesulfonyl chloride in a 6-hr. reaction period was similar (78%). In these experiments, a small amount of direct sulfonylation of the 3'-hydroxyl group in 5'-O-tritylthymidine also occurred, as shown by the presence of 3'-O-arylsulfonylthymidine on paper chromatograms after standard work-up of the reaction mixtures.

In System B, experiments were carried out using p-toluenesulfonyl chloride. The optimal yield (56–59%) was obtained within the first 2 hr. and no further increase was noted up to a period of 40 hr. The lower yield of thymidylyl(3'→5')thymidine in System B compared with that obtained above in System A is probably due to the greater extent of direct sulfonylation of the primary hydroxyl group in 3'-O-acetylthymidine. However, the possibility that the lower

yield was, in part, due to the presence of traces of moisture cannot be excluded, since these experiments were carried out on a small (0.1 mmole) scale.

While a further discussion of the use of aromatic sulfonyl chlorides in internucleotidic bond synthesis will be presented later, it may be noted that the sulfonylation of the secondary hydroxyl groups may in fact be reduced to negligible levels. A separate study of the rate of sulfonylation of 5'-O-tritylthymidine (see Experimental) using an excess of the reagent showed the reaction to be very much slower than the internucleotide bond synthesis. On the other hand, no significant difference in the rates of sulfonylation was noted when p-toluenesulfonyl chloride and mesitylenesulfonyl chloride were compared.[27]

Discussion

The survey reported in this paper has shown DCC and the arylsulfonyl chlorides to be the most efficient reagents for the synthesis of internucleotide bonds. The yields (90% or better) obtained with these reagents using stoichiometric amounts of the nucleotide and nucleoside components are highly satisfactory. The arylsulfonyl chlorides emerge as the most reactive and powerful reagents. While the previous comparative study of reagents for the polymerization reaction showed the sulfonyl chlorides to be somewhat inferior to DCC, the former may be the reagent of choice in stepwise synthesis, especially where rapidity is desired. The traces[28] of sulfonylated derivatives formed from the starting material would be removed readily from the desired products by chromatographic methods. A distinct advantage offered by the arylsulfonyl chlorides is the possibility of using the more soluble trialkyl ammonium salts of mono- and oligonucleotides in polynucleotide synthesis. This is because with these reagents only the anhydride exchange reactions are involved, whereas in activation with DCC,[29] protonation constitutes an important first step which is inhibited by the trialkyl amines.

Previously,[15] it has been shown that the reaction of a phosphomonoester with DCC leads rapidly to the formation of a trimetaphosphate (XV), and that the latter is probably the initial phosphorylating species.[30] Since the rate of synthesis of the internucleotide

XV
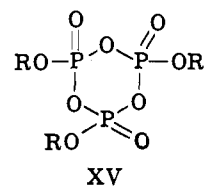
bond using the arylsulfonyl chlorides is 5–10 times higher than that using DCC, it must be concluded that a species other (more reactive) than the trimeta-

(22) For earlier literature, see G. M. Kosolapoff, "Organo-phosphorus Compounds," John Wiley and Sons, Inc., New York, N. Y., 1950.

(23) In unpublished experiments by Dr. G. Weimann, the major compound detected on paper-electrophoretic examination[15] of the ethyl metaphosphate preparations was P1,P2,P3-triethyl polyphosphate. This result shows that the major product present in the anhydrous preparation is triethyl trimetaphosphate, identical with that obtained by treatment of monoethyl phosphoric acid with DCC.[15]

(24) In another experiment on the polymerization of deoxyadenosine-5' phosphate using ethyl metaphosphate, the major reaction observed was the formation of adenine. No polymeric nucleotidic products could be detected (G. Weimann and H. G. Khorana, unpublished work).

(25) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, J. Am. Chem. Soc., 85, 3821 (1963).

(26) We have recently learned from Dr. Farkas of the Institute of Organic Chemistry and Biochemistry of the Czechoslovakian Academy of Sciences, Prague, that the reagent is, in fact, effective for the ring chlorination of certain nucleosides.

(27) The results are in agreement with those of W. S. Johnson, J. C. Collins, Jr., R. Pappo, M. B. Rubin, P. J. Kropp, W. F. Johns, J. E. Pike, and W. Bartmann, J. Am. Chem. Soc., 85, 1409 (1963).

(28) The extent of direct sulfonylation has been reduced to undetectable levels in more recent work using a larger excess of the nucleotide component and a proportionately large excess of the arylsulfonyl chloride (T. M. Jacob, H. Radloff, and H. G. Khorana, unpublished work).

(29) M. Smith, J. G. Moffatt, and H. G. Khorana, J. Am. Chem. Soc., 80, 6204 (1958).

(30) Very probably the ketoketenimines, trichloroacetonitrile at elevated temperatures, and picryl chloride [F. Cramer, R. Witmann, K. Daneck, and G. Weimann, Angew. Chem., 75, 92 (1963)] are all similar to DCC in first producing the trimetaphosphate.

phosphate is the phosphorylating species in the reactions mediated by arylsulfonyl chlorides. A number of further observations supports this conclusion. The treatment of pyridinium 3'-O-acetylthymidine-5' phosphate with 0.5 molar equiv. of an arylsulfonyl chloride gives mainly P^1,P^2-di(thymidine-5') pyrophosphate (present work and see also ref. 13). The use of 1 molar equiv. of the reagent gives, presumably, the trimetaphosphate (XV), which during the subsequent aqueous pyridine treatment breaks down to equimolar amounts of the nucleotide and the corresponding pyrophosphate.[15] However, when larger amounts (2–2.5 molar equiv.) of the arylsulfonyl chloride are used, subsequent aqueous treatment regenerates mostly the mononucleotide. The species that might be formed in the presence of an excess of the sulfonyl chlorides would be as shown in the formulas XVI–XX. All
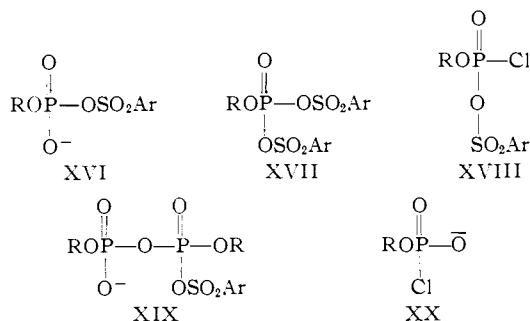
of these would be expected to be extremely reactive and would generate the free mononucleotide on hydrolysis. At the present time it does not seem possible to prefer any one of these possibilities over the others. It should be observed further that, while the tendency for the formation of a trimetaphosphate from a monoalkyl phosphate is undoubtedly great, the trimetaphosphate apparently can be further attacked by the arylsulfonyl chlorides to give species more reactive than the trimetaphosphate. In a set of experiments, pyridinium 3'-O-acetylthymidine-5' phosphate first was converted to the trimetaphosphate by treatment with DCC for 5 min.[15] Subsequent addition of 2 molar equiv. of mesitylenesulfonyl chloride followed by 5'-O-tritylthymidine gave a 60% yield of thymidylyl-(3'→5')thymidine within 1 hr. at room temperature. The minimum reaction that must be postulated between the initially formed trimetaphosphate and the arylsulfonyl chloride is the formation of XXI. The latter may undergo disproportionation reactions to

XXI
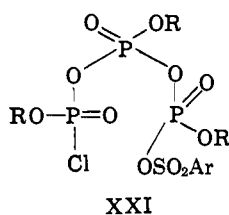
form several of the species postulated above (XVI–XX). It is worth noting that in the above experiment the conversion of the trimetaphosphate to more reactive species required the addition of a sulfonyl chloride. Equivalent amounts of pyridinium mesitylenesulfonate and pyridine hydrochloride gave only a slight effect which is attributed to the increased proton concentration.

Experimental

Methods.—Paper chromatography was performed by the descending technique using Whatman No. 1 or 3MM paper. The solvents used were isopropyl alcohol–concentrated ammonia–water (7:1:2 v./v.) (Solvent A) and ethyl alcohol–1 M ammonium acetate (pH 7.5) (5:2 v./v., Solvent B). The R_f values of different compounds are listed in Table I. Paper electrophoresis was performed in an apparatus similar to that described by Markham and Smith[31] or in a commercially available water-cooled apparatus designed on the same principle but capable of giving a potential of 4000 v. The buffers used were 0.05 M ammonium formate (pH 3.5) and 0.03 M phosphate (pH 7.1).

TABLE I
R_f Values of Compounds on Paper Chromatography in Solvent A

Compound	R_f
Thymidine-5' phosphate	0.09
P^1,P^2-di(thymidine-5') pyrophosphate	0.15
Thymidylyl(3'→5')thymidine	0.38
Nucleotidic side product from III (XIII?)	0.49
Thymidine	0.66
p-Toluenesulfonic acid	0.75
Mesitylenesulfonic acid	0.77
3'-O-(p-Toluenesulfonyl)thymidine	0.87
3'-O-(Mesitylenesulfonyl)thymidine	0.87

Estimation of the yields of thymidylyl(3'→5')thymidine most frequently was carried out spectrophotometrically after elution of spots from paper chromatograms run in Solvent A. The spots and appropriate blank areas of the paper were eluted after being cut into short pieces by soaking in 0.01 N hydrochloric acid for 24–48 hr. in sealed vessels, the contents of the vessels being shaken occasionally. The yields of thymidylyl(3'→5')-thymidine were calculated both on the basis of unchanged nucleoside and on the recovered nucleotide, partly present as the pyrophosphate. In experiments where the isolated yield was determined, the elution of paper chromatographic bands was performed with dilute (0.1 N) ammonia followed by spectrophotometric measurement of the product at an acid pH. The ϵ_{max} value at 267 mμ used for thymidylyl(3'→5')thymidine was 18,500.[5]

p-Toluenesulfonyl chloride was a commercial sample. It was recrystallized from petroleum ether (b.p. 40–80°) to a constant melting point, stoppered, and stored in a desiccator over calcium chloride. Mesitylenesulfonyl chloride was prepared from the commercially available 2,4,6-trimethylbenzenesulfonic acid (Aldrich Chemical Co., Milwaukee, Wis.) by treatment with phosphorus oxychloride. The product after crystallization from pentane had m.p. 57°. It was stoppered and stored with exclusion of moisture. Ethoxyacetylene (II) was procured commercially. It was distilled (b.p. 49° at normal pressure) prior to use. Dry Dowex-50 (pyridinium) ion-exchange resin was prepared by repeated evaporation of pyridine from the pyridinium resin and storage of the dry resin over calcium chloride in a desiccator.

Thymidylyl(3'→5')thymidine Using DCC. A. From Pyridinium 3'-O-Acetylthymidine-5' Phosphate and 5'-O-Tritylthymidine (System A).—A mixture of pyridinium 3'-O-acetylthymidine-5' phosphate (0.1 mmole), 5'-O-tritylthymidine (0.056 g., 0.1 mmole), and DCC (103 mg., 0.5 mmole) in dry pyridine (1 ml.) was kept at room temperature with exclusion of moisture. Aliquots (0.1 ml.) were removed at different intervals and each was treated with 0.5 ml. of water for several hours. For further work-up, the solvent was removed under suction and the residue was treated with 2 ml. of 80% acetic acid for 10 min. at 100°. The acetic acid then was removed with suction, and the dry residue was treated with 1 ml. of 0.5 N sodium hydroxide. The alkaline solution was kept at room temperature for 1 hr. and then was neutralized with freshly prepared pyridinium Dowex-50 ion-exchange resin. An aliquot of the aqueous solution was chromatographed in Solvent A. The yield of thymidylyl-(3'→5')thymidine was determined as described above. The results were similar to those published previously.[5b] While in this experiment the yield of thymidylyl(3'→5')thymidine leveled off at about 72%, the above experiment was later repeated on the same scale with the following modifications. Pyridinium

(31) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

3'-O-acetylthymidine-5' phosphate was passed, prior to use, through a column of pyridinium Dowex-50 ion-exchange resin and precipitated from ether. The pyridine used in evaporations in order to obtain anhydrous solution and in the condensation itself was prepared as follows. The Baker and Adam reagent grade pyridine was refluxed in the presence of p-toluenesulfonyl chloride or chlorosulfonic acid, distilled, refluxed over solid potassium hydroxide, and again distilled. The distilled sample was dried over molecular sieves (Linde Company). The yield of thymidylyl(3'→5')thymidine after 8 days at room temperature using stoichiometric amounts of 5'-O-tritylthymidine and pyridinium 3'-O-acetylthymidine-5' phosphate was estimated to be about 90% after elution of the spots. The isolated yield was 85.4%.

B. Using System A and a Mixture of DCC and Mesitylenesulfonyl Chloride.—To an anhydrous pyridine solution (2 ml.) of 0.5 mmole of 5'-O-tritylthymidine and pyridinium 3'-O-acetylthymidine-5' phosphate (0.5 mmole) was added DCC (0.515 mg., 2.5 mmoles), and the stoppered reaction mixture was kept for 5 min. at room temperature during which time crystals of dicyclohexylurea appeared. Mesitylenesulfonyl chloride (1 mmole) then was added and the sealed reaction mixture was shaken. The disappearance of the dicyclohexylurea was soon observed, and the clear solution was kept at room temperature. Aliquots (0.1 ml.) were withdrawn at intervals of 1 and 24 hr. The yield of thymidylyl(3'→5')thymidine was estimated to be 60% after 1 hr. and 90% after 24 hr.

C. Using System A and a Mixture of DCC and Different Salts. Experiment I.—Mesitylenesulfonyl chloride (0.2 mmole) was treated with 50% aqueous pyridine at room temperature for 2 hr. The solution was evaporated with pyridine and, after the addition of 5'-O-tritylthymidine (0.1 mmole) and 3'-O-acetylthymidine-5' phosphate (0.1 mmole), the pyridine solution was rendered anhydrous by the standard method. After the subsequent addition of DCC (0.5 mmole), the reaction mixture was kept stoppered at room temperature, and aliquots were removed at different intervals, worked up as in A, and analyzed by paper chromatography in Solvent A.

Experiment II.—This was the same as experiment I except that the mixture of salts obtained by the hydrolysis of mesitylenesulfonyl chloride was replaced by pyridine hydrochloride[32] (0.4 mmole).

Experiment III.—In experiment II, pyridine hydrochloride was replaced by dry pyridinium Dowex-50 ion-exchange resin (0.2 g.).

Experiment IV.—In the above standard experiment, pyridinium mesitylenesulfonate (0.4 mmole) was used as the salt. In this experiment there was detected the formation of an unidentified side product migrating in Solvent A between thymidylyl-(3'→5')thymidine and thymidine.

The results of expt. I–IV are given in Table II.

TABLE II

YIELDS OF THYMIDYLYL(3'→5')THYMIDINE USING DCC AND FOUR DIFFERENT SALTS

Expt. no.	Time, hr.	Yield, %
I	1	16
	20	73
	96	90
	168	95
II	20	70
	96	88
	168	88
III	20	61
	96	81
	168	88
IV	20	58
	96	63
	168	64

D. From Pyridinium 5'-O-Acetylthymidine-3' Phosphate and 3'-O-Acetylthymidine (System B).—The experiment was identical with that reported previously.[15] At different intervals, aliquots were removed and treated with an equal volume of water for 16 hr. or more. Each aliquot (0.1 ml. out of 1 ml. of reaction

mixture) then was treated with 0.5 ml. of 1 N sodium hydroxide. After 1 hr. at room temperature the alkali was neutralized by the addition of an excess of pyridinium Dowex-50 ion-exchange resin. The product analysis was by paper chromatography in Solvent A. The results were similar to those described previously[15] except that when, in the above work-up, the initial treatment with water was short (e.g., only 1 hr.) then during subsequent paper chromatography large amounts of products with R_f 0.84–0.90 (Solvent A) were detected,[33] and the yield of thymidylyl(3'→5')thymidine was lower. Prolonging the aqueous pyridine treatment led to the disappearance of the additional products, and the yield of the desired product was then higher.

Thymidylyl(3'→5')thymidine Using Arylsulfonyl Chlorides. A. Using System A.—A mixture of pyridinium 3'-O-acetylthymidine-5' phosphate (0.5 mmole), 5'-O-tritylthymidine (0.280 g., 0.5 mmole), and the aromatic sulfonyl chloride (1 mmole) was kept in dry pyridine (1 ml.) at room temperature with exclusion of moisture. After periods of 1–6 hr., the reaction mixtures were treated with 1 ml. of ice water with cooling, and the clear solution was kept at room temperature for 1 hr. to 1 day. The solvent was removed in vacuo and, after addition of 10 ml. of 80% acetic acid, the solution was heated at 100° for 10 min. The acetic acid was removed with suction, and the residue was taken up in 50% aqueous ethyl alcohol (the insoluble tritanol was removed by filtration). The total solution was made up to 25 ml. with the same solvent, and aliquots were treated as follows.

An aliquot (0.5 ml.) was treated with 0.5 ml. of 1 N sodium hydroxide for 1 hr. at room temperature. An excess of pyridinium Dowex-50 ion-exchange resin then was added to remove the sodium ions. A portion of the neutral solution was chromatographed in Solvent A for determination of the yield of thymidylyl-(3'→5')thymidine. The R_f values of the different ultraviolet absorbing spots observed are in Table I.

Using p-toluenesulfonyl chloride in the above experiment, the yield of thymidylyl(3'→5')thymidine after 6 hr. was estimated to be 78%. When mesitylenesulfonyl chloride was used, the yield in 1 hr. was 71% and 90% in 6 hr. The yield in a longer reaction period (24 hr.) was similar.

In the different experiments, the formation of 3'-O-arylsulfonylthymidine was looked for on chromatograms run in Solvent A. The arylsulfonyl thymidine (after removal of the trityl group) had R_f 0.84–0.90 (Table I). It was absent in 1-hr. reaction products but was detected in 6-hr. and 24-hr. reaction products.

Using System B.—p-Toluenesulfonyl chloride (0.038 g., 0.2 mmole) was added to an anhydrous pyridine solution (1 ml.) of pyridinium 5'-O-acetylthymidine-3' phosphate (0.1 mmole) and 3'-O-acetylthymidine (0.1 mole) with exclusion of moisture, and the sealed reaction mixture was kept at room temperature. Three such experiments were set up side by side. After a specified time, each reaction mixture was treated with 1 ml. of water and, after 1 hr. at room temperature, the solution was evaporated. The residue was taken up in 5 ml. of 0.6 M sodium hydroxide, and the alkaline solution was kept at room temperature for 1 hr. The alkali then was neutralized with an excess of pyridinium Dowex-50 ion-exchange resin and the clear supernatant was chromatographed in Solvent A. The yields of thymidylyl(3'→5')-thymidine was 56% in 2 hr. and 59% in 18 hr. The yield in the 40-hr. experiment was 54%.

Thymidylyl(3'→5')thymidine Using N-Ethyl-5-phenylisoxazolium Fluoroborate (III). A. Using System A.—To an anhydrous mixture of 3'-O-acetylthymidine-5' phosphate (0.1 mmole) and 5'-O-tritylthymidine (0.1 mmole) in dry pyridine (1 ml.), N-ethyl-5-phenylisoxazolium fluoroborate (0.5 mmole, 0.125 g.) was added, and the sealed reaction mixture which became warm on addition of the reagent was kept at room temperature. Aliquots (0.1 ml.) were removed and worked up exactly as described above for condensation using DCC. On paper chromatography of the reaction products in Solvent A, in addition to the nucleotide, its pyrophosphate, thymidylyl(3'→5')thymidine, and thymidine,[34] the slow formation (after a 1-day reaction) of a nucleotidic product with R_f 0.49 was noted. In addition, some ultraviolet

(32) Prepared by pipetting a known amount of hydrochloric acid into pyridine and evaporating in the presence of pyridine.

(33) Cf., ref. 16.

(34) The spot corresponding to thymidine was impure in that it contained another material with λ_{max} 252 mμ. The latter which presumably originated from the reagent could be separated by paper electrophoresis at pH 7.1 in which operation only thymidine had zero mobility. Because of the impurity contaminating thymidine, the yields of thymidylyl(3'→5')thymidine were calculated on the basis of the total products derived from the nucleotide component.

absorbing material with R_f 0.86 was present. The latter had λ_{max} (H^+) 249 mμ and was, presumably, derived from the reagent. The yield of thymidylyl($3'\rightarrow5'$)thymidine was calculated on the basis of the unchanged nucleotide which was the sum of thymidine-5' phosphate, P^1,P^2-di(thymidine-5') pyrophosphate, and the unidentified product (R_f 0.49). The yield of thymidylyl-($3'\rightarrow5'$)thymidine after 1 day was 50%, and 68% after 3 days.

The product with R_f 0.49 (Solvent A) had an ultraviolet absorption spectrum like that of thymidine and was completely converted to thymidine-5' phosphate on treatment with 1 N hydrochloric acid at room temperature for 24 hr. On paper electrophoresis at pH 7.1, it had mobility 0.64 relative to thymidylic acid; at pH 3.6 its mobility was 0.89 relative to thymidylic acid.

Repeating the above experiment, the reaction mixture was extracted with ether before the alkaline treatment, and the ultraviolet absorbing material traveling faster than thymidine mostly was absent.

B. Using System B.—An anhydrous pyridine solution (1.0 ml.) of pyridinium 5'-O-acetylthymidine-3' phosphate (0.1 mmole) and 3'-O-acetylthymidine (0.1 mmole) was treated with the reagent (III, 0.5 mmole, 0.125 g.), aliquots (0.1 ml.) were removed at intervals, and each was treated with 0.5 ml. of water for 1–16 hr. After subsequent evaporation, the residue was treated with 0.5 ml. of 1 N sodium hydroxide. After neutralization with pyridinium Dowex-50 ion-exchange resin, the products were chromatographed in Solvent A. The major products formed were as described above under A, but a number of other trace products (detectable on paper electrophoresis of bands eluted from paper chromatograms in Solvent A) were also detected. The yield of thymidylyl($3'\rightarrow5'$)thymidine after 1 day was 60%, and after 2 days it was 68%.

Attempted Synthesis of Thymidylyl($3'\rightarrow5'$)thymidine Using Ethoxyacetylene.—A reaction mixture (0.1 mmole scale) was set up using System A and freshly distilled ethoxyacetylene (0.09 ml., about 1 mmole) as the reagent. Aliquots (0.1 ml.) were removed from the reaction mixture at different intervals and, after addition of water (0.5 ml.), the solvent and the excess reagent were removed by evaporation *in vacuo*. The alkali treatment and paper chromatography were as described above. No thymidylyl($3'\rightarrow5'$)thymidine was detected up to 4 days, the only products being thymidine-3' phosphate and the corresponding pyrophosphate. The amount of the latter was 14% in 2 hr. and 82% in 22 hr.

Thymidylyl($3'\rightarrow5'$)thymidine Using Phosgene–Dimethylformamide Reagent (IV).—The reagent was prepared by passing phosgene through an anhydrous chloroform solution of dimethylformamide and subsequent removal of the solvent and excess phosgene *in vacuo* at 80°. Because of the hygroscopic nature of the reagent, the following experiment was carried out in a drybox.

To the dry reagent (IV, 0.191 g., 1.5 mmoles) was added an anhydrous pyridine solution (2 ml.) of 5'-O-tritylthymidine (0.5 mmole) and pyridinium 3'-O-acetylthymidine-5' phosphate (0.5 mmole). Aliquots were removed and worked up as described above under DCC experiments with System A. The yield of thymidylyl($3'\rightarrow5'$)thymidine was estimated to be 21.2% in 15 min., 31% in 17 hr., and 29% after 2 days at room temperature.

In another experiment, when 1 mmole of the reagent was used for 0.5 mmole of the pyridinium nucleotide, the yield (maximum in the 1-hr. aliquot) was 12.5%. When a much larger excess (4 mmoles for 0.5 mmole of the nucleotide) was used, an exothermic reaction was observed, a number of colored products were formed, and the yield of the desired product could not be determined.

Attempted Synthesis of Thymidylyl($3'\rightarrow5'$)thymidine Using Ethyl Metaphosphate as the Condensing Agent.—A mixture of phosphorus pentoxide (3.45 g.), chloroform (5 ml.), and ether (10 ml.) was heated under reflux for 48 hr. when a homogeneous solution resulted. The solvent was removed *in vacuo*, and to the resulting sirup was added an anhydrous pyridine solution (2 ml.) of 5'-O-tritylthymidine (0.1 mmole) and pyridinium 3'-O-acetylthymidine-5' phosphate (0.1 mmole). The total mobile solution was kept sealed at room temperature for 10 days. The total reaction mixture then was treated with 30 ml. of ice water. The aqueous solution was extracted with ether and then with

chloroform. The ether, chloroform, and aqueous solutions were analyzed separately by the combined techniques of paper chromatography (Solvent A) and paper electrophoresis.

The aqueous layer contained mainly a cationic compound identified as N-ethylpyridinium cation. Although other minor ultraviolet absorbing compounds were present, no thymidylyl-($3'\rightarrow5'$)thymidine could be detected.

The ether extract also contained cationic compounds and, in addition, an ultraviolet absorbing compound which after treatment with 80% acetic acid was converted to ethyl thymidine-3' phosphate. The latter product, as expected, was resistant to the action of the venom phosphodiesterase[35] but was completely hydrolyzed to thymidine-3' phosphate on incubation with the spleen phosphodiesterase.[36]

The material in the chloroform extract was treated with aqueous sodium hydroxide to remove the acetyl groups and, upon examination by paper chromatography in Solvent A, was found to contain four ultraviolet absorbing compounds. The product composition was complex as judged by a combination of paper electrophoresis and paper chromatography. After acidic treatment, ethyl thymidine-3' phosphate was identified as a product. No thymidylylthymidine could be detected.

Relative Rates of Sulfonylation of the 3'-Hydroxyl Group in 5'-O-Tritylthymidine.—Anhydrous pyridine solutions (1 ml. each) of 5'-O-tritylthymidine (0.1 mmole) were treated, each with 0.5 mmole of mesitylenesulfonyl chloride and p-toluenesulfonyl chloride at room temperature. At intervals, 0.1-ml. aliquots were removed and treated with 0.5 ml. of water with cooling. After evaporation of the solvent, the residue was dissolved in 1 ml. of 80% acetic acid at 100° for 10 min. The acetic acid then was removed under suction, and the residue was examined as its solution in aqueous ethyl alcohol by paper chromatography. The spots corresponding to thymidine and the sulfonylthymidine were eluted, and their concentrations determined spectrophotometrically. The results are in Table III.

TABLE III

RELATIVE RATES OF 3'-O-ARYLSULFONYLTHYMIDINE FORMATION

Time	Mesitylenesulfonyl-thymidine, %	p-Toluenesulfonyl-thymidine, %
5 hr.	8	15
21 hr.	30	43
2 days	55	72
5 days	82	87
7 days	86	86

The Reaction of Pyridinium 3'-O-Acetylthymidine-5' Phosphate with Mesitylenesulfonyl Chloride.—In a series of experiments anhydrous pyridinium 3'-O-acetylthymidine-5' phosphate (0.1 mmole) was treated in dry pyridine (1 ml.) with varying amounts of mesitylenesulfonyl chloride with exclusion of moisture. After 7 min. at room temperature, an excess of water was added, the products were examined by paper chromatography in the neutral Solvent B, and their amounts were determined spectrophotometrically after elution of the spots. When 0.05 mmole of the sulfonyl chloride was used, the ratio of P^1,P^2-3'-O-acetylthymidine-5' pyrophosphate to 3'-O-acetylthymidine-5' phosphate was 4:1. In the experiment using 0.1 mmole of the sulfonyl chloride, the ratio of the pyrophosphate to the mononucleotide was 1:1.5. When 0.5 mmole (5 molar equiv. to the nucleotide) of the sulfonyl chloride was used, 3'-O-acetylthymidine-5' phosphate was detected as the only nucleotidic product.

In another experiment, DCC (0.5 mmole) was first added to the anhydrous pyridine solution (1 ml.) of pyridinium 3'-O-acetylthymidine-5' phosphate (0.1 mmole). After 15 min. at room temperature, mesitylenesulfonyl chloride (0.2 mmole) was added and, after another 9 min. at room temperature, water (10 ml.) was added; the products were examined by paper chromatography. The major product (87%) was 3'-O-acetylthymidine-5' phosphate, and the other product (13%) was the corresponding pyrophosphate.

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(36) W. E. Razzell and H. G. Khorana, *ibid.*, **236**, 1144 (1961).