[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH OF THE UNIVERSITY OF WISCONSIN, MADISON, WISCONSIN]

Studies on Polynucleotides. XVII.[1] On the Mechanism of Internucleotide Bond Synthesis by the Carbodiimide Method[2]

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RECEIVED APRIL 16, 1962

In mechanistic studies of the formation of an internucleotide bond by the condensation of a protected mononucleotide with the hydroxyl group of a suitably protected nucleoside in the presence of DCC, the following two sets of experiments were carried out: 1, the study of rates of phosphorylation of 3′-*O*-acetylthymidine, 5′-*O*-acetylthymidine and 5′-*O*-trityl thymidine by a mixture of β-cyanoethyl phosphate (stoichiometric amount) and DCC (excess). 2, the rate of formation and the final yield of thymidylyl-(3′ → 5′)-thymidine in condensation of 3′-*O*-acetylthymidine with different 5′-*O*-substituted thymidine-3′ phosphates (5′-*O*-trityl-, 5′-*O*-mesitoyl-, 5′-*O*-trimethylacetyl- and 5′-*O*-acetyl-thymidine-3′ phosphate). The results showed that the size of the protecting groups in the nucleoside (the hydroxylic component) does not have very significant effect but that the size of the organic groups in the phosphomonoester component (the nucleotide) has a profound influence on the rate and the yields of the phosphodiester. The reaction of monoethyl phosphoric acid with DCC in dioxane rapidly gave a product which is shown to be triethyl trimetaphosphate. The reaction of 3′-*O*-acetylthymidine-5′ phosphate or P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate with DCC in anhydrous pyridine also rapidly gave a product which is concluded to be the corresponding trimetaphosphate. The reaction of β-cyanoethyl phosphate with DCC in pyridine followed by alkaline treatment gave inorganic tripolyphosphate as the major product. The reaction of monobenzyl phosphoric acid with DCC in pyridine followed by heating at 100° gave inorganic trimetaphosphate as the main product. The reaction of monobenzyl phosphoric acid with DCC in acetonitrile followed by treatment with sodium iodide at 55° again gave inorganic trimetaphosphate. The previously described product from the reaction of bis-tri-*n*-butylammonium orthophosphate with DCC in pyridine is shown to be inorganic trimetaphosphate. The mechanism of phosphodiester bond synthesis from phosphomonoesters is discussed in the light of the above findings, and it is concluded that the initial phosphorylating species is probably an alkyl trimetaphosphate. The mechanism of reaction of phosphomonoesters with DCC in the presence and absence of trialkylamines [M. Smith, J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958)] is further discussed.

Introduction

A prerequisite to the elaboration of polynucleotide synthesis is an efficient method for the formation of the naturally occurring $C_{3'}$–$C_{5'}$ internucleotide linkage. In principle the synthesis of such a linkage may be approached in two ways. In the first approach, two nucleosides, one bearing a free 3′-hydroxyl function and the second a free 5′-hydroxyl function, may be phosphorylated in successive steps by a bifunctional phosphorylating agent. Although this approach has been used with considerable success in the phospholipid field,[3] there are severe practical limitations in the application of this approach in the polynucleotide field.[4,5] The second general approach to the internucleotide bond synthesis which so far has been much the more satisfactory involves the activation of a monoester of phosphoric acid (a mononucleotide) so as to cause the phosphorylation of the hydroxyl function of a second suitably protected nucleoside or nucleotide. The process affords in this manner the desired diester of phosphoric acid directly. Carbodiimides, especially dicyclohexylcarbodiimide (DCC), and certain reactive anhydrides, for example, *p*-toluenesulfonyl chloride,[4–6] have so far been the most effective reagents although a number of others have been studied.[7] Very early experiments had indicated the complexity of the mechanism of phosphodiester bond synthesis by this general method,[4–6,8,9] and the formation of symmetrical P^1,P^2-dialkyl pyrophosphates was recognized as the first step in the activation process. All the subsequent work with the different reagents used has similarly demonstrated the involvement of the pyrophosphates when stoichiometric amounts of the hydroxylic component are used in the synthesis of diesters of phosphoric acid.[4,10] The actual phosphorylating species involved have been postulated to be linear polyphosphates or metaphosphates,[5,11,12] the postulate being based on an appreciation of the properties of phosphate esters in general. More recently, the monomeric metaphosphate has been implicated[13] by Todd as the phosphorylating agent in these methods. Since an intimate knowledge of the mechanism of the diester bond synthesis is vital to the problem of polynucleotide synthesis and since DCC has so far been the reagent of choice, we have carried out a closer study of the formation of internucleotide bonds using this reagent. The present paper reports on (a) the results that we have obtained regarding the influence of the structure of the nucleotides and nucleosides on the rate of formation and the final yield of the internucleotide bonds, and (b) the experiments which show that, at least initially, the actual phosphorylating species is very probably a trialkyltrimetaphos-

(1) Paper XVI, D. H. Rammler and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 3112 (1962).

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

(3) See *e.g.*, E. Baer, *Can. J. Biochem. and Physiol.*, **34**, 288 (1955).

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

(5) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6212 (1958).

(6) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *ibid.*, **80**, 6223 (1958).

(7) See *e.g.* (a) F. Cramer and G. Weimann, *Chem. Ber.*, **94**, 996 (1961). (b) F. Cramer, *Angew. Chem.*, **72**, 718 (1960).

(8) H. G. Khorana, G. M. Tener, J. G. Moffatt and E. H. Pol, *Chem. and Ind.* (London), 1523 (1956).

(9) M. Smith, J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 6204 (1958).

(10) For reactions involving trichloroacetonitrile, see F. Cramer and H. J. Baldauf, *Angew. Chem.*, **72**, 627 (1960); P. T. Gilham, unpublished work.

(11) P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4647 (1959).

(12) See pages 22 and 140 in ref. 4.

(13) A. R. Todd, *Proc. Natl. Acad. Sci.*, (U. S.), **45**, 1389 (1959); *Proc. Chem. Soc.* (London), 187 (1961).

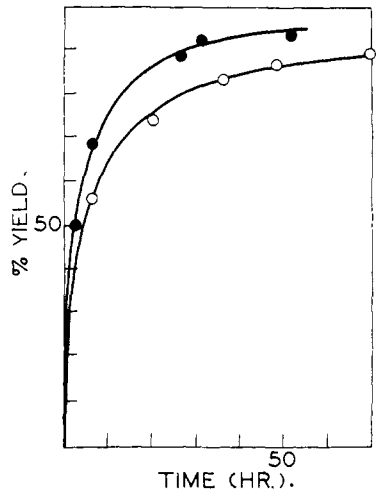


Fig. 1.—The rate of phosphorylation of 3′-*O*-acetylthymidine (solid circle curve) and of 5′-*O*-tritylthymidine (hollow circle curve) using stoichiometric amounts of β-cyanoethyl phosphate and an excess of DCC in pyridine at room temperature. The yields shown are those of the nucleotides (thymidine-5′ phosphate or thymidine-3′ phosphate) obtained after removal of protecting groups (see text).

phate. A brief report of these results has already appeared.[14]

Influence of the Structure of the Phosphomonoester on the Rate and Yield of the Product.—Early experiments[15] using simple alkyl phosphates such as monoethyl phosphate and alcohols such as ethyl alcohol showed that the yield of the diesters from equimolar concentrations of the two components and an excess of DCC were essentially quantitative at room temperature. Precise analysis of the results was complicated, however, by a side reaction, namely, the nucleophilic attack of pyridine, the medium of reaction, on the alkyl group of the activated phosphomonoester component. For this reason, and because of the much higher reactivity of the hydroxylic groups in simple alcohols than that of the hydroxylic groups in nucleosides, the model experiments were not pursued further. With the development of the phosphorylating agent consisting of a mixture of β-cyanoethyl phosphate and DCC,[16,17] experiments much more relevant to the theme of internucleotide bond synthesis became possible. In the present work, the rates of phosphorylation of the 5′-hydroxyl group in 3′-*O*-acetylthymidine (I) and of the 3′-hydroxyl groups in 5′-*O*-trityl- (III)[18] and 5′-*O*-acetyl-thymidine (IV) were studied using stoichiometric amounts of β-cyanoethyl phosphate and an excess of DCC. The reaction was followed by analyzing for the formation of thymidine-5′ phosphate (II) (from I), and thymidine-3′ phosphate (VI) (from III and IV) after removal of the protecting groups from the initial products (Chart I).

Figure 1 gives the results obtained with 3′-*O*-acetylthymidine and 5′-*O*-tritylthymidine. The phosphorylation of both the primary and the secondary hydroxyl groups in I and III respectively, virtually went to completion, the phosphorylation of the secondary hydroxyl group being somewhat slower. The method is thus capable of giving quantitative yields of nucleotides using stoichiometric amounts of the two components when a small-sized phosphomonoester such as cyanoethyl phosphate is used. Of particular interest also is the finding that the rates of phosphorylation of 5′-*O*-tritylthymidine and 5′-*O*-acetylthymidine (IV) were very similar. Thus after a 5.5 hour reaction time, the yield of the nucleotide from 5′-*O*-acetylthymidine was 59%

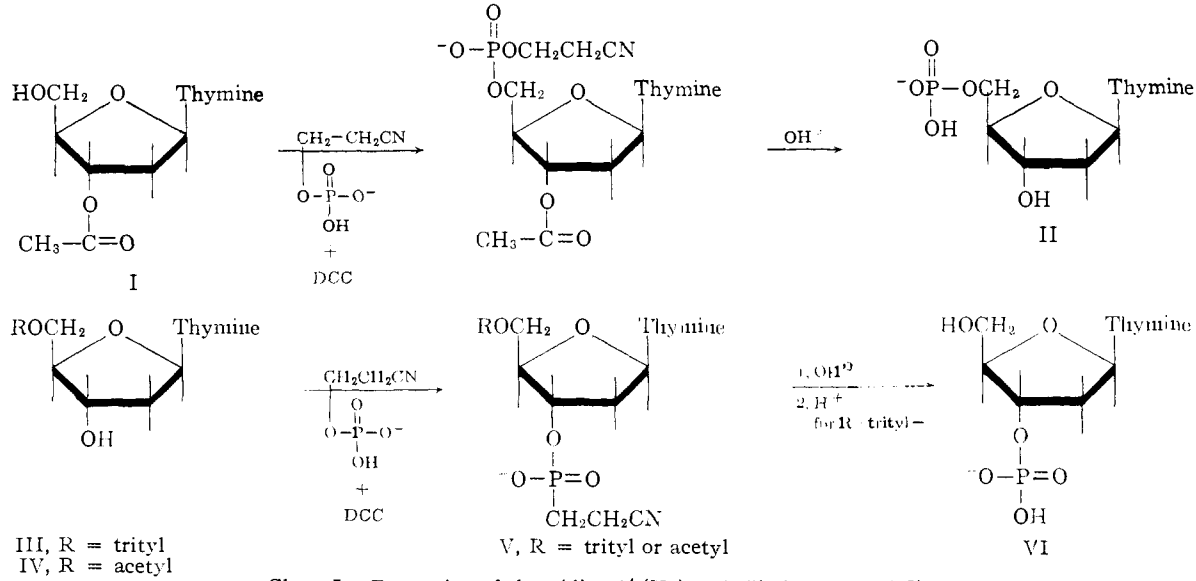


Chart I.—Formation of thymidine-3′ (VI) and -5′ phosphates (II).

(14) G. Weimann and H. G. Khorana, *Chem. and Ind.* (London), 271 (1962).

(15) P. T. Gilham and H. G. Khorana, unpublished work (experiments carried out in 1956).

(16) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).

(17) The activated intermediates derived from β-cyanoethyl phosphate or from the nucleotides do not suffer nucleophilic attack by pyridine on the alkyl groups involved in the ester linkages.

(18) The rate of phosphorylation of 5′-*O*-tritylthymidine has previously been studied by Tener.[16]

while that from 5′-*O*-tritylthymidine was 56.5%. Thus, in the phosphorylation of the 3′-hydroxyl group, there is no marked effect of the size of the substituent on the 5′-hydroxyl group. This is in contrast, as shown below, to the effect observed for a similar change in the phosphomonoester component.

Previously[5] it was determined that in the reaction of equimolar amounts of 3′-*O*-acetylthymidine-5′ phosphate (VII) and 5′-*O*-acetyl- or 5′-*O*-tritylthymidine (III) in pyridine at room temperature the yield of thymidylyl-(3′→5′)-thymidine (VIII), (Route 1, Chart II), isolated after removal of the protecting groups, leveled off at about 60% (Fig. 2).[18a] The maximum yield using 5′-*O*-tritylthymidine was 67%.[18a] The similarity in yield using 5′-*O*-acetyl or 5′-*O*-tritylthymidine may again be noted. The rate of reaction of 5′-*O*-tritylthymidine-3′ phosphate (IX; R = trityl) with 3′-*O*-acetylthymidine (Route 2; Chart II) using stoichiometric amounts of the two components is shown in Fig. 2. Thus, there was a marked reduction in the rate and in the final yield,[19] although the phosphorylation of a primary hydroxyl group rather than that of a more hindered secondary hydroxyl group was involved. The conclusion was, therefore, drawn that the rate of phosphorylation and the final yield was markedly influenced by the size of the phosphomonoester group and hence that of the phosphorylating species derived from it. This conclusion also follows from the results reported above in which when going from β-cyanoethyl phosphate to the bulkier 3′-*O*-acetylthymidine-5′ phosphate the ultimate yield of the phosphorylation product fell from near quantitative to about 67%. The influence of the size of the phosphomonoester on the yield of the phosphodiester bond was examined further by varying the substituent on the 5′-hydroxyl group in thymidine-3′ phosphate.

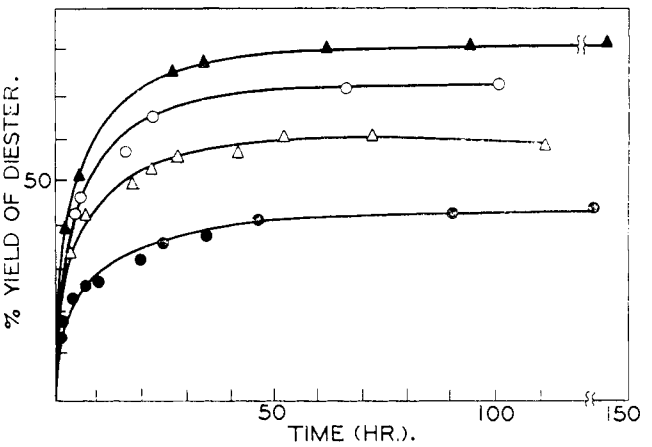


Fig. 2.—The rates of formation of thymidylyl-(3′ →5′) thymidine by the condensation of protected nucleotide (0.1 *M*) and protected nucleoside (0.1 *M*) in pyridine at room temp. in the presence of DCC (0.5 *M*). For work-up and estimation of yield see text: △-△-△-△, from 3′-*O*-acetylthymidine-5′ phosphate and 5′-*O*-acetylthymidine; ●-●-●-● from 5′-*O*-tritylthymidine-3′ phosphate and 3′-*O*-acetylthymidine; O-O-O-O, from 5′-*O*-trimethylacetylthymidine-3′ phosphate and 3′-*O*-acetylthymidine; ▲-▲-▲ from 5′-*O*-acetylthymidine-3′ phosphate and 3′-*O*-acetylthymidine.

The protected derivatives of thymidine-3′ phosphate used were 5′-*O*-acetylthymidine-3′ phosphate,[20] 5′-*O*-trimethylacetylthymidine-3′ phosphate and 5′-*O*-mesitoylthymidine-3′ phosphate. The starting materials for the last two derivatives were, respectively, 5′-*O*-trimethylacetylthymidine and 5′-*O*-mesitoylthymidine, both of which were prepared in satisfactory yields by the reaction of thymidine with slightly more than stoichiometric amounts of the corresponding acid chlorides. The subsequent steps in the preparation of the protected nucleotides were as illustrated in the formulae (X → XI → IX). Of particular note is the selective removal of the β-cyanoethyl group by very mild alkaline treatment of the diesters (XI). It may also be added that an acetyl group

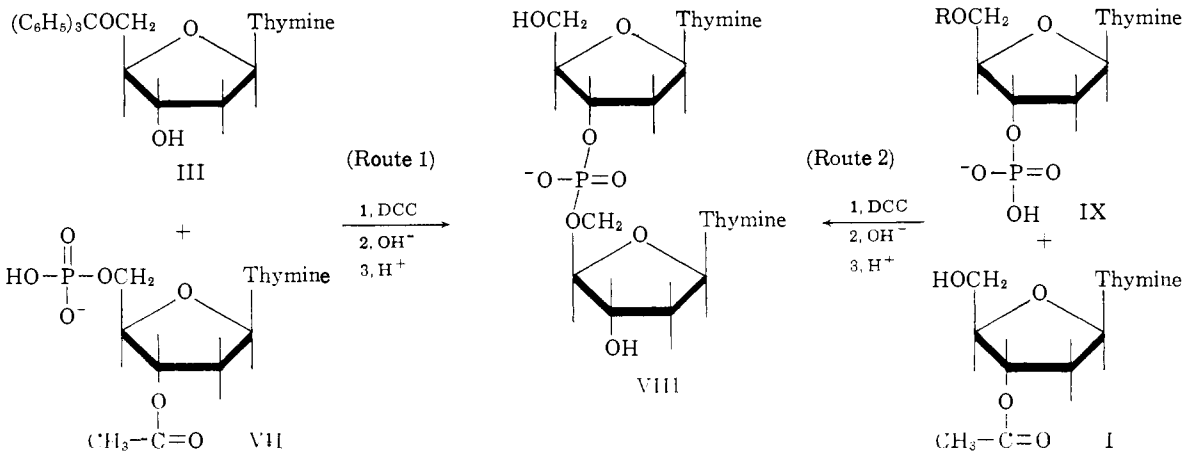


Chart II.—Synthesis of thymidylyl-(3′ → 5′)-thymidine.

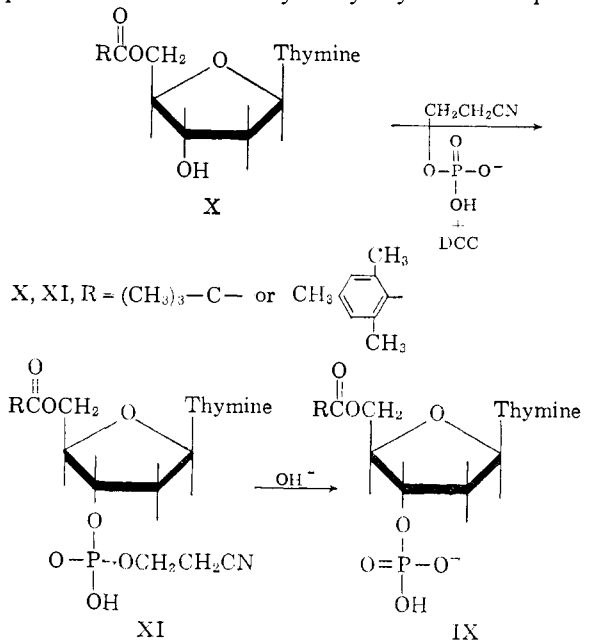
(18a) In recent experiments (H. Schaller and H. G. Khorana, to be published) the yield of thymidylyl- (3′ → 5′) thymidine from the reaction of equimolar amounts of 3′-*O*-acetylthymidine-5′ phosphate and 5′-*O*-tritylthymidine has been around 75%. The distinctly lower yield using 5′-*O*-acetylthymidine (Fig. 2) is to be ascribed to the omission of the acidic step in work-up.

(19) G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 419 (1962).

(20) Prepared from thymidine-3′ phosphate by acetylation as described for thymidine-5′ phosphate: H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

present at another portion of an oligonucleotide chain can be selectively removed without significantly affecting the trimethylacetyl or mesitoyl group. While the use of these groups in the polynucleotide synthesis will be reported subsequently, it may be noted here that the general principle in our previous work on polynucleotide synthesis[4] has been to use an acid-labile group (*e.g.*, a trityl or a substituted trityl) at one end and an alkali-labile group at the other end. The development now reported aims at the use of alkali-labile groups only, which nevertheless, offer selectivity in removal.

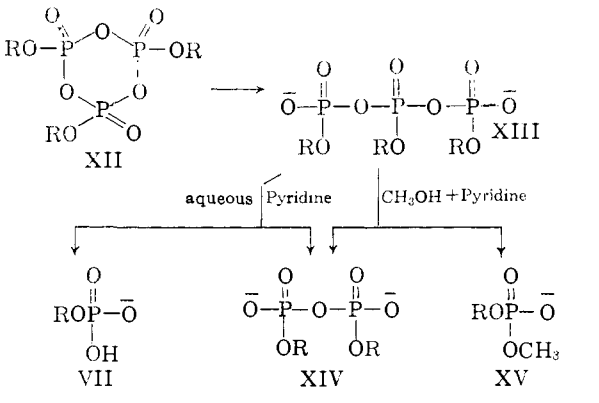
Included in Fig. 2 are the rates of formation of thymidylyl-(3′→5′)-thymidine by the condensation of, respectively, 5′-*O*-acetylthymidine-3′ phosphate and 5′-*O*-trimethylacetylthymidine-3′ phosphate with 3′-*O*-acetylthymidine. A similar kinetic study using 5′-*O*-mesitoylthymidine-3′ phosphate as the nucleotide component in this set of experiments (see Experimental) showed that the yield of thymidylyl-(3′→5′)-thymidine levelled off at 60%. The use of 5′-*O*-acetylthymidine-3′ phosphate thus gave the highest rate and ultimate yield (81%) of the internucleotidic bond that has so far been obtained using stoichiometric amounts of the two components.



The total of experiments described above[21] on the phosphorylation of hydroxyl groups in nucleosides by activation of phosphomonoesters clearly demonstrate the profound influence of the size of the organic group in the phosphomonoester on the ultimate yield of the phosphorylation product.

It should also be emphasized here that as reported previously,[4,19,22,23] when an excess of either one of the two components over the second one is used, the yields of the desired products are quantitative with respect to the component used in lesser amount. While the bearing of these findings on the mechanism of phosphodiester bond synthesis will be discussed elsewhere,[23] the following experiments were carried out to obtain insight into the precise nature of the phosphorylating agent involved in these reactions.

The Nature of the Initial Phosphorylating Agent.—Pyridinium 3′-*O*-acetylthymidine-5′ phosphate was reacted with DCC at room temperature in dry pyridine for a period of 10 minutes during which time the diester bond synthesis is not appreciable. The nucleotidic product was freed from the excess of DCC and pyridine by rapid precipitation with ether. Dissolution of the product in water followed by paper-electrophoresis at pH 7.5 in triethylammonium bicarbonate buffer revealed one major product (about 80%) shown below to be P¹,P²,P³-tri-3′-*O*-acetylthymidine-5′ triphosphate (XIII; R = 3′-*O*-acetylthymidine-5′). (The two other minor products detected were 3′-*O*-acetylthymidine-5′ phosphate, VII, and P¹,P²-di-3′-*O*-acetylthymidine-5′ pyrophosphate, XIV). The formulation of the major product as (XIII; R = 3′-*O*-acetylthymidine-5′) is based on the following evidence. Consistent with the assigned structure, it travelled slower than P¹,P²-di-3′-*O*-acetylthymidine-5′ pyrophosphate (XIV) on paper electrophoresis. Treatment with aqueous pyridine at room temperature for 0.5 hr. caused complete degradation and gave equimolar amounts of 3′-*O*-acetylthymidine-5′ phosphate (VII) and P¹,P²-di-3′-*O*-acetylthymidine-5′ pyrophosphate (XIV). Finally, treatment of the product immediately after purification by paper-electrophoresis with an excess of methyl alcohol and pyridine gave equimolar amounts of the pyrophosphate (XIV) and methyl 3′-*O*-acetylthymidine-5′ phosphate (XV), the latter product being characterized further after removal of the acetyl group.



R = 3′-*O*-acetylthymidine-5′

When pyridinium P¹,P²-di-3′-*O*-acetylthymidine-5′ pyrophosphate (XIV)[24] was treated with DCC in dry pyridine for 10–30 min., the major product isolated again was XIII and the nature and the

(21) A further striking result reinforcing the present conclusion has been obtained in the condensation of N,3′-*O*-bis-dimethoxytrityldeoxyguanosine-5′ phosphate with 5′-*O*-dimethoxytritylthymidine by the standard method. The ultimate yield of the internucleotidic bond [thymidylyl-(3′ → 5′)-deoxyguanosine] was only 12%. H. Schaller and H. G. Khorana, *ibid.*, in press.

(22) H. G. Khorana, *J. Cellular Comp. Physiol.*, **54**, 5 (1959).

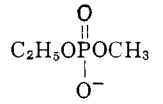
(23) H. Schaller and H. G. Khorana, *Chem. and Ind.* (London), 699 (1962).

(24) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959).

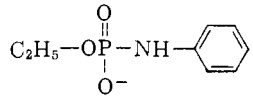
proportions of the minor products formed were also similar to those obtained when 3′-*O*-acetylthymidine-5′ phosphate was the starting material. The result shows that the activation of the pyrophosphate (XIV) and the nature of the phosphorylating species derived from it is identical with that derived from the phosphomonoester. This is confirmation of the view expressed earlier[25] that the dialkyl pyrophosphates can serve equally well as starting materials in the synthesis of diesters of phosphoric acid. The possible intermediates involved in the formation of XII when XIV is used as the starting material are discussed below.

Since the linear triphosphate (XIII) is not expected to be a powerful phosphorylating agent, the actual phosphorylating species is concluded to be (XII), and it seems probable, in view of the previous work,[26] that XIII arises from it on exposure to water. Attempts to demonstrate the presence of XII in the nucleotidic material precipitated with ether were unsuccessful. The failure is ascribed to a number of technical difficulties. For example, these experiments were carried out on a very small scale, and the complete removal of pyridine and DCC from the product necessitated repeated washing with ether and the complete exclusion of moisture during these operations was probably not realized. Further experiments to demonstrate the formation of XII were carried out on a larger scale using the more readily available ethyl phosphoric acid.[27]

Brief treatment of ethyl phosphoric acid in dry dioxane with an excess of DCC followed by careful work-up to ensure complete removal of DCC gave a product which was proven to be mainly ethyl trimetaphosphate (XII; R=ethyl). (The latter previously has been prepared by the reaction of silver trimetaphosphate with ethyl iodide.[26,28]) Thus, on treatment with methyl alcohol, it gave ethyl methyl phosphate (XV) in 85–90% yield,[29] and, further on being refluxed with an excess of



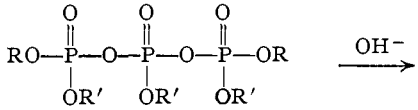
XV



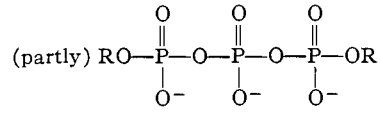
XVI

aniline[30] it gave ethyl phosphoroanilidate (XVI) in over 90% yield. On dissolution in water, triethyl metaphosphate (XII; R = ethyl) would be rapidly converted to XIII (R = ethyl) and paper electrophoresis, indeed, showed the presence of a single major product having mobility consistent with the structure XIII.

In the above experiments, the possibility was considered that monomeric metaphosphate was the species actually present and that the large increase in concentration resulting from precipitation caused its polymerization to XII. To rule out this possibility, an experiment was devised on the basis of a finding reported previously. It had been noted[19] that when after phosphorylation of 5′-*O*-tritylthymidine with a mixture of β-cyanoethyl phosphate and DCC, the reaction mixture was directly treated with alkali, a side product concluded to be XVIII was encountered. This could only have arisen from XVII by the base-catalyzed elimination of β-cyanoethyl groups in preference to the cleavage of the pyrophosphate linkages. It was, therefore, hoped that in compounds of the type XII or XIII obtained by the

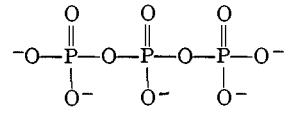


XVII; R = 5′-*O*-tritylthymidine-3′,R′ = CH_2CH_2CN



XVIII, R = 5′-*O*-tritylthymine-3′

reaction of β-cyanoethyl phosphate with DCC, elimination might again occur to give inorganic poly- or meta-phosphate. After the reaction of β-cyanoethyl phosphate with DCC in dry pyridine for 10 min. the total mixture was directly treated with aqueous sodium hydroxide at room temperature. The major product obtained was identified



XIX

as inorganic tripolyphosphate (XIX). It is important to note that no β-cyanoethyl phosphate or inorganic phosphate was detected as a product. Any monomeric metaphosphate derived from β-cyanoethyl phosphate would have generated the latter or inorganic phosphate on alkaline treatment. The result shows that all of the phosphomonoester was, indeed, converted to a polymeric anhydride on reaction with DCC. Again, because of the extreme sensitivity of the trimetaphosphate (XII) to water, it is believed that the metaphosphate was instantaneously converted to XIII (R = cyanoethyl), and because of the relative stabilization of pyrophosphate linkages due to the phosphoryl dissociations in the latter, elimination of the cyanoethyl groups superseded the pyrophosphate bond cleavage and resulted in the formation of XIX.

Benzyl esters of phosphoric acid are especially susceptible to nucleophilic attack by tertiary bases,[31] and further experiments were carried out with monobenzyl phosphoric acid with a view to

(25) A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4651 (1959).

(26) Among other earlier studies, there are the as yet unpublished investigations of V. J. Reilly and D. Lipkin (V. J. Reilly, Ph.D. thesis, Washington University 1949). These elegant studies included the kinetics of the ring opening of triethylmetaphosphate (XII; R = ethyl) and related compounds. XII (R = ethyl) was shown to hydrolyze almost instantaneously in water at 0° to the linear triphosphate (XIII; R = ethyl).

(27) Experiments were carried out in a dry box maintained under slightly positive dry nitrogen pressure.

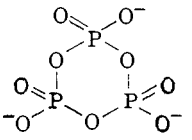
(28) F. Cramer and H. Hettler, *Chem. Ber.*, **91**, 1181 (1958).

(29) We believe this result to be consistent with the formulation of the activated product as trimetaphosphate (XII; R = ethyl). It should be pointed out that the result is not in agreement with that reported previously on the methanolysis of methyltrimetaphosphate,[28] in which a 50% yield of tetramethylpyrophosphate and a 35% yield of monomethyl phosphate was recorded.

(30) G. Schramm and H. Wissmann, *Chem. Ber.*, **91**, 1073 (1958).

(31) See *e.g.*, J. Baddiley, V. M. Clark, J. J. Michalski and A. R. Todd, *J. Chem. Soc.*, 815 (1949); V. M. Clark and A. R. Todd, *ibid.*, 2023, 2030 (1950).

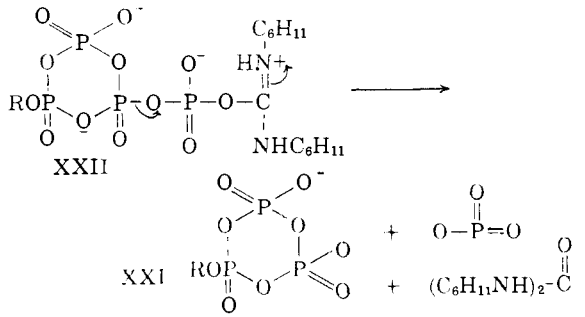
the isolation of trimetaphosphate (XX) *in situ* after activation of the phosphomonoester with DCC.



XX

The reaction of pyridinium monobenzyl phosphate in pyridine with DCC at room temperature followed by heating at 100° for 0.5 hr. gave a product in about 50% yield which was identical on paper electrophoresis with authentic inorganic trimetaphosphate (XX). Again, the reaction of free monobenzyl phosphoric acid with DCC in acetonitrile at room temperature followed by treatment of the product with sodium iodide at 55° for 30 min. gave inorganic trimetaphosphate (XX). It seems reasonable to conclude that in both these experiments debenzylation of the initially formed benzyl trimetaphosphate (XII, R = benzyl) occurred to give XX and that the formation of the latter did not occur on further activation of an open chain debenzylated intermediate such as XIX.[32]

It was reported previously[33] that the reaction of bis-tri-*n*-butylammonium orthophosphate with DCC in pyridine gives a single, chromatographically homogeneous product. This product has now been characterized as the trimetaphosphate (XX), again by comparison with a sample of the crystalline silver salt prepared by the method of Thilo and Rätz.[34] In fact, the carbodiimide method provides an excellent means of preparation of XX. The result is perhaps relevant to the present findings in that it is indicative of the tendency for the formation of the trimetaphosphate ring.[35]

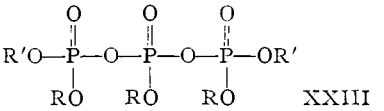


XXII XXI

Discussion

Internucleotide Bond Synthesis.—All the evidence presented above makes it highly probable that the trimetaphosphate (XII) derived from the starting material is the initial phosphorylating species. This result, which was predicted earlier,[4] follows from the expected higher nucleophilicity of phosphate ester anions than that of hydroxyl groups in nucleosides. The hypothetical monomeric metaphosphate may well be formed as a transient intermediate, but it would be expected to be attacked by a phosphate anion faster than by a neutral hydroxyl group.

Although the nature of the phosphorylating species at the start of the reaction may now be regarded as established, an analysis of the total sequence of events as the phosphodiester bond synthesis gets under way is difficult. Continual anhydride exchange reactions and the formation of new anhydride bonds by further activation with the carbodiimide would be expected to occur and, indeed, the complex kinetics of phosphodiester bond synthesis supports the conclusion drawn earlier (ref. 4, p. 140) that a number of phosphorylating species varying in "vigor" may actually be involved.[36] The process of phosphodiester bond synthesis would slow down with the accumulation of species such as XXIII, since compounds of this type are not expected to be powerful phosphorylating agents.



XXIII

An increase in the size of the phosphomonoester would be expected to have pronounced steric influence on the rate of diester bond synthesis, especially since the phosphomonoester is utilized as a polymeric anhydride. However, the final yields of the phosphodiesters should be the same. The fact that the phosphodiester bond synthesis came to a halt at different levels (Fig. 2) shows that other factors must be operating. Two possibilities can be conceived. The first one is that concurrently with the phosphorylation process anhydride exchange reactions occur which convert the activated intermediates to less reactive species. The slower the rate of phosphorylation, the higher would be the "loss" of the reactive species by means of the anhydride exchange reactions.[37] The second possibility is pointed out by the experiments to be reported separately.[23] In brief, in these experi-

(32) Like all the previously studied reactions of phosphate esters with DCC,[4] further reaction of XIX with DCC to form trimetaphosphate (XX) would require protons. No protons would be expected to be present after the debenzylation reactions employed above which lead to the formation, respectively, of benzyliodide and the sodium salt of the acid, and of the benzylpyridinium salt of the acid present in solution.

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

(34) E. Thilo and R. Rätz, *Z. anorg. u. allgem. Chem.*, **272**, 333 (1953).

(35) In the previously reported study[33] of the reaction of trialkylammonium nucleoside-5′ phosphates and inorganic phosphate with DCC, substantial amounts of nucleoside-5′ tetraphosphate and even higher polyphosphates were formed initially but with increase in time, the 5′-triphosphates increased at the expense of the higher polyphosphates. It was postulated that the triphosphate was stabilized by the formation of the trimetaphosphate (XXI). This postulate is further supported by the present work. Two alternatives seem reasonable regarding the formation of XXI. First, there may be disproportionation of, for example, a 5′-pentaphosphate, to XXI and inorganic pyrophosphate. Alternatively, the cyclization could follow activation of the terminal phosphoryl group as shown (XXII–XXI). (The other phosphoryl groups bearing only primary dissociations would be inert to further reaction in presence of a trialkylamine.[9])

(36) That the formed phosphodiester anions participate in mixed anhydride bond formation with activated intermediates derived from the phosphomonoesters is supported by the results obtained in the previous comprehensive study on the synthesis of the dinucleotide from 5′-*O*-tritylthymidine-3′ phosphate and β-cyanoethyl thymidine-3′ phosphate.[19] The pattern of yields obtained was radically different from that obtained in the condensation of a protected nucleotide with a *nucleoside* component.

(37) These would be of the general kind previously shown to occur by N. S. Corby, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 1234 (1952).

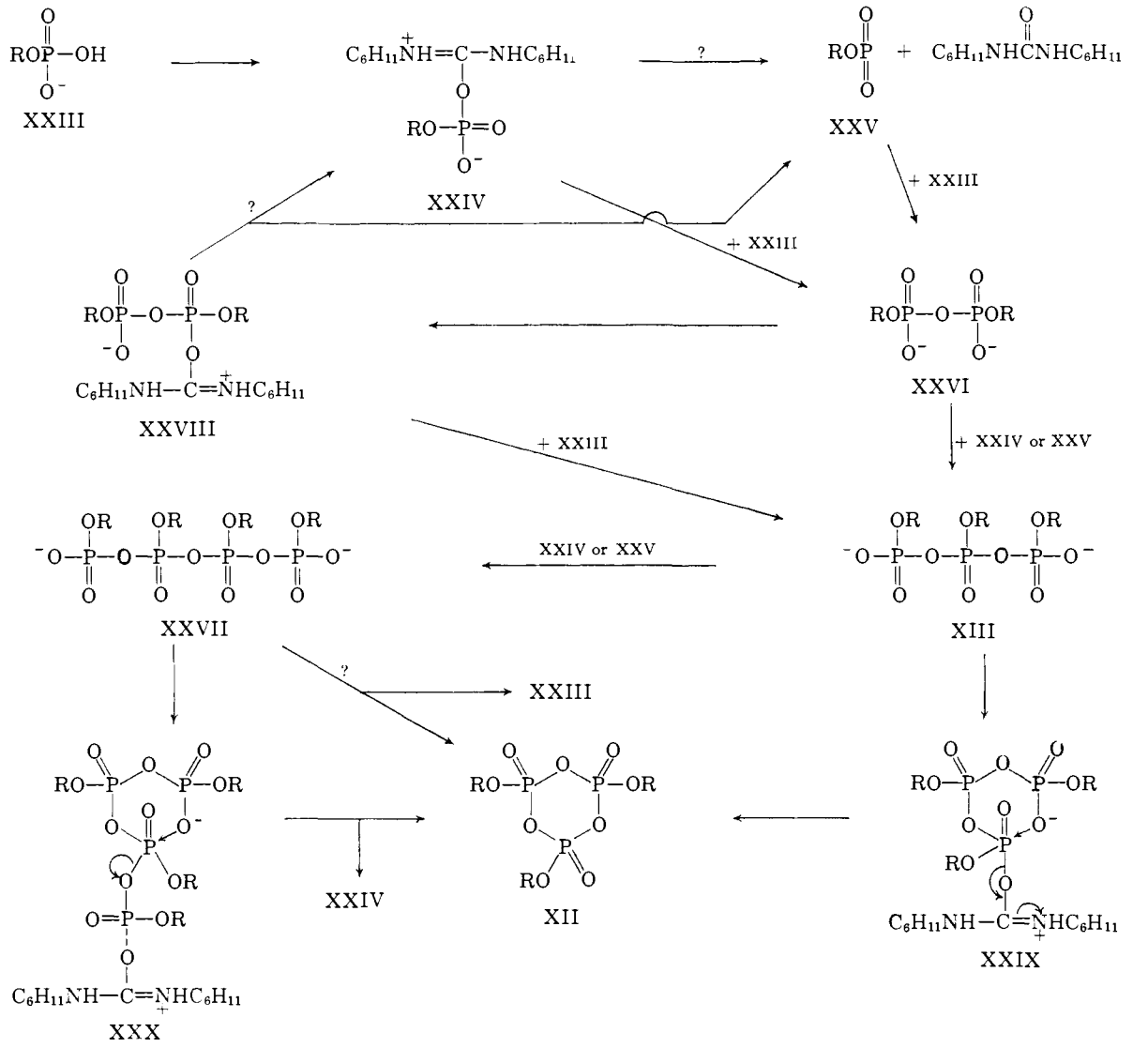


Chart III.—Formation of trimetaphosphate (XII) from monoalkyl phosphate (XXIII).

ments, a second mole of the protected nucleotide or nucleoside was added after the phosphodiester bond synthesis had levelled off using stoichiometric amounts of the two components. Whereas the addition of a second mole of the nucleotide did not cause any significant increase in the yield of the phosphodiester, the addition of a second mole of the protected nucleoside increased the yield of the phosphodiester to near quantitative with respect to the nucleotide component. It has, therefore, been tentatively concluded that the nucleoside becomes unavailable because of a side reaction. The loss of the hydroxylic component (nucleoside) because of this side reaction[38] could then be inversely proportional to the rate of phosphodiester bond synthesis.

The above conclusion on the nature of the initial phosphorylating species using DCC as the reagent can probably be extended to some other reagents. The differences in the different activating agents would reflect themselves in the rapidity with which the steps leading to the formation of the trimetaphosphate proceed. For example, at *room temperature*, trichloroacetonitrile,[19] ethoxyacetylene,[39] phosphoroamidates[40] and phosphoroimidazoles[41] appear to be effective in converting the phosphomonoesters to the corresponding pyrophosphates, but, with them, the subsequent steps seem to be sluggish. On the other hand, *p*-toluenesulfonyl chloride[6,42] appears to be the most effective reagent so far studied for phosphodiester bond synthesis.

(38) The side reaction could be assumed to be proceeding at a constant rate.

(39) T. M. Jacob and H. G. Khorana, *Chemistry and Industry*, 932 (1962).

(40) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 649 (1961).

(41) H. Schaller, H. A. Staab and F. Cramer, *Chem. Ber.*, **94**, 1621 (1961). A. F. Turner and H. G. Khorana, unpublished work.

(42) H. G. Khorana, J. P. Vizsolyi and R. K. Ralph, *J. Am. Chem. Soc.*, **84**, 414 (1962). Recent unpublished experiments by Dr. T. M. Jacob in this Laboratory show that the use of *p*-toluenesulfonyl chloride in the condensation of 3'-*O*-acetylthymidine-5' phosphate with 5'-*O*-tritylthymidine gives an excellent yield (90% using stoichiometric amounts of the two components) of thymidylyl-(3' → 5')-thymidine within 6 hr. at room temperature. Detailed studies on the mechanism of phosphodiester bond synthesis using aromatic sulfonyl chlorides will be reported subsequently.

The Formation of a Trimetaphosphate from a Phosphomonoester.—The possible intermediates in the conversion of a monoalkyl phosphate to a trimetaphosphate on treatment with DCC in pyridine will now be discussed. The starting point in all discussions of the carbodiimide reactions is the activation of the monoalkyl phosphate (XXIII) in the form of the protonated adduct (XXIV). The latter or the monomeric metaphosphate (XXV) derived from it will be attacked by another phosphomonoester anion to form the symmetrical pyrophosphate (XXVI). Although the preferred reaction initially would be the formation of the pyrophosphate (XXVI), it may be reasonably expected that as the concentration of the phosphomonoester (XXIII) diminishes and that of the pyrophosphate increases, the latter would also attack[43] the activated intermediate (XXIV or XXV) to form the linear triphosphate (XIII) and likewise the higher homologs (*e.g.*, XXVII) could also be formed. Experimental support for this conclusion was provided by carrying out an experiment with limited amount of DCC. At a stage when 44% of the starting material was recovered and the pyrophosphate (XXVI) amounted to 49%, there was present XIII in 7%. It is possible that some of the pyrophosphate (XXVI) arose by the rapid hydrolysis of XXVII (see below).

Further activation of the pyrophosphate (XXVI) would give the intermediate (XXVIII) which could eithr collapse to a mixture of XXIV and the metaphosphate (XXV)[44] or be attacked by anions to form linear tri- (XIII) or tetra-polyphosphate (XXVII). Further activation of XIII to form XXIX would result in the trimetaphosphate (XII). The formation of the trimetaphosphate (XII) from the tetrapolyphosphate (XXVII) would probably occur by further activation of the latter to form XXX which would collapse to XII and XXIV. It is conceivable but not likely that XXVII might also disproportionate to XII and the monophosphate anion (XXIII). In all the alternative paths that may be conceived, the ultimate product would be the trimetaphosphate XII. The total scheme in Chart III also readily explains that the net result is the same when XXIII or the pyrophosphate (XXVI) is used as the starting material.

The Reaction of Phosphomonoesters with DCC in the Presence of Trialkylamines.—Finally some observations made during the course of this work on the reaction of phosphomonoesters with DCC in the presence of a trialkylamine are herein recorded. These findings contribute further to the understanding of the mechanism of reactions of phosphate esters with carbodiimides (*cf.* ref. 9). It has been established previously[9] that the reaction of a monoalkyl phosphate with DCC at room temperature in the presence of two equivalents of a strong base such as tri-*n*-butylamine gives the pyrophosphate (XXVI) as the sole product. In the present work, the highly significant observation was made that when only one equivalent of the trialkylamine was used, the major product as detected by paper electrophoresis after 24 hr. at room temperature was XIII (R = 3′-*O*-acetylthymidine-5′). In a shorter time (2.5 hr.) the major product (54%) observed by the same technique was the pyrophosphate (XXVI; R = 3′-*O*-acetylthymidine-5′), while the tripolyphosphate (XIII, R = 3′-*O*-acetylthymidine-5′) was present in the amount of 26%, the remainder being an unidentified product with mobility less than that of XIII. None of the starting material (mononucleotide) was present among the products, either, of the short, or, of the long reaction period. In repetitions of the above experiments an excess of methyl alcohol was added to the mixtures after the two reaction periods mentioned above, and the products were separated after an alkaline treatment. In both cases, the products were methyl thymidine-5′ phosphate and P^1,P^2-di-thymidine-5′ pyrophosphate, there being in the short reaction period experiment a small amount (4%) of thymidine-5′ phosphate in addition. The yield of methyl thymidine-5′ phosphate was similar, it being 35% in the long reaction period and 29% in the short reaction period experiment.

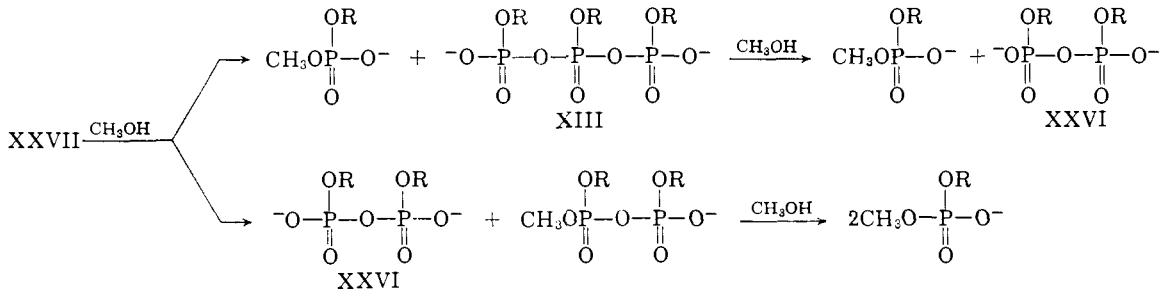
The yields of methyl thymidine-5′ phosphate obtained above were higher than those to be expected if the pyrophosphate observed on paper electrophoresis was present as such in the reaction mixtures. It is concluded that the products present in the short reaction period were, in fact, the pyrophosphate (XXVI), the tripolyphosphate (XIII) and the tetrapolyphosphate (XXVII). The last compound hydrolyzed rapidly during work-up to the pyrophosphate (XXVI). The total yield of methyl thymidine-5′ phosphate, thus, was derived from methanolysis of the tripolyphosphate (XIII) to equimolar amounts of the methyl ester and the pyrophosphate (XXVI) as well as from the methanolysis of linear tetraphosphate proceeding in either of the two ways shown in the following formulas. The results of the methanolysis of the long reaction period experiment again are explained by postulating that the products present then were, in fact, tri- and tetra-polyphosphate, the latter being equivalent to the pyrophosphate (24%) observed on paper-electrophoresis. The presence of considerable amount of the tetrapolyphosphate (XXVII) among the products of the short period experiment is also necessary to explain the subsequent formation of the tripolyphosphate (XIII) as the major product since the pyrophosphate (XXVI) as the trialkylammonium salt would be inert to further reaction with DCC at room temperature.[9,45] The tripolyphosphate thus arose as the ultimate major product as a result of disproportionation of the pyrophosphate (XXVI) and the tetrapolyphosphate (XXVII).

The formation of the tri- and tetra-polyphosphates in the presence of one equivalent of a trialkylamine shows that accumulating pyrophos-

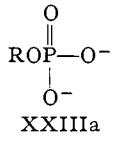
(43) As proposed earlier,[4,9] the order of nucleophilicity of the anions is not as important in attacking the activated intermediates (*e.g.*, XXIV) as it is in attacking the protonated carbodiimide itself to form the activated intermediates.

(44) This has previously been proposed by Todd.[18]

(45) This has been again checked by keeping bis-tri-*n*-butylammonium P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate in dry pyridine with DCC at room temperature. No reaction was observed up to 24 hr.



phate anions and, later, the triphosphate anions, can compete with the monoester anions for attack on the activated intermediate (XXIV) derived from the monoester.[46] On the other hand, in the presence of two equivalents of a trialkylamine, the pyrophosphate (XXVI) is the sole product at room temperature (present work and ref. 9). Since under these conditions the phosphomonoester would be expected to be present as the diionized species (XXIIIa), it is concluded that the latter, being much more nucleophilic than the monoionized species (XXIII), *does compete effectively* with a



XXIIIa

high concentration of the pyrophosphate anions, and consequently the only product formed is the pyrophosphate. It is recalled that the enhanced nucleophilicity of the diionized species (XXIIIa) was also invoked previously[4,9] to explain results of the rates of reactions using pyridine and the strongly basic trialkylamines with DCC and di-*p*-tolylcarbodiimide.

Experimental

General Methods and Materials.—Paper chromatography was performed by the descending technique using Whatman No. 1, 40 or 3 MM paper. The solvent systems used were Solvent I, isopropyl alcohol–ammonia–water (7–1–2); Solvent II, ethyl alcohol–1 *M* ammonium acetate (pH 7.5)(7–3 v./v.). Solvent III, isopropyl alcohol (75 ml.)–water (25 ml.)–trichloroacetic acid (5 g.)–ammonia (0.3 ml. of 25%)[47a]; Solvent IV, isopropyl alcohol (40 ml.)–isobutyl alcohol (20 ml.)–water (39 ml.)–ammonia (1 ml. of 25%).[47] Solvent V, isopropyl alcohol (75 ml.)–water (30 ml.)–trichloroacetic acid (4 g.)–ammonia (0.3 ml. of 25%). The R_f's of different compounds are listed in Tables I and II.

Paper electrophoresis was performed by using 0.05 *M* triethylammonium bicarbonate (pH 7.5) buffer[47b] or 0.03 *M* phosphate buffers in an apparatus similar to that of Markham and Smith[48] or in a high voltage (5000 volts) commercially available apparatus of similar design. The relative electrophoretic mobilities are listed in Table III.

(46) It would appear likely that the formation of the tri- and tetraphosphates occurs only by the nucleophilic attack,[42] respectively, of the pyrophosphate and the triphosphate anions on the activated intermediate (XXIV) derived from the mononucleotide. However, the possibility of further activation of the pyrophosphate to XXVII must also be considered when only one equivalent of trialkylamine is used. Although the pyrophosphate as the trialkylammonium salt is inert to further reaction with DCC,[45] it is possible that in the reaction of the mononucleotide as monotrialkylammonium salt, as long as the mononucleotide survives, the proton concentration is such as to permit some activation of the pyrophosphate.

(47) (a) J. P. Ebel, *Bull. soc. chim.* (France), 991 (1953). (b) J. Porath, *Nature*, **175**, 478 (1955).

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

TABLE I

PAPER CHROMATOGRAPHY OF DIFFERENT COMPOUNDS

Compound	R_f in solvent II
Thymidine	0.75
5′-*O*-Tritylthymidine	.88
5′-*O*-Acetylthymidine	.78
3′-*O*-Acetylthymidine	.81
3′,5′-Di-*O*-acetylthymidine	.83
5′-*O*-Trimethylacetylthymidine	.85
5′-*O*-Mesitoylthymidine	.85
Thymidine-5′ phosphate	.31
Thymidylyl-(3′ → 5′)-thymidine	.60
Methyl thymidine-5′ phosphate	.62
5′-*O*-Trimethylacetylthymidine-3′ phosphate	.58
5′-*O*-Mesitoylthymidine-3′ phosphate	.61
5′-*O*-Trimethylacetylthymidine-3′ β-cyanoethyl phosphate	.81
5′-*O*-Mesitoylthymidine-3′ β-cyanoethyl phosphate	.84
5′-*O*-Mesitoylthymidylyl-(3′ → 5′)-thymidine	.75
Monobenzyl phosphoric acid	.64
Ethylphosphoroanilidate	.86
3′-*O*-Acetylthymidine-5′ phosphate	.48
P^1,P^2-Di-3′-*O*-acetylthymidine-5′ pyrophosphate	.71
Monoethyl phosphate	.66
P^1,P^2-Di-ethyl pyrophosphate	.82

TABLE II

PAPER CHROMATOGRAPHY OF DIFFERENT COMPOUNDS

Compound	R_f[a] Solvent III	Solvent IV	Solvent V
Orthophosphate	0.61	0.63	0.21
Pyrophosphate	.26		.13
Tripolyphosphate (XIX)	.12	.16	.06
Trimetaphosphate (XX)	.08	.13	.28
Tetrametaphosphate	.03	.07	.23
Monoethyl phosphate	.79	.77	.35
P^1,P^2-Diethylpyrophsophate	.57	.62	.53

[a] Descending chromatography for 15 hr. of sodium salts on Whatman paper I.

TABLE III

RELATIVE PAPER-ELECTROPHORETIC MOBILITIES OF DIFFERENT COMPOUNDS (*p*H 7.5)

Compound	Mobility relative to Trimetaphosphate (XX)
Tetrametaphosphate	0.73
Tri-polyphosphate (XIX)	.71
Orthophosphate	.85
Monoethylphosphate	.80
Diethylpyrophosphate	.73
Inorganic pyrophosphate	.53

Phosphorus analysis was performed by the method of King[49] or by the method of Chen, *et al.*[50] Thymidine and

(49) E. J. King, *ibid.*, **26**, 292 (1932).

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

thymidine-5′ phosphate were commercial preparations which were checked carefully for purity as described earlier.[20]

Rate of Phosphorylation of 3′-*O*-Acetylthymidine, 5′-*O*-Acetylthymidine and 5′-*O*-Tritylthymidine by a Mixture of β-Cyanoethyl Phosphate and Dicyclohexylcarbodiimide.—The general method described was employed: an anhydrous pyridine (1 ml.) solution of the protected thymidine (0.1 mmole), β-cyanoethyl phosphate (0.1 mmole) and DCC (0.5 mmole) was kept sealed at room temperature. Aliquots (0.1 ml.) were removed at intervals, diluted with 5 ml. of water and kept at room temperature for several hours. After evaporation *in vacuo*, 5 ml. of 1 *N* sodium hydroxide was added and the resulting mixture heated at 100° for 1 hr. Dowex-50 (H^+) ion exchange resin was added to neutralize the alkali and the solutions obtained after removal of the resin were concentrated and examined by paper chromatography in Solvent I. The spots corresponding to thymidine and thymidine phosphate were eluted with water and their optical densities determined at 267 mμ.

The reaction mixture using 5′-*O*-tritylthymidine was worked up as described previously.[18]

The results obtained with 3′-*O*-acetylthymidine (formation of thymidine-5′ phosphate) and 5′-*O*-tritylthymidine[16] (formation of thymidine-3′ phosphate) are shown in Fig. 1. The rate of phosphorylation of 5′-*O*-acetylthymidine was similar to that of 5′-*O*-tritylthymidine. For example, the yield of thymidine-3′ phosphate from the two protected derivatives after a 5.5 hr. reaction period was: 5′-*O*-acetylthymidine, 59%; 5′-*O*-tritylthymidine, 56.5%.

5′-*O*-Trimethylacetylthymidine.—Trimethylacetyl chloride (1.4 ml.) was added to a suspension of thymidine (2.42 g., 10 mmole) in dry pyridine (15 ml.). The clear solution, which resulted, was kept at room temperature for 12 hr. and then poured into an excess of ice-water. The mixture was extracted with ether three times and the total ether extract washed with water and then dried. Paper chromatography of the ether extracts in Solvent II showed only one major spot (R_f, 0.95) corresponding to 5′-*O*-trimethylacetylthymidine and a small amount of material travelling at the front. The desired product present in ether extracts was crystallized from hot benzene. The yield of the shiny crystals was 1.95 g. (65%).

The aqueous phase contained unreacted thymidine (about 10%) and some of the 5′-*O*-trimethylacetylthymidine which could not be extracted out into ether. The aqueous phase was evaporated to dryness and the residue further dried in a vacuum. It was then dissolved in a mixture of chloroform and methyl alcohol and the solution applied on a silicic acid (20 cm. × 2 cm.) column. Elution with 300 ml. of chloroform at a flow rate of 10–12 ml./15 min. did not give any ultraviolet absorbing material. Subsequent elution with chloroform containing 5% methyl alcohol gave 5′-*O*-trimethylacetylthymidine. The total yield of this product was 82%. It melted at 140–141° after softening at 96°.

Anal. calcd. for $C_{15}H_{22}N_2O_6$: C, 55.21; H, 6.75; N, 8.58. Found C, 55.55; H, 6.90; N, 8.75.

The rate of hydrolysis of 5′-*O*-trimethylacetylthymidine was studied in 1 *N* sodium hydroxide at 0°. The half life of the trimethylacetyl group was found to be around 3 minutes, complete hydrolysis occurring in 30 min. Under these conditions, 5′-*O*-acetylthymidine was completely hydrolyzed to thymidine within 30 sec., the time the first aliquot was taken.

β-Cyanoethyl 5′-*O*-Trimethylacetylthymidine-3′ Phosphate and 5′-*O*-Trimethylacetylthymidine-3′ Phosphate.—A solution of 5′-*O*-trimethylacetylthymidine (330 mg., 1 mmole) in dry pyridine (20 ml.) was treated with a mixture of β-cyanoethyl phosphate (5 mmole) and DCC (15 mmole) for two days at room temperature. Water (20 ml.) then was added, and after allowing the mixture to stand at room temperature for several hr. it was extracted several times with petroleum ether to remove DCC. The residual aqueous pyridine mixture was filtered from dicyclohexylurea. The clear solution was divided into two equal parts.

One part was passed through a column of Dowex-50 (H^+) ion exchange resin column and the column washed with water. The total acidic effluent was neutralized with 0.05 *M* barium hydroxide solution to pH 7.5 in the cold. The neutral solution was concentrated to a small volume and the excess of β-cyanoethyl phosphate (barium salt) removed by precipitation with three volumes of ethyl alcohol and centrifugation. The clear supernatant contained pure barium salt of β-cyanoethyl 5′-*O*-trimethylacetylthymidine-3′ phosphate which was lyophilized. The yield was 210 mg. (85%). The product was homogeneous on paper chromatography in Solvent II, and paper electrophoresis.

The second part was evaporated to dryness *in vacuo* and the residue was treated with 20 ml. of 1 *M* lithium hydroxide for 5 min. at 0°. Lithium hydroxide was then neutralized by the addition of pyridinium Dowex-50 ion exchange resin and the solution filtered from resin, the latter being washed with water. The total product was separated by preparative paper chromatography in Solvent II. Two major bands (R_f's, 0.47 and 0.74) were obtained, there being in addition four weak bands. The band with R_f 0.74 corresponding to 5′-*O*-trimethylacetylthymidine-3′ phosphate was eluted with water to give 0.21 mmole (40%) of the product. The total eluate was passed through a pyridinium Dowex-50 ion exchange column and the resulting eluate lyophilized repeatedly to remove pyridinium acetate. This product was used in the phosphorylation of 3′-*O*-acetylthymidine. The material present in the band with R_f 0.47 corresponded to thymidine-3′ phosphate. An aliquot of the latter was incubated with the crude snake venom (*Crotalus adamanteus*). No dephosphorylation to thymidine was observed, showing the absence of any thymidine-5′ phosphate.

5′-*O*-Mesitoylthymidine.—A mixture of thymidine (1.2 g.; 5 mmole) and freshly distilled mesitoyl chloride (1 ml.) was kept in dry pyridine (5 ml.) at room temp. for 2 days. Water (10 ml.) was then added and the mixture kept for several hr. at room temp. The product was extracted several times with chloroform and the extracts washed with water. The chloroform solution contained only one ultraviolet absorbing material as judged by paper chromatography. The aqueous layer contained unreacted thymidine (0.52 mmole, about 10%). The chloroform solution was evaporated and the residue soon began to crystallize. Trituration with some ether afforded 1.35 g. of a yellowish powder which was recrystallized from chloroform–cyclohexane or acetone–cyclohexane to give transparent needles with m.p. 170–171°.

Anal. Calcd. for $C_{20}H_{24}N_2O_6$: C, 61.86; H, 6.18; N, 7.22. Found: C, 62.51; H, 6.35; N, 7.18.

β-Cyanoethyl 5′-*O*-Mesitoylthymidine-3′ Phosphate.—A mixture of 5′-*O*-mesitoylthymidine (370 mg.), pyridinium β-cyanoethyl phosphate (644 mg.) and DCC (2.2 g.) in dry pyridine (5 ml.) was kept for 4 days at room temperature. Water (20 ml.) was then added and the mixture kept for several hours before filtration to remove dicyclohexylurea. The latter was washed thoroughly with aqueous pyridine, and the combined filtrate and washings were concentrated *in vacuo* to a small volume. The solution was then passed through a column of Dowex-50 (H^+) ion exchange resin, the column being washed thoroughly with an acetone–water mixture. The acidic effluent was neutralized carefully with 0.05 *M* barium hydroxide solution in the cold. The solution was concentrated and barium cyanoethyl phosphate then precipitated by the addition of three volumes of ethyl alcohol. After removal of the precipitate, the clear supernatant was concentrated and then lyophilized to give 450 mg. of a faintly yellow powder of the desired product. The preparation was homogeneous by paper chromatography in Solvent II and by paper electrophoresis.

5′-*O*-Mesitoylthymidine-3′ Phosphate.—Two hundred milligrams of the above cyanoethyl ester were dissolved in a mixture of pyridine (2 ml.) and 1 *N* sodium hydroxide (5 ml.) and the solution kept at room temp. for 10 min. Pyridinium Dowex-50 ion exchange resin was then added to remove the alkali. Paper chromatography of the total clear aqueous pyridine solution on two full sheets of Whatman 3 MM paper in Solvent A showed a weak ultraviolet absorbing band at origin corresponding to thymidine-3′ phosphate product. The latter was eluted with water and lyophilized. The yield as estimated spectrophotometrically was 80%.

The rate of alkaline hydrolysis of the mesitoyl group in the product was determined by keeping a sample (0.02 mmole) in 1.5 ml. of 1 *N* sodium hydroxide at room temp. (23°). Aliquots (0.2 ml.) were removed at intervals, treated with an excess of pyridinium Dowex-50 ion exchange resin and the aqueous pyridine solution chromatographed in Solvent II. Elution of the spots and determination of

their absorbance at 267 mμ gave the following rate of formation of thymidine-3′ phosphate: 18 hr., 19%; 45.5 hr., 34.5%; 90 hr., 53.8%; 114 hr., 63%. The half-life for hydrolysis of the mesitoyl group was thus determined to be around 80 hr.

Rate of Formation of C_3'–C_5' Internucleotidic Linkage Using Protected Thymine-5′ or -3′ Phosphate and Protected Thymidine.—A solution of the protected nucleotide (0.1 mmole) and the protected thymidine (0.1 mmole) in anhydrous pyridine (1 ml.) was treated with DCC (103 mg.; 0.5 mmole) and the sealed mixture kept at room temperature. Aliquots (0.1 ml.) were removed at intervals, diluted with water (10 ml.) and the resulting mixtures kept overnight at room temperature. The subsequent work-up varied depending upon the nature of the protecting groups. Thus, the aliquots of reaction mixtures involving condensation of 3′-*O*-acetylthymidine-5′ phosphate with 5′-*O*-acetylthymidine and that of 5′-*O*-acetylthymidine-3′ phosphate with 3′-*O*-acetylthymidine were worked up as described previously.[5] In the study using 5′-*O*-mesitoylthymidine-3′ phosphate, the aliquots after treatment with water were lyophilized and then treated with 1 ml. of 1 *N* sodium hydroxide for 45 min. at 100°. After cooling, Dowex-50 (H^+) ion exchange resin was added to take up the alkali and the supernatants applied on paper chromatograms as described below. The aliquots from the reaction mixture involving 5′-*O*-tritylthymidine-3′ phosphate and 3′-*O*-acetylthymidine was first treated with alkali at about pH 13 for 0.5 hr. Dowex-50 (H^+) resin was then added to neutralize the mixture to *p*H 7 and the mixture was filtered, the resin being washed with water and some ethyl alcohol. The total filtrate was evaporated to dryness and the residue treated with 10 ml. of 80% acetic acid at 100° for 10 min. Acetic acid was then removed by evaporation and then repeated co-evaporation with water. Finally, the residue was dissolved in 0.5 ml. of water and some drops of ethyl alcohol. After work-up, suitable portions of the mixtures were chromatographed in Solvent I. Spots corresponding to thymidine phosphate, P^1,P^2-dithymidine pyrophosphate, thymidylyl-(3′→5′)-thymidine and thymidine were observed. The spot corresponding to P^1,P^2-dithymidine pyrophosphate was very weak in reaction mixtures with long reaction periods. The spots were all eluted with water in standard volumes, and optical densities of the solutions were determined at 267 mμ using appropriate blanks. The results in respect of the yields of thymidylyl-(3′→5′)-thymidine are given in Fig. 2.

The yields of thymidylyl-(3′→5′)-thymidine obtained in the reaction between 5′-*O*-mesitoylthymidine-3′ phosphate and 3′-*O*-acetylthymidine, which are not shown in Fig. 2, were as follows: 17 hr., 63 (60)%; 25 hr., 58%; 41 hr., 59% (59); 84 hr., 60%; 115.5 and 144 hr., 60.5%. The first figures are yields as based on the nucleoside, whereas those given in parenthesis are those based on the nucleotide material.

The Reaction of Bis-tri-*n*-butylammonium Orthophosphate with Dicyclohexylcarbodiimide.—An anhydrous pyridine (5 ml.) solution of crystalline anhydrous orthophosphoric acid (495 mg., 5 mmole), tri-*n*-butylamine (2.4 ml., 10 mmole) and DCC (5.1 g., 25 mmole) was kept at room temperature. Examination of an aliquot after 4.5 hr. by chromatography in Solvents III–V showed only a minor spot corresponding to inorganic pyrophosphate while the main phosphorus-containing product had mobility identical with that of authentic inorganic trimetaphosphate. After a total reaction time of 15 hr., ether (20 ml.) was added and after removal of the ether layer, the residue was retreated with 20 ml. of ether. The process was repeated after removal of ether. To the white residue finally obtained was added water (5 ml.), and dicyclohexylurea (1.14 g.) was removed by filtration. The turbid aqueous solution was extracted once with ether. A solution of silver nitrate (1.35 g.) in water (2 ml.) was prepared, and a portion (0.2 ml.) of this solution was added to the aqueous solution obtained above. The small amount of precipitate which formed was quickly filtered off, and to the filtrate was added the bulk of the silver nitrate solution. On storage at 0°, the solution deposited the crystalline silver salt of trimetaphosphate. The yield was 580 mg. (60%).

Anal. Calcd. for $Ag_3P_3O_9 \cdot 1H_2O$: P, 16.19. Found: P, 15.81.

The above preparation had mobilities on paper chromatograms and paper electrophoresis identical with those of a sample prepared by the method of Thilo and Raetz.[34]

Tripolyphosphate (XIX) from the Reaction of β-Cyanoethyl Phosphate and Dicyclohexylcarbodiimide.—A solution of pyridinium β-cyanoethyl phosphate (0.1 mmole) and DCC (1 mmole) in dry pyridine (1 ml.) was kept at room temp. for 15 min. Sodium hydroxide (10 ml. of 1 *N*) was then added and the mixture extracted with ether. After 15 min., the solution was neutralized in the cold to pH 7 with Dowex-50 (H^+) resin. Chromatography in Solvent IV showed the complete absence of inorganic phosphate and β-cyanoethyl phosphate. The main product was identical in mobility with inorganic tripolyphosphate in three solvent systems (III-V). Two other minor products, which were present and which remain unidentified, had R_fs in Solvent System IV, 0.30 (very weak) and 0.11. The major product, tripolyphosphate, had R_f 0.16, identical with that of an authentic sample.[51]

The Reaction of 3′-*O*-Acetylthymidine-5′ Phosphate with Dicyclohexylcarbodiimide in Pyridine.—A solution of pyridinium 3′-*O*-acetylthymidine-5′ phosphate (0.2 mmole) in anhydrous pyridine (1 ml.) was treated with DCC (206 mg., 1 mmole). Aliquots (0.2 ml.) were withdrawn after 3, 6.5, 15 and 30 minutes and each added to 10 ml. of cold anhydrous ether. The sealed mixture was shaken and the precipitate which formed was spun down. The supernatant was poured off and the precipitate washed twice with 10 ml. portions of ether and the washed precipitate again collected by centrifugation. It was dissolved in about 0.3 ml. of water and the solution quickly applied to Whatman 3 MM paper, and electrophoresis was performed in 0.05 *M* triethylammonium bicarbonate at 15 volts/cm. for 3 hr. There was found a small amount of 3′-*O*-acetylthymidine-5′ phosphate, some P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate (mobility 0.79 relative to that of 3′-*O*-acetylthymidine-5′ phosphate) and a slower travelling product (mobility, 0.67 relative to that of 3′-*O*-acetylthymidine-5′ phosphate), which accounted for about 80% of the total ultraviolet absorbing material. In various runs, the amount of the latter product varied between 60 and 80%. The following experiments were performed with the above major product collected by preparative paper-electrophoresis and elution with water. (1) Hydrolysis in aqueous pyridine. A portion was kept in aqueous pyridine (20%) for 0.5 hr. at room temp. Paper electrophoresis then showed the production of 3′-*O*-acetylthymidine-5′ phosphate and P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate. The optical density at 267 mμ of these products after elution was found to be 0.285 and 0.55 thus showing a molar ratio close to 1.

(2) **Methanolysis.**—A band of the above product as obtained on paper electrophoresis was cut out, the strip was dried over phosphorus pentoxide and the dried strip kept in an equal mixture of pyridine and methyl alcohol for 3 days at room temperature. Subsequent paper electrophoresis showed the products to be P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate (XIV) and methyl 3′-*O*-acetylthymidine-5′ phosphate (XV). The molar ratio of the two products as determined spectrophotometrically was respectively 1:0.985. Methyl 3′-*O*-acetylthymidine-5′ phosphate (XV) band was treated with alkali and the product subjected to paper chromatography and paper electrophoresis. It had mobilities identical with authentic methyl thymidine-5′ phosphate.

Reaction of P^1,P^2-Di-3′-*O*-acetylthymidine-5′ Pyrophosphate with Dicyclohexylcarbodiimide in Pyridine.—A solution of 0.025 mmole of P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate in dry pyridine (0.5 ml.) was treated with DCC (50 mg.) and the mixture kept at room temperature for 30 min. Paper electrophoresis as described above showed the products to be the same as obtained in the preceding experiment with 3′-*O*-acetylthymidine-5′ phosphate. The major product, P^1,P^2,P^3-tri-3′-*O*-acetylthymidine-5′ triphosphate, was estimated spectrophotometrically to be 84% of the total ultraviolet absorbing material.

The Reaction of 3′-*O*-Acetylthymidine-5′ Phosphate with Limited Amount of DCC in Pyridine.—An anhydrous pyridine (1 ml.) solution of 3′-*O*-acetylthymidine-5′ phosphate (0.2 mmole) was treated with 0.1 mmole of DCC at room temperature for 15 min. Precipitation of the product with ether and then paper electrophoresis as described above

(51) Crystalline sodium tripolyphosphate ($Na_5P_3O_{10} \cdot 6H_2O$) was a gift fron Dr. W. A. Darlington of Monsanto Chemical Co., St. Louis.

revealed the spots corresponding to 3′-*O*-acetylthymidine-5′ phosphate, P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate (XIV) and P^1,P^2,P^3-tri-3′-*O*-acetylthymidine-5′ triphosphate (XIII; R = 3′-*O*-acetylthymidine-5′). Their relative intensities as determined spectrophotometrically were, respectively, 43%, 49 and 7%.

The Reaction of 3′-*O*-Acetylthymidine-5′ Phosphate with Dicyclohexylcarbodiimide in the Presence of One Equivalent of Tri-*n*-butylamine. (a) **Two and one-half hour reaction.**—A solution of anhydrous 3′-*O*-acetylthymidine-5′ phosphate (0.2 mmole), tri-*n*-butylamine (0.05 ml., 0.2 mmole) and DCC (350 mg.) in dry pyridine (1 ml.) was kept sealed at room temperature for 2.5 hr. Direct paper electrophoresis of an aliquot in triethylammonium bicarbonate buffer showed three bands. In the order of decreasing mobility, they were P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate (54%),[52] P^1,P^2,P^3-tri-3′-*O*-acetylthymidine-5′ triphosphate (XIII)(37%) and an unidentified band in the amount of 9%. (The slowest band did not have the characteristic thymidine ultraviolet absorption spectrum but hydrolyzed slowly in water to give mainly 3′-*O*-acetylthymidine-5′ phosphate.)

In an exact repeat of the above experiment, 2 ml. of anhydrous methyl alcohol was added to the reaction mixture after 2.5 hr. reaction period and the resulting solution kept sealed at room temperature. After 20 and 120 hr., portions of the mixture were worked up by addition of water, alkaline treatment after extraction of DCC with ether and paper electrophoresis at pH 7.1 in phosphate buffer. In the aliquot removed after 20 hr. reaction with methyl alcohol, the products were P^1,P-di-thymidine-5′ pyrophosphate (64%), methyl thymidine-5′ phosphate (27%) and thymidine-5′ phosphate (9%). After 120 hr., the pyrophosphate amounted to 69%, methyl thymidine-5′ phosphate was 27% and thymidine-5′ phosphate was 4%.

From the proportion of methyl thymidine-5′ phosphate obtained, it is calculated that the products present originally in anhydrous pyridine were linear tetraphosphate (XXVII) (28%), linear triphosphate (XIII)(37%), the pyrophosphate (26%) and the unidentified product mentioned above (9%).

(b) **Twenty-hour Reaction.**—The experiment under (a) above was repeated exactly on a 0.1 mmole scale of the nucleotide. Examination of the reaction mixture by paper electrophoresis after 20 hr. showed the products to be P^1,P^2-di-3′-*O*-acetylthymidine-5′ pyrophosphate (24%) and the linear triphosphate (XIII)(76%). Treatment of the mixture at this stage with water for 24 hr. followed by paper electrophoresis gave 3′-*O*-acetylthymidine-5′ phosphate (32%) and the pyrophosphate (68%). (These amounts are in good agreement with those expected from hydrolysis of the linear triphosphate ascertained to be present.) In another repeat of the above experiment, the reaction mixture after 20 hr. was treated with 2 ml. of dry methyl alcohol for 3 days and the products analyzed by paper electrophoresis after a work-up including alkaline treatment. The products were methyl thymidine-5′ phosphate (35%) and P^1,P^2-di-thymidine-5′ pyrophosphate (65%). From the amount of methyl thymidine-5′ phosphate found, it may be concluded that the 24% of the pyrophosphate observed above on direct paper-electrophoresis actually was present in the reaction mixture as the linear tetraphosphate (XXVII).

The Reaction of Monoethyl Phosphoric Acid with Dicyclohexylcarbodiimide in Dioxane.—Barium monoethyl phosphate[24] (1.89 g., 6 mmole) was dissolved in water and the solution passed through a column of Dowex-50 (H^+) ion exchange resin. The total acidic effluent was lyophilized. The residue of the free ethyl phosphoric acid was dissolved in dry dioxane and the solution taken to dryness *in vacuo* using an oil pump. The process of dissolution in dioxane and evaporation was repeated three times. The dry residue was taken up in 2.5 ml. of dioxane, DCC (3.09 g.; 15 mmole) was added to the solution and the mixture sealed. All the subsequent operations were carried out in a dry box maintained under slightly positive pressure with dry nitrogen. After 1 hr. at room temperature, the solvent was evaporated from the reaction mixture *in vacuo*, and to the residue were added 30 ml. of a 1:1 mixture of dry petroleum ether and diethyl ether. The resulting precipitate was centrifuged and the supernatant separated by decantation into a dry flask. The precipitate was further washed twice with the petroleum ether–ether mixture (30 ml. portions), there resulting in all three supernatants.

The first supernatant was found to contain 35% of the total phosphate and the bulk of the excess of DCC. The second supernatant contained 12% of the total phosphate and 250–300 mg. of DCC. The third supernatant contained 3% of the total phosphate and 25–40 mg. of DCC. The final precipitate was treated further as follows: Dioxane (10 ml.) and chloroform (5 ml.) were added and the insoluble dicyclohexylurea was removed by filtration. The yield of the urea was 1.43 g.[53]; (theoretical for trimetaphosphate, XII, R = ethyl, formation from ethyl phosphoric acid, 1.33 g.; for the linear P^1,P^2,P^3-triethyltriphosphate (XIII), 0.88 g.; for P^1,P^2-diethylpyrophosphate, 0.66 g.[53]) The dioxane–chloroform solution was concentrated *in vacuo* to about 1 ml. and the precipitation repeated with petroleum ether–ether mixture (30 ml.). The resulting semisolid precipitate (corresponding to 10% of the starting material as determined by phosphorus analysis) was dissolved in chloroform (10 ml.) and used in the following experiments. The supernatant (No. 4) from this ether–petroleum ether precipitation contained the remainder of the phosphorus-containing material.[54] (Anilidate formation with this solution is described below.)

An aliquot was subjected to paper electrophoresis at pH 7.5. The main band corresponded to P^1,P^2,P^3-triethyl triphosphate (XIII; R = ethyl) with mobility, 0.9 compared to that of marker P^1,P^2-diethylpyrophosphate and 0.81 compared to that of marker monoethyl phosphate. There was also a small amount of material present with mobility less than that of the major band.

A portion (1 ml.) of the chloroform solution was treated under gentle reflux for 2.5 hr. with 4 ml. of anhydrous methyl alcohol. A part of the resulting solution was applied as a band on Whatman 3 MM paper and chromatographed in Solvent I. Two minor bands corresponding to monoethyl-phosphate and P^1,P^2-diethylpyrophosphate and a major band corresponding to methyl ethyl phosphate were observed. Their relative amounts were determined by elution with water and phosphorus analysis of the total eluates. In three parallel experiments, methyl ethyl phosphate accounted for 85–90% of the total phosphorus-containing products.

An aliquot (1 ml.) of supernatant No. 4[54] above was kept in 1 ml. of aniline for 2.5 hr. at 80°. Subsequent chromatography of the reaction mixture showed ethylphosphoroanilidate to be the sole product.

Monobenzyl phosphoric acid was prepared by a modification of the procedure previously described.[7] A solution of crystalline, anhydrous orthophosphoric acid[55] (1.96 g.) and triethylamine (5.6 ml., 40 mmole) in dry benzyl alcohol (50 ml.) was treated with 14 ml. of trichloroacetonitrile for 5 hr. at room temperature. The solution was then vigorously shaken with 50 ml. of water for 30 hr. The aqueous layer was separated and the organic phase further extracted three times with water. The combined aqueous extracts were concentrated *in vacuo* and the concentrate passed through a short column (externally cooled) of Dowex-50 (H^+) ion exchange resin. The total acidic effluent was extracted with ether several times and the combined ether solution dried over sodium sulfate. Ether was evaporated and the residue was crystallized from a mixture of chloroform and petroleum ether. The yield of pure monobenzyl phosphoric acid was 2.3 g.

The Reaction of Monobenzyl Phosphoric Acid with Dicyclohexylcarbodiimide. Isolation of Inorganic Trimetaphosphate (XX).—A solution of monobenzyl phosphoric acid (60 mg.) in dry pyridine (1 ml.) was treated with DCC (135 mg.) for 12 hr. at room temperature. Direct paper-electro-

(52) In another experiment, examination of the products by direct paper electrophoresis after a 90 min. reaction period, showed the pyrophosphate to be even higher in amount (64%), none of the starting material being present.

(53) An exactly identical experiment was carried out in dry ether. Because of the insolubility of P^1,P^2-diethyl pyrophosphoric acid in this solvent, the reaction did not go furrher and the yield of dicyclohexylurea was correspondingly less.

(54) It is clear from the experimental results that the low yield of the trialkyltrimetaphosphate (XII) was due to incomplete precipitation. There is, however, little doubt that the same product was present in the supernatants. This was shown, for example, by the quantitative formation of the phosphoranilidate from supernatant No. 4.

(55) Fluka, A. G., Switzerland.

phoresis of the reaction mixture at pH 7.5 showed little of inorganic trimetaphosphate (XX). Subsequent heating of the pyridine mixture at 100° for 0.5 hr. gave a product in about 50% yield identical in mobility on paper electrophoresis with authentic inorganic trimetaphosphate. The latter was clearly separated from inorganic pyrophosphate, linear triphosphate and orthophosphate.

In another experiment 60 mg. of monobenzyl phosphoric acid was treated with DCC (135 mg.) in 2 ml. of dry acetonitrile for 0.5 hr., and to the reaction mixture was added a solution of sodium iodide (300 mg.) in acetonitrile (2 ml.). Subsequent heating of the reaction mixture at 55° for 30 min. again gave inorganic trimetaphosphate in about 50% yield.

COMMUNICATIONS TO THE EDITOR

DIISOPINOCAMPHEYLBORANE AS A REAGENT FOR THE PRODUCTION OF OPTICALLY ACTIVE OLEFINS AND THE ESTABLISHMENT OF THEIR CONFIGURATION

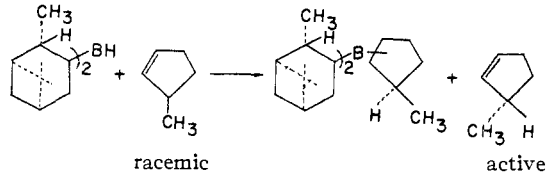
Sir:

It has been reported recently that the optically active stereoisomers of *trans*-cycloöctenes can be isolated by utilizing an olefin amine platinum complex.[1] We wish to report that the partial hydroboration of a racemic mixture of olefins by diisopinocampheylborane provides a highly convenient means for the isolation of the individual optically active isomers. Moreover, the method permits one to assign the absolute configuration to the products.

It was observed previously that diisopinocampheylborane exhibits a remarkable asymmetric stereoselectivity when applied to the hydroboration of *cis*-olefins[2] and to the reduction of ketones.[3] Thus, hydroboration of *cis*-olefins with the reagent, and then oxidation of the intermediate organoborane, yielded alcohols in optical purities of 70–90%. The configuration of the alcohols obtained can be rationalized in terms of an optimal steric fit of the reagent involved in the transition state.[4]

Inspection of models indicates that the asymmetric reagent should differentiate between enantiomeric olefins. Accordingly, hydroboration of an olefin racemate with a deficient amount of diisopinocampheylborane should result in an accumulation of one enantiomer in the reaction mixture as a consequence of the more reactive enantiomer being converted into the organoborane.

For example, on the basis of the model, one would predict that the R enantiomer of 3-methylcyclopentene should react more readily with diisopinocampheylborane derived from (−)α-pinene, leaving an excess of the S enantiomer.

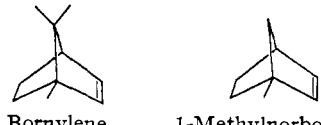


racemic active

In a typical esperiment 8.47 g. of α-pinene (62 mmoles, $[\alpha]^{20}D$ −47.8°) was added to 23.4 ml. of a 1.0 *M* solution of sodium borohydride in diglyme. Boron trifluoride, 8.5 ml. of 3.65 *M* solution in diglyme, was added to the olefin–sodium borohydride mixture. The flask was maintained for 4 hours at 0–5°. The diisopinocampheylborane thus obtained (32 mmoles) was treated with 5.08 g. of 3-methylcyclopentene (62 mmoles). The reaction was permitted to proceed for 4 hours at 0–5°. Water was added to decompose residual hydride. The unreacted 3-methylcyclopentene was recovered from the reaction mixture by distillation at 0–20° at reduced pressure. Redistillation yielded 1.91 g. of 3-methylcyclopentene (75% yield), b.p. 63–64° at 750 mm., $n^{20}D$ 1.4205, $[\alpha]^{26}D$ − 34.6°, indicating an optical purity of 45%.[5] (−)3-Methylcyclopentene has the S configuration,[6] in agreement with the prediction.

Under similar experimental conditions racemic 3-ethylcyclopentene yielded 80% of optically active 3-ethylcyclopentene, b.p. 99–99.5° at 765 mm., $n^{20}D$ 1.4305, $[\alpha]^{26}D$ −45.2°, indicating an optical purity of 37%.[7] The (−)enantiomer has the S configuration[6] which is in agreement with the one deduced from inspection of models.

Similarly, partial hydroboration of racemic 1-methylnorbornene yielded optically active 1-methylnorbornene in 67% yield, b.p. 104–105° at 760 mm., $n^{20}D$ 1.4513[8], $[\alpha]^{26}D$ −6.51°. The configuration of (−)1-methylnorbornene is not known. However, (−)bornylene, which has a *gem* dimethyl group at the bridge carbon atom, is related to (+)-camphor, since both give (+)camphoric acid on oxidation. Consequently, (−)bornylene has the 1S:4R configuration. Assuming that the *gem*-dimethyl group does not affect the sign of rotation, (−)1-methylnorbornene must have the 1R:4S configuration.



(−) Bornylene 1S:4R 1-Methylnorbornene 1R:4S

Inspection of models predicts the same configuration for the 1-methylnorbornene obtained by selective hydroboration with the diisopinocampheylborane from (−)α-pinene.

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(2) H. C. Brown and G. Zweifel, *ibid.*, **83**, 486 (1961).

(3) H. C. Brown and D. B. Bigley, *ibid.*, **83**, 3166 (1961).

(4) G. Zweifel, N. R. Ayyangar and H. C. Brown, *ibid.*, **84**, 4342 (1962).

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(8) P. v. R. Schleyer, "Abstracts of Papers, A.C.S.," Sept., 1956, p. 29, reports b.p. 104.3°, $n^{22}D$ 1.4519.