ml. fractions. The phosphor[11] was attached to an RCA 6810A photomultiplier tube operated at 1800 volts. With suitable discriminator settings, both S^{35} and P^{32} radioactivity peaks were sensitively recorded using a linear count rate meter and strip chart recorder.

The deacylated alfalfa lipids were fractionated on the larger columns in the presence of glycerophosphoryl glycerol-P^{32} and sulfoglycosyl glycerol-S^{35} as described above. Phosphate-containing contaminant was found in the product and found to be due to the cyclic 1,2-glycerophosphate. Subsequent separations were performed after treating the lipid deacylate with Dowex-50·H^+ and traces of hydrochloric acid which effected hydrolysis of the cyclic ester. Fractions containing S^{35} activity were decationized with Dowex-50·H^+ and concentrated *in vacuo*. Neutralization of the concentrate with cyclohexylamine and crystallization from ethanol–toluene gave colorless prisms, m.p. 191–192°, $[\alpha]^{25}D$ + 74.5° in water (*p*H 4, *c* 18), $[\alpha]^{25}_{436}$ + 127° in water (*p*H 4, *c* 1.75), $[\alpha]^{25}_{436}$ + 38° in cupra B (*c* 0.706). The yield from 2 kg. of dry leaves was 180 mg. of crystalline salt. Products from independent separations were analyzed.

Anal. Calcd. for $C_{15}H_{31}O_{10}NS$: C, 43.2; H, 7.44; N, 3.36; S, 7.66. Found: C, 43.22, 43.15; H, 7.44, 7.37; N, 3.20, 3.26; S, 7.44, 7.37.

1-Sulfo-2,3-propanediol.—The reaction of allyl alcohol with sulfuric acid according to the method of Friese[12] gave 1-sulfo-2,3-propanediol which was converted to its cyclohexylammonium salt in the usual manner. The infrared absorption spectrum of the salt revealed strong absorption bands at 1042 and 1200 cm.$^{-1}$ and a moderately strong band at 792 cm.$^{-1}$.

(11) Plastic phosphor, NE 501, was obtained from Nuclear Enterprises, Ltd., 1750 Pembina Highway, Winnipeg, Canada.

(12) H. Friese, *Ber.*, **71**, 1303 (1938).

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER 8, B. C., CAN.]

2-Cyanoethyl Phosphate and its Use in the Synthesis of Phosphate Esters[1]

BY G. M. TENER[2]

RECEIVED AUGUST 2, 1960

A new method for the synthesis of phosphomonoesters has been developed. 2-Cyanoethyl phosphate (V) is coupled to an alcohol using dicyclohexyl carbodiimide (DCC) as the condensing agent and then the cyanoethyl group is removed from the resulting phosphodiester by very mild alkaline hydrolysis to give the desired phosphate ester. The method has been applied to the synthesis of a number of nucleotides in good yield. An improved method for the synthesis of 2-cyanoethyl phosphate is reported as well as a method for the synthesis of P^{32}-labeled 2-cyanoethyl phosphate and its application to the synthesis of P^{32}-labeled phosphate esters.

Many reagents[3,4] have been employed for the synthesis of phosphate esters but none has proved to be completely general and, indeed, for the phosphorylation of certain compounds no satisfactory reagent exists. The limitations of existing reagents are particularly evident in the synthesis of nucleotides where such considerations as acid lability of purine glycosidic bonds, alkaline lability of the 6-amino group of cytosine[5] and the catalytic reduction of pyrimidine rings[6] must be taken into account. Multifunctional reagents such as phosphorus oxychloride[7–9] and polyphosphoric acid[10] are of limited value because of the complex mixture of products they produce and their use is practical only in those cases where the reaction products can withstand hydrolysis. The most successful reagent so far employed in the nucleotide field is dibenzyl phosphorochloridate.[11] It readily phosphorylates most primary alcoholic functions and the benzyl groups can be removed from the intermediate phosphotriesters by mild catalytic hydrogenolysis using a palladium catalyst. The reagent is, however, unstable and very sensitive to traces of water and during the prolonged reaction time required to phosphorylate secondary alcoholic functions the reaction solvent (pyridine) causes some debenzylation[12] of the intermediates thus giving lower yields of the desired products. Further, dibenzyl phosphorochloridate is not extremely powerful as shown by its inability to phosphorylate guanosine nucleosides.[8] Tetra-*p*-nitrophenyl pyrophosphate[9] was developed to fill this latter need, but in this case the *p*-nitrophenyl protecting groups must be removed from the intermediate phosphotriester by drastic alkaline hydrolysis or by a specific enzymic procedure. Likewise, *O*-benzylphosphorous-*O,O*-diphenyl phosphoric anhydride[13] was developed to provide a more powerful phosphorylating agent but because of the difficulties encountered[14] in removing benzyl groups from cytosine-containing nucleotides by hydrogenolysis, even with palladium catalysts, alternate reagents were sought.

In view of the above limitations, it is apparent that any new phosphorylating agent for use in the nucleotide field should satisfy the following requirements; it should be very powerful; and it should be a monofunctional reagent from which the protecting groups can be removed by very mild and specific methods. In addition, it is desirable to have a simple procedure for both the preparation of

(1) This work has been supported by a grant from the National Research Council of Canada, Ottawa.

(2) Dept. of Biochemistry, University of B. C., Vancouver 8, B. C., Canada.

(3) For a recent review of phosphorylating agents see F. Cramer, *Angew. Chem.*, **72**, 236 (1960).

(4) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 3741 (1957).

(5) R. Hurst and A. Kuksis, *Can. J. Biochem. Physiol.*, **36**, 931 (1958).

(6) W. E. Cohn and D. G. Doherty, THIS JOURNAL, **78**, 2863 (1956).

(7) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934).

(8) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 2476 (1949).

(9) R. W. Chambers, J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, 3747 (1957).

(10) R. H. Hall and H. G. Khorana, *ibid.*, **78**, 1871 (1956).

(11) F. R. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945).

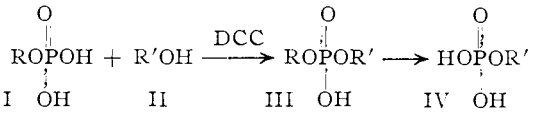
(12) V. M. Clark and A. R. Todd, *T. Chem. Soc.*, 2023 (1950).

(13) N. S. Corby, G. W. Kenner and A. R. Todd, *T. Chem. Soc.*, 3669 (1952).

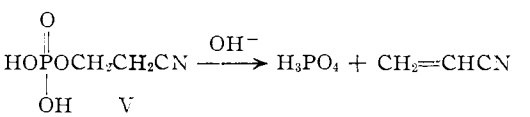
(14) P. T. Gilham and H. G. Khorana, THIS JOURNAL, **81**, 4647 (1959).

the reagent and its use. Since nucleotides are unaffected by mild alkali, it was considered that a phosphorylating agent with protecting groups sensitive to mild alkali would prove useful.

In a recent communication[15] we reported a new approach to the synthesis of phosphate esters. The method was based on the earlier observations from this Laboratory[16] that phosphodiesters (III) can be readily prepared from a monoalkyl phosphate (I) and an alcohol (II) by allowing them to react in anhydrous pyridine with dicyclohexylcarbodiimide (DCC). In the new phosphorylation

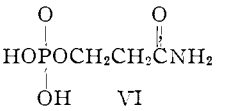


procedure an alkyl phosphate (I) is chosen, such that after coupling it to the alcohol, as shown in the above reaction scheme, its alkyl group can be selectively removed. The net result of the reaction is then the conversion of the alcohol II to its phosphate ester IV. This method lends itself to the use of a wide variety of monoalkyl phosphates. In the previous communication,[15] preliminary results with two suitable alkyl phosphates were described. With the first reagent, monobenzyl phosphate (I, benzyl), phosphodiesters were formed from which the benzyl groups could be removed by catalytic hydrogenolysis. Although this reaction went in good yield, it has not been explored further since our main interest was to find an alkyl phosphate with a protecting group that could be removed by mild alkaline hydrolysis. The reagent which satisfied this requirement was 2-cyanoethyl phosphate (V). It was chosen because it had been shown by Cherbuliez and Rabinowitz[17] to break down under very mild alkaline treatment and liberate orthophosphate, presumably by the reaction

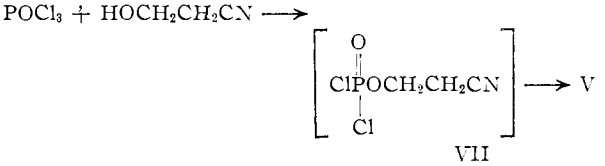


This paper gives a detailed account of the synthesis and properties of 2-cyanoethyl phosphate and its use in the synthesis of several nucleotides.

The initial problem was to prepare the reagent in large quantities. Cherbuliez and Rabinowitz[17] had reported its synthesis by heating a mixture of hydracrylonitrile and polyphosphoric acid at 100° for four hours. Attempts to use their procedure with a polyphosphoric acid of undetermined degree of hydration gave low yields of a compound which from analysis, infrared spectrum and chromatographic studies appears to be the phosphate ester of 3-hydroxypropionamide (VI). The formation of



the amide is in accord with the studies of Snyder and Elston[18] who showed polyphosphoric acid to be an excellent reagent for the conversion of nitriles to amides. However, a small yield of the desired product was obtained when the polyphosphoric acid–hydracrylonitrile mixture was allowed to react overnight at room temperature. Here again, some of the amide VI was detected chromatographically and could be separated from the nitrile only by repeated recrystallization of the mixed cyclohexylammonium salts. In view of these difficulties a new synthesis was devised which gave a pure product in good yield. In this method an equimolar mixture of hydracrylonitrile and pyridine was added slowly to a cold solution of phosphorus oxychloride in ether. The reaction intermediate, presumably VII, was decomposed by pouring the ethereal solution into a mixture of aqueous pyridine and ice and the product collected as its



crystalline barium salt in 60% yield. Attempts to decompose the intermediate by pouring the reaction mixture into a suspension of barium carbonate in ice-water or into cold aqueous barium acetate led to little or none of the desired product. The aqueous pyridine apparently favors hydrolysis of the phosphorus–chlorine bond, whereas the use of the barium salts leads to a β-elimination reaction with the formation of acrylonitrile, inorganic phosphate and chloride ion.

The phosphorylation reactions with this reagent are carried out in anhydrous pyridine. In general, phosphorylating agents are very sensitive to traces of water; however, 2-cyanoethyl phosphate offers the advantage of being completely stable and is converted into the active phosphorylating species only when brought into reaction with DCC.[19] Water will decompose this activated intermediate and care is therefore taken to remove as much water as possible from the reaction mixture. However, in practice absolutely anhydrous conditions are not required because an excess of DCC is normally added and last traces of water are removed by the acid-catalyzed hydration of DCC to form dicyclohexylurea. One other precaution necessary when using this reagent is to exclude the presence of strong bases, since they prevent the formation of the activated intermediate. For this reason, and because of solubility problems, barium 2-cyanoethyl phosphate is converted to the pyridinium salt before use.

Initial studies on this new phosphorylating agent were carried out with 5′-O-tritylthymidine (VIII).

(15) P. T. Gilham and G. M. Tener, *Chemistry & Industry*, 542 (1959).

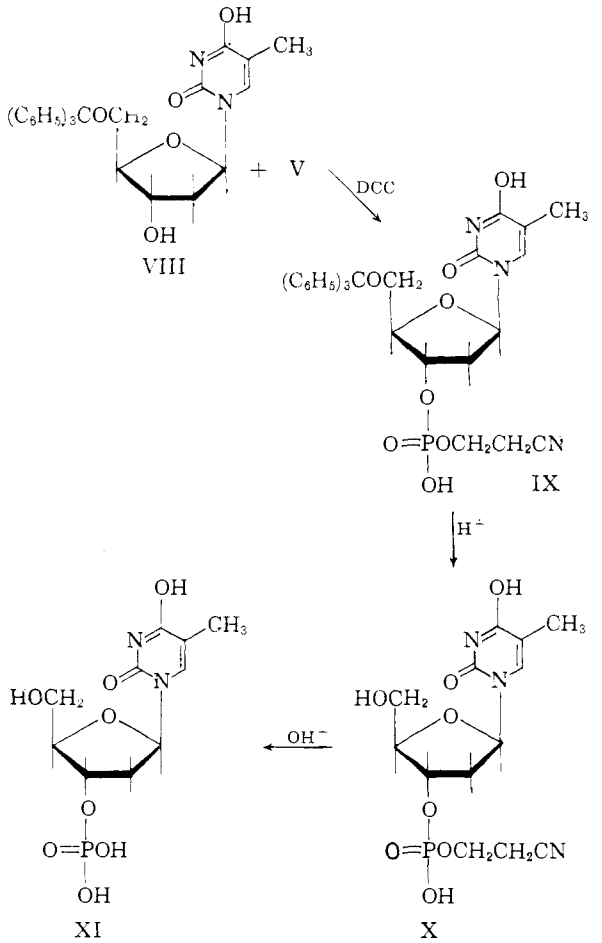
(16) (a) H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener and E. H. Pol, THIS JOURNAL, **79**, 1002 (1957); (b) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).

(17) (a) E. Cherbuliez and J. Rabinowitz, *Helv. Chim. Acta*, **39**, 1461 (1956). (b) After this manuscript had been completed, a paper appeared by E. Cherbuliez, G. Cordahi and J. Rabinowitz, *ibid.*, **43**, 863 (1960), in which they showed that the material reported earlier as being 2-cyanoethyl phosphate was in reality the corresponding amide. In this paper they report the syntheses of 2-cyanoethyl phosphate in 16% yield using phosphorus oxychloride and a tertiary base.

(18) H. R. Snyder and C. T. Elston, THIS JOURNAL, **76**, 3039 (1954).

(19) For a discussion of the mechanism of action of DCC, see M. Smith, J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 6204 (1958).

This compound was chosen not only because reactions with it can be followed by its ultraviolet light absorption but also because the phosphorylation of the hindered secondary alcoholic group is an excellent test of the power of a phosphorylating agent. The reaction sequence going from 5′-O-tritylthymidine (VIII) to thymidine-3′ phosphate (XI) is.



The various factors affecting the reaction were then studied. First, an experiment was set up to determine the rate of the reaction at room temperature (higher temperatures have not yet been explored). Equal molar amounts of the two reactants V and VIII in anhydrous pyridine (0.1 mmole of each per ml.) were allowed to react with five equivalents of DCC. Aliquots were removed from the reaction mixture at various time intervals and treated with acid and alkali to remove the protecting groups from the various derivatives as described in the Experimental. The products were then chromatographed on Whatman 1 paper in solvent A, the ultraviolet-absorbing spots eluted quantitatively and the amount of each determined spectrophotometrically at 267 mμ. Only two spots were detected, untreated thymidine and the product thymidine-3′ phosphate. The results are recorded in Fig. 1. It can be seen that the reaction can go to completion under these conditions but that it is slow.

Next, a series of experiments was set up to find the best ratio of phosphorylating agent to nucleoside. Reaction mixtures similar to that above, but containing various proportions of 2-cyanoethyl phosphate to 5′-O-tritylthymidine were used. The reactions were left two days at room temperature, worked up as described in the Experimental and assayed as before. The results are shown in Table I.

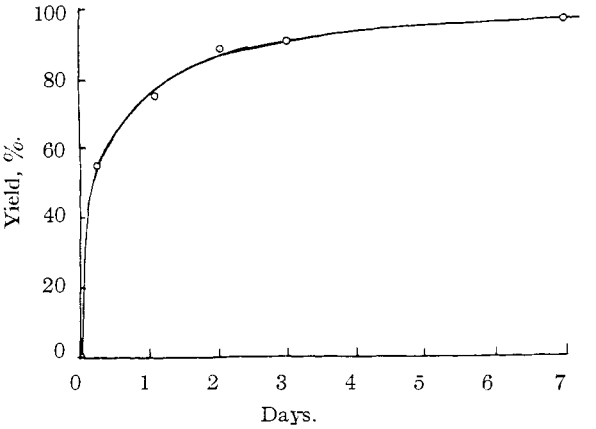


Fig. 1.—The yield of thymidine-3′ phosphate with time, using equimolar quantities of 5′-*o*-tritylthymidine and 2-cyanoethyl phosphate (see text).

TABLE I

Ratio of 2-cyanoethyl phosphate to 5′-O-tritylthymidine	Thymidine-3′ phosphate, %	Thymidine, %
0.5	50	50
1.0	82	18
1.25	91	9
1.5	96	4
2.0	100	0

These experiments show that with a one mole excess of either reactant, the reaction goes to completion under the specified conditions of temperature and time. In the light of these results, an excess of 2-cyanoethyl phosphate has been used in most of the phosphorylations reported here. However, in those cases where it is desirable to use all the phosphorylating agent, for example in the synthesis of P^{32}-labeled nucleotides, an excess of the nucleoside can be used.

On the basis of these observations, a synthesis of thymidine-3′ phosphate was carried out using 2 millimoles of 2-cyanoethyl phosphate, 1 millimole of 5′-*O*-tritylthymidine and 6 millimoles of DCC. After reaction for two days at room temperature followed by the appropriate work-up, no trace of thymidine remained— the sole ultraviolet absorbing spot on paper chromatograms was thymidine-3 phosphate. The product was isolated as its barium salt in better than 90% yield.

Next, studies were undertaken to compare the alkaline stability of the cyanoethyl group in a phosphomonoester (2-cyanoethyl phosphate) with it in a phosphodiester (2-cyanoethyl thymidine-3′ phosphate (X)). This latter product was obtained by detritylating the reaction intermediate IX in 80% acetic acid at 100° for 15 minutes followed by chromatography on Whatman 3 MM paper using solvent A. The compound with an R_f of *ca.* 0.5 was eluted with water and used directly. In agree-

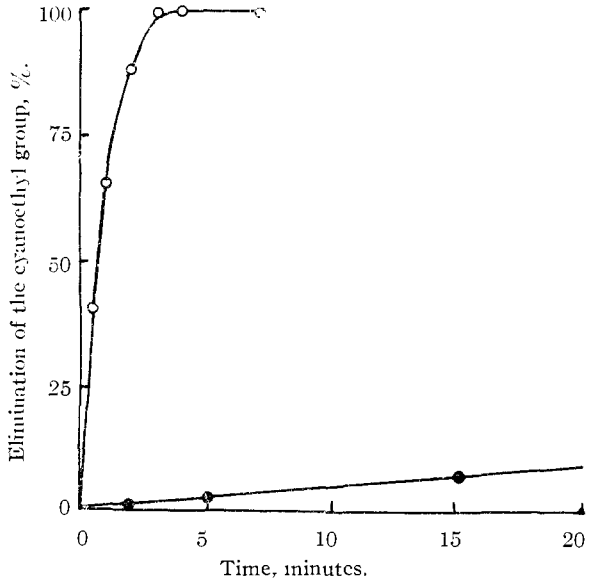


Fig. 2.—The rate of elimination of the cyanoethyl group from 2-cyanoethylthymidine-3′ phosphate (open circles) and 2-cyanoethyl phosphate (solid circles) in 0.1 *N* sodium hydroxide at 50°.

ment with Cherbuliez and Rabinowitz[17] we have found the half-life of 2-cyanoethyl phosphate to be very short—in the order of 1 minute in 1.0 *N* sodium hydroxide at 100°. It was completely destroyed in 5 minutes. In 0.1 *N* sodium hydroxide at 100° the half-life was about 5 minutes and the reaction was complete in 30 minutes.

On the other hand, 2-cyanoethyl thymidine-3′ phosphate was completely converted to thymidine-3′ phosphate in less than 2 minutes at 100° in 1.0 *N* sodium hydroxide. In order to get a better idea of the relative stabilities of the two compounds, less drastic conditions were studied. The hydrolysis curves are shown in Fig. 2 for degradation at 50° by 0.1 *N* sodium hydroxide. The breakdown of 2-cyanoethyl phosphate was followed by the rate of release of inorganic phosphate and that of 2-cyanoethyl thymidine-3′ phosphate by the formation of thymidine-3′ phosphate as shown by quantitative paper chromatography. The diester was degraded many times faster than the monoester. This markedly increased lability of the diester is an advantage when phosphorylating alkali-sensitive compounds and in the synthesis of P^{32}-labeled 2-cyanoethyl phosphate discussed below. In the normal phosphorylation procedure, the separation of the product from excess reagent can be accomplished in several ways. If the reaction product is completely stable to alkali, *e.g.*, thymidine-3′ phosphate the excess 2-cyanoethyl phosphate is destroyed by prolonged boiling with alkali, to give inorganic phosphate which is then separated from the product as lithium or barium phosphate. In those cases where the product is not stable to alkali, the excess reagent can be separated by chromatography on ion exchange resins (*cf.* deoxycytidine-3′ phosphate synthesis) or by precipitating the excess reagent as the barium salt while the nucleotide is still at the diester level. Barium salts of phosphodiesters are generally very soluble in aqueous alcohol, whereas barium 2-cyanoethyl phosphate is not. This latter method has been used successfully by Heidelberger and Sunthankar[20] in the synthesis of 5-fluorodeoxyuridylic acid.

Besides thymidine-3′ phosphate, several other nucleotides have been prepared. These include examples from both the ribo- and deoxyribonucleotide series, as well as dinucleotides. The synthesis of thymidine-5′ phosphate from 3′-O-acetylthymidine was straightforward and it was isolated in 87% yield as the barium salt. If, however, the reaction mixture was left for several days, a new spot, which increased with time, could be observed on paper chromatograms (R_f 0.9 in solvent A). This compound has not yet been fully characterized, but its behavior is reminiscent of the N-phosphorylureas described by Dekker and Khorana.[21]

The phosphorylation of nucleosides containing amino groups on the purine or pyrimidine rings presents an additional problem, since these groups compete for the phosphorylating agent. A detailed study of the extent of phosphorylation of these groups is discussed below (see Table III), but for preparative purposes sufficient 2-cyanoethyl phosphate is used to completely phosphorylate both the alcoholic and amino groups, and then the phosphate is selectively removed from the amino group during work-up. For example, isopropylidine guanosine was readily phosphorylated using four moles of the reagent to give guanosine-5′ phosphate. The O, N-diphosphate which formed as an intermediate could be demonstrated by paper electrophoresis, but the phosphoamide proved to be very labile to acid and broke down even under neutral conditions. This lability held also in the adenosine series (*cf.* ref. 3a). On the other hand, with the cytosine nucleotides the phosphoamide is much more stable. In the case of deoxycytidylic acids, the acidic conditions necessary to cleave the phosphoamide bond also cleaves some of the glycosidic linkage. This difficulty could be circumvented, however, since it was observed that a solution at *p*H 7 of the ammonium salt of N-phosphodeoxycytidine-3′ phosphate was unstable and slowly liberated deoxycytidine-3′ phosphate and inorganic phosphate. The process could be accelerated by heating and was complete after 40 minutes at 100°. No glycosidic cleavage occurred under these conditions. Analogous adenosine and guanosine derivatives can also be cleaved by this method.

The yield of deoxycytidine-3′ phosphate was only 35%, which was much lower than with other nucleotides. Chromatographic studies on the crude reaction mixtures showed deoxycytidine-3′ phosphate to be by far the major reaction product absorbing ultraviolet light and containing phosphorus. All the starting material could not be accounted for, but it is probable that in the reaction mixture the deoxycytidine forms an insoluble derivative which is removed along with dicyclohexylurea during the work-up. This reaction requires further investigation. The phosphorylation of deoxycytidine with an excess of 2-cyanoethyl phosphate to give deoxycytidine-3′,5′ diphosphate

(20) Private communication.

(21) C. A. Dekker and H. G. Khorana, THIS JOURNAL, **76**, 3522 (1954).

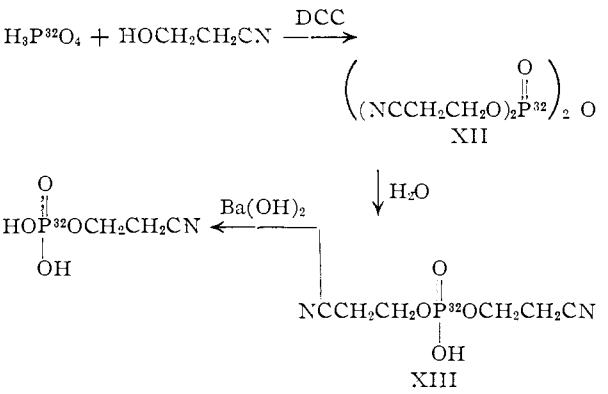
and with a limited amount of the reagent to give primarily the 5′-phosphate in better yields proceeded without difficulty.

Cyanoethyl phosphate is also useful for phosphorylating terminal hydroxyl groups of polynucleotide chains. The phosphorylation of 5′-O-tritylthymidylyl-(3′→5′)-thymidine gave thymidylyl-(3′→5′)-thymidylic-(3′) acid[16b] in much better yield than the previous method. Likewise the method was used to prepare 5′-*O*-phosphoryl-thymidylyl-(3′→5′)-thymidylic-(3′) acid by phosphorylation of both hydroxyl groups of thymidylyl-(3′→5′)-thymidine.

Methods for the Synthesis of P^{32}-Labeled Nucleotides.—In recent years P^{32}-labeled nucleotides have proved very useful for elucidating the mechanisms of biochemical reactions. However, more extensive use of these compounds has been limited by their availability. Generally they must be prepared by biological means[22] which limits not only the specific activity of the desired compounds but also the types of esters which may be prepared. These limitations would not apply to chemical methods. There are at present two readily available reagents that can be used in the synthesis of P^{32}-labeled compounds. These reagents are phosphorus oxychloride and polyphosphoric acid, which is prepared from labeled phosphoric acid.

Phosphorus oxychloride has been used for the synthesis of P^{32}-labeled adenosine-2′ and -3′ phosphates with a yield of 18%,[23] but this reagent is subject to the objections mentioned earlier and, as well, it is very sensitive to traces of water and presents a hazard because of its volatility. It is not considered suitable for small-scale experiments. Labeled polyphosphoric acid has been used successfully in the synthesis of labeled pyrimidine nucleotides,[24] but its use is limited because of its strong acidity and, further, with it the radioactive yields are very low, which necessitates handling large amounts of radioactivity when preparing products of high specific activity.

In view of the general nature of the 2-cyanoethyl phosphate procedure and of the fact that it gives good yields of product, it was decided to adapt the method to the synthesis of P^{32}-labeled nucleotides. It was considered desirable to prepare the reagent directly from P^{32}-labeled phosphoric acid. The method devised was based on two observations: firstly, that a phosphodiester loses a cyanoethyl group much more readily than a monoester does (see Fig. 2), and secondly, that a mixture of phosphoric acid and an excess of alcohol in pyridine will condense in the presence of DCC to give a quantitative yield of a diester. As expected, phosphoric acid and hydracrylonitrile readily condensed in pyridine with DCC to form tetra-(2-cyanoethyl) pyrophosphate (XII). This compound was a bit more stable in aqueous pyridine than other pyrophosphates studied earlier, but it could be hydrolyzed to di-(2-cyanoethyl) phosphate (XIII) by brief heating. As demonstrated by chromatography, XIII was the only phosphate ester present in the reaction mixture. It was, in turn, readily converted to the desired 2-cyanoethyl phosphate by treatment with barium hydroxide at room temperature for five minutes. The over-all scheme is



The product was isolated as the crystalline barium salt in about 60% yield and was converted to the pyridinium salt before use.

The studies done so far with this reagent have been concerned with the synthesis of P^{32}-labeled nucleoside-5′ phosphates, since these esters are of primary interest to the biochemist. A different approach to these syntheses was required from that used in the standard phosphorylations described above. Here it is desirable to incorporate as much of the labeled phosphorus as possible and, to achieve this, an excess of the alcohol (nucleoside) must be employed (see Table I). In addition, one should ideally use nucleosides protected on all functional groups, except the group to be phosphorylated. In practice though, these intermediates can be prepared only with difficulty and were found to be unnecessary for the synthesis of the 5′-phosphates. The commercially available deoxyribonucleosides and 2′,3′-O-isopropylidene ribonucleosides have been used directly.

In order to use these unprotected or partially protected nucleosides, a study was required on the rates of phosphorylation of their various functional groups, namely, the 3′- and 5′-hydroxyl and the ring amino groups. The rate of phosphorylation was studied by allowing 2-cyanoethyl phosphate plus DCC in pyridine to compete for the groups in the presence of a large excess (ninefold) of the nucleoside and then to isolate and determine the amount of each phosphorylated product. Two major ultraviolet-absorbing bands were separated by chromatography in solvent A—one was the unreacted nucleoside (R_f 0.6–0.8) and the other (R_f 0.05–0.2) contained phosphorylated derivatives. In each case this latter band was eluted with water and analyzed. When thymidine was phosphorylated, this band contained a mixture of thymidine-3′ and-5′ phosphates. This latter nucleotide is readily dephosphorylated by the 5′-nucleotidase of crude rattlesnake venom (*Crotalus adamanteus*) whereas the 3′-isomer is unaffected. By rechromatographing an enzymic digest of the eluate again in solvent A and by quantitatively eluting the products, it was possible to find their relative amounts

(22) See, for example, (a) L. V. Eggleston, *Biochem. J.*, **58**, 503 (1954); (b) C. Heidelberger, E. Harbers, K. Liebman, Y. Takagi and V. R. Potter, *Biochem. et Biophys. Acta*, **20**, 445 (1956); (c) R. Lehman, M. J. Bessman, E. S. Simms and A. Komberg, *J. Biol. Chem.*, **233**, 163 (1958).

(23) G. R. Barker, *J. Chem. Soc.*, 3396 (1954).

(24) R. W. Chambers, THIS JOURNAL, **81**, 3032 (1959).

TABLE II

RATIO OF PRODUCTS FROM DIRECT PHOSPHORYLATION OF NUCLEOSIDES

Reactant	3′-Phosphate	5′-Phosphate	N-Phosphate
Thymidine	1	7	...
Deoxycytidine	1	7	6
Deoxyadenosine	1	16	Not detd.
Isopropylideneadenosine	..	4	1
Isopropylideneguanosine	..	9	1
Isopropylidenecytidine	..	7	6

and thereby the ratio of isomers in the original mixture (see Table II).

With nucleosides containing ring amino groups, the eluted "mononucleotide" band was heated at 100° for an hour to break the phosphoamide linkages. The hydrolysate was then rechromatographed and the ratio of nucleoside to nucleotide determined. This ratio showed the rate of attack of the phosphorylating agent on the ring amino group relative to the hydroxyl groups. In the deoxynucleoside series, the resulting mixture of 3′- and 5′-phosphates was assayed as before after digestion with snake venom. The results of these studies with a number of nucleosides are shown in Table II.

It can be seen that, even in the most unfavorable case, that of the cytosine nucleosides, the loss of phosphorylating agent due to phosphorylation of the amino group is less than 50%. In view of the simplicity in using the readily available nucleosides directly for these syntheses, this loss is not considered serious.

In the deoxynucleotide series, a mixture of the 3′- and 5′-isomers was produced. In all cases, except with thymidine, these isomers were readily separated by ion exchange chromatography on Dowex 1 resin. Even with the thymidine nucleotides, some separation was achieved, since the product was found to be at least 95% thymidine-5′ phosphate. And for most studies this small amount of contaminating 3′-isomer is of little consequence. However, the pure 5′-isomer can be prepared when necessary by phosphorylating the readily synthesized 3′-O-acetylthymidine.[29] It is interesting to note that the 3′-hydroxyl group of deoxyadenosine is much less susceptible to phosphorylation than the corresponding group in either thymidine or deoxycytidine.

Two side reactions are apparent in these phosphorylations. As mentioned in the case of the large scale synthesis of thymidine-5′ phosphate, products having the properties of phosphorylureas slowly formed. These compounds are very fast running on paper chromatograms in solvent A and break down slowly in acid to give back the nucleotide. The second by-product arises from experimental manipulations. In most experiments water is added to terminate the phosphorylation reaction. The water presumably breaks down "metaphosphates" and tetraalkyl pyrophosphates, but in some cases this reaction is slower than in others. For example, in the synthesis of deoxyadenosine-5′ phosphate, it was found necessary to boil the reaction mixture for a short period after adding water; otherwise in the following step when ammonia is added to remove the cyanoethyl groups an additional product is formed which from paper chromatographic and electrophoretic studies appears to be deoxyadenosine-5′ phosphoamidate. This amidate could arise by attack of ammonia on the pyrophosphate bond. The alternate possibility, that it arose by the DCC-catalyzed addition of ammonia to the nucleotide,[25] appears unlikely in view of the absence of these amidates in the synthesis of other nucleotides.

For obvious reasons, these methods for the syntheses of the P^{32}-labeled nucleotides have been developed using unlabeled phosphoric acid and unlabeled 2-cyanoethyl phosphate. However, one synthesis of P^{32}-labeled 2-cyanoethyl phosphate starting with 0.14 m*M* phosphoric acid containing 0.56 mc. of P^{32} and its conversion to P^{32}-labeled uridine-5′ phosphate has been carried out and, as expected, the synthesis of the labeled material proceeded in a manner identical to that for unlabeled uridine-5′ phosphate. Because of the short half-life of P^{32}, labeled nucleotides must be prepared at frequent intervals. The cyanoethyl phosphate procedure provides a convenient method for doing this.

Experimental

General Methods.—Paper chromatography was carried out using a descending technique on Whatman 1 or 3 MM paper: solvent A, isopropyl alcohol–concentrated ammonium hydroxide-water, 7:1:2; solvent B, butanol–acetic acid–water, 5:3:2.

Reagent grade pyridine was kept anhydrous by storing it over calcium hydride. It was used directly without distillation. Phosphorus was determined quantitatively by the method of King[26] and qualitatively on chromatograms by the method of Hanes and Isherwood.[27] All evaporations were carried out *in vacuo* (10 mm.) at a temperature of 40° or less. Paper electrophoreses were done with standard buffers by the technique of Markham and Smith.[28]

The syntheses of P^{32}-labeled nucleotides are best carried out in a long-necked flask (Kjeldahl flask) since the neck helps to confine splashing and foaming encountered during evaporations. The evaporations were conveniently done on a portable flash evaporator (Buchler Instruments Inc., New York 31, N.Y.).

Synthesis of Cyanoethyl Phosphate. (A) Polyphosphoric Acid Method.—A mixture made by brief heating at 100° of 20 g. of phosphorus pentoxide and 20 g. of 85% phosphoric acid was cooled to room temperature and 15 g. of hydracrylonitrile added. The solution was stirred well and left 15 hours in a stoppered flask. The reaction mixture was then dissolved in 2 l. of water and the solution neutralized by the addition of solid barium carbonate. The *p*H of the solution was finally adjusted to 7.5 with barium hydroxide, then filtered to remove insoluble barium salts. The product crystallized from the clear filtrate on the addition of three volumes of ethanol. Crystallization was completed by cooling to 0° and the product collected by centrifugation. It was washed twice with 50% aqueous ethanol and then with 95% ethanol and finally air dried—yield about 10%. This product was not chromatographically homogeneous, so it was converted to the cyclohexylammonium salt in the following manner: the barium salt (2 g.) was dissolved in water with the aid of about 2 cc. of Dowex 50 (H^+) resin and passed through a small column of the same resin to ensure complete removal of barium ions. The effluent was neutralized to *p*H 7.5 with cyclohexylamine and the solution concentrated to dryness *in vacuo*. The white residue was dissolved in about 80 ml. of absolute methanol, filtered, and then heated to boiling. As the methanol boiled off, ethyl acetate was slowly added, keeping the volume constant.

(25) R. W. Chambers and J. G. Moffatt, THIS JOURNAL, **80**, 3752 (1958).

(26) E. J. King, *Biochem. J.*, **26**, 292 (1932).

(27) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

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

When most of the methanol had evaporated, the solution started to become turbid, 1 ml. of methanol was added and the solution set aside to allow crystallization. The product (first crop 1.3 g.) was collected as colorless needles, m.p. 165° (with prior softening). The infrared spectrum in a Nujol mull showed major bands at 2,500, 2,210, 1,623 and 1,548 cm.$^{-1}$; R_f in solvent A, 0.25.

Anal. Calcd. for $C_{15}H_{32}N_3O_4P \cdot 1/2H_2O$: C, 50.2; H, 9.27; N, 11.7. Found: C, 50.01; H, 9.05; N, 11.95.

When the above synthesis was modified by heating the phosphorylation mixture at 100° for 1 hour, a trace of 2-cyanoethyl phosphate could be detected chromatographically, but the major component was a new spot, isolated as the crystalline cyclohexylammonium salt by the above procedure; m.p. 180–190°. The infrared spectrum determined in a Nujol mull showed peaks at 2,500, 2,200, 1,675, 1,628 and 1,550 cm.$^{-1}$; R_f in solvent A, 0.18.

Anal. Calcd. for $C_{15}H_{34}N_3O_5P$: C, 49.1; H, 9.34; N, 11.45. Found: C, 48.93; H, 9.42; N, 11.47.

(B) **Phosphorus Oxychloride Method.**—Phosphorus oxychloride (30.6 g.,-0.2 *M*, 18.4 ml.) was mixed with 200 ml. of anhydrous ether in a three-necked flask containing a thermometer, a sealed stirrer and a pressure-equalizing dropping funnel stoppered with a silica gel drying tube. The solution was cooled to −13° in an ice–salt–bath, then a mixture of 15.8 g. of anhydrous pyridine (0.2 *M*, 16.1 ml.) and 14.2 g. (0.2 *M*) of hydracrylonitrile was added dropwise with vigorous stirring. The addition took about an hour in order to maintain a reaction temperature of −10° or below. Then it was stirred an additional hour at −10°. Pyridine hydrochloride crystallized out, but was not removed. The mixture was then slowly poured with stirring into a mixture of 750 ml. of water, 80 ml. of pyridine and 300 g. of ice. To this reaction mixture was added a solution of 100 g. of barium acetate in 300 ml. of water. The solution was set aside 2 hours to allow aggregation of the barium phosphate and then filtered on a Büchner funnel. The remaining ether evaporated at this stage. To precipitate the product, two volumes of 95% ethanol was added slowly with stirring to the clear filtrate. Gleaming platelets of the barium salt separated; these were collected after 1 hr. at 0°. The crystals were washed with 50% ethanol followed by pure ethanol and then air-dried to give 41 g. of product. A sample recrystallized as described below was analyzed.

Anal. Calcd. for $C_3H_4NO_4PBa \cdot 2H_2O$: C, 11.18; H, 2.50; N, 4.35; P, 9.6. Found: C, 11.6; H, 2.40; N, 4.34; P, 9.7.

The product decomposed on drying at 100°. Drying *in vacuo* over P_2O_5 for four days removed one additional mole of water but the product was hydroscopic. A small second crop of material could be obtained by adding acetone to the mother liquors from the above filtration.

When necessary, last traces of inorganic phosphate were removed by dissolving the product (but not the barium phosphate) in water (40 g. in 1.5 l.) by careful addition of acetic acid, then readjusting the solution to *p*H 7 with barium hydroxide. The solution was quickly filtered or centrifuged to remove the small amount of insolubles and the product recrystallized by the addition of two volumes of ethanol. On occasion, the barium salt commenced to crystallize even before the addition of alcohol.

A standard solution containing 1 mmole/ml. for use in phosphorylation runs was prepared in the following way: 16.1 g. of the dried barium salt was dissolved in water (add Dowex 50 [H^+] resin to aid solution) and washed through a column of Dowex 50 [H^+] resin; 20 ml. of pyridine was added to the effluent and the solution concentrated *in vacuo* to about 20 ml. This solution was transferred to a 50-ml. volumetric flask and diluted to the mark with pyridine. The reagent thus prepared showed no sign of decomposition after 1 month at 0°; however, after 4 months traces of inorganic phosphate could be detected.

Thymidine-3′ Phosphate.—A solution of 567 mg. of 5′-O-tritylthymidine[16b] (1 mmole containing one benzene of crystallization) and 2 mmoles of 2-cyanoethyl phosphate (2 ml. of the standard solution) in 20 ml. of pyridine was concentrated to dryness *in vacuo* at 30°. The residue was taken up in 20 ml. of anhydrous pyridine and again concentrated to dryness. The process was repeated a second time to ensure removal of water. Finally, the residue was dissolved in 10 ml. of anhydrous pyridine and 1.67 g. of DCC added. The reaction was left in a well-stoppered flask for 2 days at room temperature, during which time dicyclohexylurea separated. One ml. of water was then added and after an hour at room temperature the solution was concentrated to dryness *in vacuo*. The residue was treated with 30 ml. of 10% acetic acid at 100° for 20 minutes and then concentrated to dryness. Last traces of acetic acid were removed by a second evaporation after adding 20 ml. of water. The residue was next treated with 40 ml. of 0.5 *N* lithium hydroxide at 100° for 45 minutes. The reaction mixture was cooled and the precipitate removed by centrifugation. The supernatant was then passed through a column (2 × 20 cm.) of Dowex 50 [H^+] resin and the effluent adjusted to *p*H 7.5 with barium hydroxide. The solution was concentrated *in vacuo* to about 15 ml., filtered to remove a trace impurity and the barium thymidine-3′ phosphate precipitated by the addition of two volumes of ethanol. The precipitate was collected by centrifugation, washed with 50% ethanol followed by ethanol, acetone and then ether. Finally it was dried *in vacuo* over P_2O_5. The yield was 88%, 450 mg., of a product which analyzed as the trihydrate. The product was chromatographically pure and behaved identically with a sample of thymidine-3′ phosphate prepared by the method of Michelson and Todd.[29]

Thymidine-5′ Phosphate.—A solution of 284 mg. of 3′-acetylthymidine[29] (1 mmole) and 2 mmoles (2 ml. of stock solution) of 2-cyanoethyl phosphate in 10 ml. of pyridine was concentrated *in vacuo* at 30° to an oil. The oil was then taken up in 20 ml. of anhydrous pyridine and the solution concentrated to dryness. The process was repeated. Next, 10 ml. of anhydrous pyridine and about 1.2 g. of DCC (an excess, about 6 mmoles) were added, the reaction flask tightly stoppered and set aside for 2 days at room temperature.

Three ml. of water was then added and the solution left a half-hour at room temperature to destroy "metaphosphates." Pyridine was then removed by concentrating the solution to dryness *in vacuo* and the residue taken up in 10 ml. of water and filtered to remove dicyclohexylurea. The urea was washed well with an additional 10 ml. of water. Twenty ml. of 1 *N* sodium hydroxide was added to the combined filtrate and washings which was then heated under reflux. The diester was converted to the monoester in less than 2 minutes at 100°; however, the excess 2-cyanoethyl phosphate required an additional 40 minutes heating to destroy it completely (see Fig. 2). The solution was cooled and then passed through a column of Dowex 50 [H^+] resin to remove sodium ions. Paper chromatography at this stage revealed the presence of only one ultraviolet-absorbing material. The *p*H of the effluent was adjusted to 7.5 with barium hydroxide and the barium phosphate removed by centrifugation and washed with water in the same manner. The addition of two volumes of alcohol brought down the barium thymidine-5′ phosphate. The product was dried by washing with ethanol, followed by acetone, then ether and finally by removing traces of solvent *in vacuo*. It was redissolved in water, centrifuged to remove a trace of impurity and again precipitated in the above manner; yield of vacuum dried material 428 mg. which analyzed as the decahydrate (67% yield). The product was chromatographically homogeneous and was completely degraded by crude snake venom to thymidine. It was identical in all respects to a commercial sample of thymidine-5′ phosphate. A second crop of product can be obtained by reworking the mother liquors to give an over-all yield of 87%.

Thymidylyl-(3′ → 5′)-thymidylic-(3′) Acid.—A solution of the pyridinium salt of 5′-tritylthymidylyl-(3′→5′)-thymidine[16b] (0.1 mmole) and 2-cyanoethyl phosphate (0.3 mmole) in 10 ml. of pyridine was concentrated to dryness *in vacuo* at 25%. The residue was dissolved in 10 ml. of anhydrous pyridine and again concentrated to dryness. This process was repeated once more and finally the residue was dissolved in 2 ml. of anhydrous pyridine. Two grams of DCC was added and the flask stoppered and shaken until the solution became homogeneous (1 minute). The reaction was then left 2 days at room temperature, during which time dicyclohexylurea separated; 1 ml. of water was added and after a half-hour at room temperature the mixture was concentrated to dryness. The residue was then treated with 20 ml. of 80% acetic acid under reflux for 20 minutes and again concentrated to dryness. Last traces of acetic acid were removed by adding 10 ml. of water to the residue and again concentrating the solution to dryness. To the residue was

(29) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 951 (1953).

added 20 ml. of 9 *N* ammonium hydroxide and the mixture was then heated at 60° for 1 hour. The solution was filtered to remove trityl alcohol and dicyclohexylurea, concentrated to *ca.* 2 ml. and streaked onto a sheet of Whatman 3MM chromatographic paper. The chromatogram was developed in solvent A for two days using a descending technique (the solvent was allowed to run off the end of the paper). The major band near the origin was eluted with water and passed through a small column (1 × 3 cm.) of Dowex 50 [H^+] resin. The effluent was adjusted to *p*H 7.5 with barium hydroxide and the small precipitate removed by centrifugation. The barium salt of the product was precipitated by adding two volumes of ethanol. It was collected by centrifugation, washed with ethanol and then acetone and ether and finally dried *in vacuo*. The yield, determined spectrophotometrically, was 55%. The product was identical with the material prepared earlier.[16b]

5′-Phosphoryl-thymidylyl-(3′ → 5′)-thymidylic-(3′) Acid.—An analogous reaction to that described above, starting with thymidylyl-(3′→5′)-thymidine,[15b] 5 equivalents of 2-cyanoethyl phosphate and 15 equivalents of DCC gave 5′-phosphoryl-thymidylyl-(3′→5′)-thymidylic-(3′) acid. It was isolated as its barium salt; specific extinction at 267 mμ per phosphorus 6,200.

Anal. Calcd. for $C_{20}H_{24}N_4O_{18}P_3Ba_{1/2}\cdot 7H_2O$: P, 7.9. Found: P, 7.88.

Guanosine-5′ Phosphate.—A solution of 323 mg. (1 mmole) of 2′,3′-*O*-isopropylideneguanosine in 10 ml. of 50% aqueous pyridine and 4 mmole of 2-cyanoethyl phosphate (4 ml. of stock solution) was concentrated to dryness *in vacuo* at 30°. The residue was dissolved in 10 ml. of anhydrous pyridine and again concentrated to dryness. This process was repeated twice more and, finally, the oily residue was dissolved in 10 ml. of anhydrous pyridine. Two grams of DCC was added and the solution left under anhydrous conditions at room temperature for 18 hours. The reaction was terminated by the addition of 1 ml. of water and after a half-hour at room temperature the solution was concentrated to dryness *in vacuo*. Twenty ml. of water was added and the solution concentrated once more; 40 ml. of 0.4 *N* lithium hydroxide was added to the residue and the mixture was heated under reflux for 1 hour. The solution was cooled, filtered to remove dicyclohexylurea, lithium phosphate and acrylonitrile polymers and the filtrate passed through a column (3 × 4.2 cm.) of Dowex 50 [H^+] ion exchange resin. The column was washed until the effluent was neutral (final volume 320 ml.). This solution was left 2 hours at room temperature (or alternately, adjusted to *p*H 2.8 and heated under reflux for 1.5 hours) and then concentrated *in vacuo* at 30° to 50 ml. and neutralized to *p*H 7.5 with saturated barium hydroxide. The precipitate of barium phosphate was removed by centrifugation and repeatedly washed. The final volume was 180 ml. Two volumes of ethanol was then added and the precipitated barium salt of guanosine-5′ phosphate collected by centrifugation, washed with ethanol, acetone and then ether. The product, dried *in vacuo*, weighed 538 mg. (81%) (9,900 O.D. units, at 257 mμ, 0.1 *N* hydrochloric acid) and analyzed as the nonahydrate. This product was converted to the more soluble sodium salt by suspending it in 10 ml. of water, adding 3 ml. of Dowex 50 [H^+] resin and stirring the suspension until all the barium salt dissolved. The solution was passed through a small column of the same resin. The combined effluent and washings (200 ml.) was neutralized to *p*H 7.5 with sodium hydroxide and the solution concentrated to dryness. The residue was dissolved in 5 ml. of water and precipitated by the addition of 3 volumes of acetone. The precipitate was collected by centrifugation, washed with alcohol, then acetone and finally ether. The white solid was dried *in vacuo* over phosphorus pentoxide for 24 hours (yield 318 mg., 72%); specific extinction at 256 mμ in 0.1 *N* hydrochloric acid per phosphorus, 12,900.

Anal. Calcd. for $C_{10}H_{12}N_5O_8PNa_2\cdot 2H_2O$: P, 7.0. Found: P, 7.05.

Deoxycytidine-3′ Phosphate.—A solution of 96 mg. (0.2 mmole) of 5′-*o*-trityldeoxycytidine[30] and 0.8 mmole of 2-cyanoethyl phosphate in 10 ml. of pyridine was concentrated to dryness *in vacuo* at 30°. Ten ml. of anhydrous pyridine was added and the solution again concentrated to dryness. The process was repeated. Finally the residue was taken up in 2 ml. of anhydrous pyridine and 550 mg. of DCC added. The flask was stoppered and set aside at room temperature for 2 days. One ml. of water was then added and after a half-hour at room temperature the solution was concentrated to dryness *in vacuo*. Last traces of pyridine were removed by twice adding 10 ml. of water and concentrating to dryness. The product was detritylated by adding 20 ml. of 80% acetic acid and heating the solution under reflux for 10 minutes, after which it was concentrated to dryness *in vacuo*. To the residue was added 20 ml. of 9 *N* ammonium hydroxide and the solution heated at 55° for 1 hour. The precipitate was removed by filtration and the filtrate gently boiled to drive off excess ammonia and finally boiled under reflux for 45 minutes (final *p*H 7). The solution was cooled, filtered to remove a trace of solid and run onto a Dowex 1 (formate: 200–400 mesh) resin column (1 × 8 cm.), the column washed well with water and the product eluted with 0.05 *M* formic acid. Elution of deoxycytidine-3′ phosphate began after 140 ml. of acid had passed through the column and was complete after an additional 120 ml. had passed. This fraction was concentrated to dryness *in vacuo* and taken up in 2 ml. of water. A slight precipitate was removed by centrifugation and 10 ml. of alcohol was added. The solution was set aside for 1 hour at 0°. The crystalline deoxycytidine-3′ phosphate was collected by filtration and washed with ethanol; yield 21 mg. of product identical to commercial samples.

Deoxycytidine-3′,5′ Diphosphate.[31]—Deoxycytidine hydrochloride (271 mg., 1.03 mmoles) was deionized by absorbing it onto a column (1 × 10 cm.) of Dowex 50 [H^+] resin and then washing the column free of chloride ion. The nucleoside was eluted by 50 ml. of 9 *N* ammonium hydroxide. The eluate was concentrated to dryness *in vacuo*, the residue dissolved in 10 ml. of pyridine and again concentrated to dryness; 6 ml. of the standard solution of 2-cyanoethyl phosphate (6 mmoles) was added along with 20 ml. of pyridine. The solution was concentrated to dryness *in vacuo*, the residue redissolved in 20 ml. of dry pyridine and again concentrated to dryness. Finally the residue was dissolved in 10 ml. of dry pyridine, 3.6 g. of DCC added and the solution set aside for 2 days at room temperature in a well-stoppered flask; 2 ml. of water was added and the mixture left one hour. Then an additional 100 ml. of water was added and the precipitate removed by filtration and washed. One ml. of concentrated ammonium hydroxide was added and the solution brought to a boil and allowed to concentrate to about 40 ml. It was then boiled under reflux for 45 minutes, and cooled (14,000 O.D. units at 280 mμ, *p*H 1). It was streaked onto sheets of Whatman 3MM paper and chromatographed in solvent A. (The purification would perhaps be more easily done by ion exchange chromatography on Dowex 2 resin.[31]) The nucleoside diphosphate remained at the origin even after 24 hours development time. This band was cut out and eluted with water. The solution was filtered and an aliquot checked to ensure the absence of inorganic phosphate. Then saturated barium acetate was added until no further precipitation occurred. One volume of alcohol was added and the precipitate collected by centrifugation. It was washed with alcohol, then acetone and finally ether. The precipitate was then triturated with water and isolated and dried as before. The final product after drying at room temperature *in vacuo* weighed 651 mg. (71%). It was chromatographically (solvents A and B) and electrophoretically homogeneous; specific extinction 6,200 at 280 mμ in 0.1 *N* HCl per phosphorus.

Anal. Calcd. for $C_9H_{11}O_{10}N_3P_2Ba_2\cdot 15H_2O$: P, 6.67. Found: P, 6.71.

Reactivity of Functional Groups in Nucleosides.—One mmole each of isopropylidene adenosine, isopropylidene guanosine and benzylidene cytidine were phosphorylated in 5 ml. of anhydrous pyridine in the usual manner with 0.1 mmole of 2-cyanoethyl phosphate and 206 mg. of DCC. The reaction mixtures were left 2 days at room temperature and then 3 ml. of water was added to each. After 1 hour at room temperature, 5 ml. of concd. ammonium hydroxide was added and the solutions heated at 60° for 90 minutes and then chromatographed on sheets of Whatman 3MM paper in solvent A. No heat was used while applying the samples to the paper and the spots were not dried in order to minimize cleavage of the phosphoamide bond. (With these precautions no inorganic phosphate and no 2-cyano-

(30) A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 34 (1954).

(31) C. A. Dekker, A. M. Michelson and A. R. Todd, *ibid.*, 947 (1953).

ethyl phosphate could be seen when test strips were sprayed with the Hanes–Isherwood spray.[27]) The slow-moving band (R_f about 0.1) in each case was eluted with water and worked up in the following manner:

(a) **Adenosine-5′ and Guanosine-5′ Phosphates.**—To 0.5 ml. of eluate was added 0.5 ml. of 20% acetic acid and the solution heated at 100° for 90 minutes. The resultant solutions were chromatographed in solvent A on Whatman 40 paper.

(b) **Cytidylic Acid.**—The eluate (0.5 ml.) was heated at 100° for 1 hour and then 0.5 ml. of 0.1 *N* HCl was added and the solution heated at 100° for an additional hour. The resultant solution was chromatographed in solvent A on Whatman 40 paper. Spots corresponding to nucleotide and nucleoside in each case were eluted with 0.1 *N* HCl and the optical density in each determined—the amount of nucleoside corresponds to the amount of N-phosphorylation. Similar experiments were carried out with thymidine, deoxycytidine and deoxyadenosine. In addition to determining the ratio of O- to N-phosphorylation as described above, the extent of phosphorylation of the 3′-hydroxyl and 5′-hydroxyl group was found. For example, the phosphorylated thymidine was chromatographed and the mononucleotide band eluted from the chromatograms and digested with *Crotalus adamanteus* venom[32] to hydrolyze the thymidine-5′ phosphate. A stock solution of the venom was prepared by dissolving 10 mg. of the commercially available material in 1 ml. of 0.1 *N* tris-(hydroxymethyl)-aminomethane buffer at *p*H 9 and the digestion carried out with 0.1 ml. of the nucleotide solution and 0.1 ml. of the stock solution at 37° for 2 hours. The resultant thymidine and thymidine-3′ phosphate were separated by chromatography as before. The results of these studies are shown in Table II.

Synthesis of P^{32}-Labeled 2-Cyanoethyl Phosphate.—A solution of P^{32}-labeled phosphoric acid containing 1 mmole of phosphate was concentrated to dryness *in vacuo* at 40° to remove traces of hydrochloric acid usually found in commercial samples of labeled phosphoric acid. Then 10 ml. of anhydrous pyridine and 1.0 ml. of hydracrylonitrile were added and the solution concentrated *in vacuo* at 40° to an oil (the crystalline pyridinium phosphate which sometimes separated dissolved as the solution became more concentrated). A second 10-ml. portion of anhydrous pyridine was added and the solution again concentrated to an oil. Then 5 ml. of anhydrous pyridine and 2.1 g. of DCC were added and the reaction set aside overnight at room temperature. Water (5 ml.) was added to stop the reaction and the resulting solution heated in a boiling water-bath for 20 minutes. The solution was then concentrated to dryness *in vacuo* and 10 ml. of water and 10 ml. of saturated barium hydroxide were added to the residue. After 5 minutes at room temperature the solution was adjusted to *p*H 7.5 with acetic acid (5 drops of glacial acetic acid) and filtered to remove dicyclohexylurea and barium phosphate. Two volumes of ethanol was added to precipitate the barium 2-cyanoethyl phosphate which was collected after 1 hour at 0° by centrifugation. The crystals were redissolved in 5 ml. of water, centrifuged to remove a trace of insoluble material and recrystallized by adding 10 ml. of ethanol. The product was collected by centrifugation, washed with ethanol, then acetone and finally ether; yield, after air-drying, 192 mg. (60%). For use in phosphorylations the product was dissolved in 5 ml. of water (with the addition of 0.3 ml. of Dowex 50 (pyridinium) resin) and passed through a small column (8 × 30 mm.) of Dowex 50 (pyridinium) resin. The effluent was used directly.

Methods for the Synthesis of P^{32}-Labeled Nucleotides. (A) Thymidylic Acid.—Thymidine (484 mg.) was dissolved in 10 ml. of pyridine and 1 mmole of P^{32}-labeled 2-cyanoethyl phosphate (prepared as above) was added. The solution was concentrated to an oil *in vacuo* at 40° and then 10 ml. of anhydrous pyridine was added and the solution again concentrated to dryness. The process was repeated once more and the residue was dissolved in 5 ml. of anhydrous pyridine and 620 mg. of DCC added. The solution was kept in a well-stoppered flask for 18 hours at room temperature, and then 5 ml. of water was added. After 30 minutes at room temperature, 10 ml. of concentrated ammonium hydroxide was added and the solution warmed at 60° for 1 hour. The reaction mixture was concentrated to dryness *in vacuo* and 10 ml. of water added to the residue. The resultant solution was filtered to remove dicyclohexylurea and the filtrate (25 ml. including washings) was chromatographed to separate components.

(a) **On Paper.**—Aliquots of the solution were spotted on Whatman No. 40 paper and developed in solvent A. Two major and several minor bands were observed. The major band with R_f 0.15 was eluted. It comprised 30% of the total optical density on the paper, *i.e.*, a 60% yield. By spraying the paper with phosphate spray a small amount of unreacted 2-cyanoethyl phosphate moving just ahead of thymidylic acid was seen. (If the reaction mixture was left 48 hours, no cyanoethyl phosphate remained.) On incubating the thymidylic acid with crude snake venom at *p*H 9, 37°, for 4 hours, only 9% of the material remained as unchanged mononucleotide [thymidine-3′ phosphate] and 91% was converted to thymidine.

(b) **On Dowex 1-X8 (Formate, 200 to 400 Mesh).**—The reaction mixture from above was run onto a Dowex 1-X8 (formate) column (2.2 × 16 cm.) and after washing the column with water the product was eluted using a linear gradient of formic acid (2 l. of 2 *N* formic acid into 2 l. of 1 *N* formic acid). The column was run at 2 ml. per minute and 20-ml. fractions were collected. Thymidylic acid emerged in tubes 106–140. The tail end (optical density below 1.6) of the peak was discarded (this is probably thymidine-3′ phosphate). Snake venom digestion showed the product to contain at least 95% thymidine-5′ phosphate.

(B) **Deoxycytidylic Acid.**—One mole of deoxycytidine hydrochloride[33] was dissolved in 5 ml. of water and 1 mmole of P^{32}-labeled 2-cyanoethyl phosphate and 10 ml. of pyridine were added. The solution was concentrated to an oil *in vacuo* and the residue redissolved in 10 ml. of anhydrous pyridine. This solution was again concentrated to dryness and the process repeated once more. The residue was taken up in 5 ml. of anhydrous pyridine and 650 mg. of DCC was added (an oil separated). The reaction mixture was shaken vigorously at room temperature for 2 days and the reaction stopped by the addition of 5 ml. of water; 10 ml. of concd. ammonium hydroxide was added and the mixture heated at 60° for 75 minutes and then concentrated to dryness *in vacuo*. The residue was extracted with water (3 × 10 ml.) and filtered free of dicyclohexylurea. The filtrate was heated at 100° for 45 minutes, cooled and run onto a column of Dowex 1-X8 (formate) (2.2 × 12 cm.). The column was washed well with water to remove deoxycytidine (5,700 O.D. units) and the nucleotide eluted with 0.02 *M* formic acid. Fractions of 20 ml. each were collected. A small fore peak (tubes 7 and 8) was discarded. Deoxycytidine-5′ phosphate (4,240 O.D. units) was eluted in tubes 16–26 and deoxycytidine-3′ phosphate (574 O.D. units) in tubes 28–34. The fractions were concentrated to dryness *in vacuo*, and the residue dissolved in 1 ml. of water. If desired, the product can be crystallized by adding ethanol to the aqueous solution, the crystals collected by centrifugation, washed with ethanol and dried *in vacuo*.

(C) **Deoxyadenosine-5′ Phosphate.**—A solution of 0.325 g. of labeled 2-cyanoethyl phosphate in *ca.* 5 ml. of water and 10 ml. of pyridine was concentrated to dryness; 10 ml. of dry pyridine was added and the solution again concentrated to dryness. Then 10 ml. of anhydrous pyridine and 0.5 mmole of deoxyadenosine (153 mg. of the trihydrate) were added and, after the adenosine had dissolved, the solution was concentrated to dryness *in vacuo* at 20°. The residue was taken up in 2.5 ml. of dry pyridine, 325 mg. of DCC added and the mixture left in a well-stoppered flask for 20 hours at room temperature. Water (5 ml.) was then added and the solution heated in a boiling water-bath for 30 minutes. Then 7.5 ml. of concd. ammonium hydroxide was added and the mixture heated at 60° for 75 minutes. The solution was concentrated to dryness *in vacuo* and the residue extracted thoroughly with water and filtered (total volume 40 ml.). The filtrate was run onto a column (2.2 × 16 cm.) of Dowex 1–X8 (chloride, 200–400 mesh). Elution was carried out using a linear gradient of 1 liter of 0.01 *N* HCl into 1 liter of water. Fractions of about 15 ml. were collected and the optical density of each determined at 260 mμ. Unreacted deoxyadenosine came off at the front, followed by a small peak (310 O.D. units) of an unidentified compound. The deoxyadenosine-5′ phosphate came off in tubes 42–55 (2,700 O.D. units). These fractions were combined and immedi-

(32) Ross Allen's Reptile Institute, Silver Springs, Fla.

(33) Initially deoxycytidine was used, but it was found that the hydrochloride would serve as well. Pyridine hydrochloride comes out of solution but it does not interfere with the reaction.

ately neutralized[34] to *p*H 7.5 with calcium hydroxide. The solution was then concentrated to about 4 ml. (check *p*H), filtered to remove impurities and the product precipitated by adding three volumes of ethanol. The precipitate was collected by centrifugation, washed with alcohol and acetone and dried *in vacuo*.

(D) Ribonucleoside-5′ Phosphates.—The methods for the preparation of the P^{32}-labeled ribonucleoside-5′ phosphates are analogous to those described above in the deoxyribonucleoside series. However, the readily available isopropylidene derivatives have been used to prevent reaction with the 2′- and 3′-hydroxyl groups. The tabulated proportions of reactants have been used. The nucleoside and the 2-cyanoethyl phosphate were dried by repeated evaporation with dry pyridine in the usual manner and then dissolved in 0.5 ml. of dry pyridine and the DCC added. After 20 hours at 25°, the well-stoppered flask was opened and 1 ml. of water added. After an hour the solution was concentrated to dryness *in vacuo*. The residue was hydrolyzed 90 minutes in 10% acetic acid (40 ml.) at 100° to remove the isopropylidene group and cleave phosphoamide bonds. The acetic acid was then removed by evaporating the solution to dryness with last traces being removed by a second evaporation after adding 10 ml. of water. The residue was next heated with 40 ml. of 9 *N* ammonium hydroxide at 60° for 90 minutes and the ammonia removed by concentrating the mixture to dryness. Ten ml. of water was added to the residue and the insoluble dicyclohexylurea removed by filtration. The precipitate was well washed with water. The nucleotides were isolated from the filtrate in 40–60% yield by ion exchange chromatography[35a,b] on Dowex 1 resin, by preparative paper chromatography on Whatman 3MM paper in solvent A or by barium salt precipitation.

For 1 mmole of nucleoside	2-Cyanoethyl phosphate, mmole	DCC, mmoles
Isopropylideneuridine[a]	0.5	2.0
Benzylidenecytidine	1.0	4
Isopropylideneadenosine[a]	0.65	3.0
Isopropylideneguanosine[a]	0.6	2.5

[a] Aldrich Chemical Co. Inc., 2369 N. 29th St., Milwaukee 10, Wis.

(34) The product is chromatographically and electrophoretically pure. For most purposes this solution can be neutralized (*p*H 7), concentrated to a convenient volume and used directly.

Acknowledgments.—I would like to thank Dr. P. Townsley for his help in the synthesis of P^{32}-labeled nucleotides.

(35) (a) W. E. Cohn, THIS JOURNAL, **72**, 1471, 2811 (1950); (b) W. E. Cohn and J. X. Khym, in Shemin, "Biochemical Preparations," Vol. 5, John Wiley and Sons, Inc., New York, N. Y., 1957, pp. 40–48.

[CONTRIBUTION FROM THE STAMFORD LABORATORIES, CHEMICAL RESEARCH DEPARTMENT, CENTRAL RESEARCH DIVISION, AMERICAN CYANAMID CO., STAMFORD, CONN.]

Reactions of Phosphine with Aliphatic Aldehydes[1]

BY SHELDON A. BUCKLER AND V. P. WYSTRACH

RECEIVED AUGUST 8, 1960

The reaction of phosphine with aliphatic aldehydes in aqueous mineral acid solution has been found to be quite useful as a method of C–P bond formation. The nature of the product varies considerably with different types of aldehydes. Of particular interest are spiro-phosphonium salts obtained from dialdehydes and derivatives of 1,3-dioxa-5-phosphacyclohexane obtained from α-branched aldehydes. *n*-Alkyl aldehydes give tetrakis-(1-hydroxyalkyl)-phosphonium salts, and chloral and dichloroacetaldehyde give 1-hydroxyalkyl secondary phosphines. A study of possible catalysts other than aqueous mineral acid is also reported.

Introduction

Relatively few publications have appeared in the literature describing the addition of phosphine to carbonyl groups. The most comprehensive study is that of Messinger and Engels who examined the reaction of phosphine with several aldehydes and ketones in ether solution using anhydrous HCl or HBr as catalyst.[2] Acetaldehyde and propionaldehyde reacted readily to give tetrakis-(1-hydroxyalkyl)-phosphonium halides as the major products (eq. 1, R = CH_3, C_2H_5; X = Cl, Br). In addition, oils were usually obtained which appeared to be tertiary phosphine hydrohalides of the type $(RCHOH)_3P\cdot HX$. Isobutyraldehyde and acrolein gave uncharacterized sirupy masses as products under these conditions.

$$4RCHO + PH_3 + HX \longrightarrow \underset{I}{(RCHOH)_4P^+X^-} \quad (1)$$

More recently, attention has been directed to the reactions of formaldehyde and phosphine. Excellent yields of tetrakis-(hydroxymethyl)-phosphonium chloride have been obtained from reactions carried out in concentrated aqueous HCl.[3,4] The use of a metal salt as catalyst has led to faster reactions and, in the absence of acid, to a product of lesser aldehyde addition, tris-(hydroxymethyl)-phosphine.[5]

$$3HCHO + PH_3 \xrightarrow{PtCl_4} (HOCH_2)_3P \quad (2)$$

Although nothing further appears in the literature relating to the reactions of phosphine with aliphatic aldehydes, certain reactions observed with phosphonium iodide in non-aqueous media will be cited here since these may well proceed by addition of phosphine rather than phosphonium ion. De Girard studied these reactions and found that tetrakis-(1-hydroxyalkyl)-phosphonium iodides and some lower substitution products were obtained from *n*-alkyl aldehydes.[6] In addition, he found that chloral and trichlorobutyraldehyde gave crystalline derivatives which probably have the structures $(CCl_3CHOH)_2PH$ and $(CH_3CHClCCl_2CHOH)_2PH$, respectively.

Recent reports from this Laboratory have described the reactions of phosphine with ketones[7] and aromatic aldehydes[8] which differ from those observed with aliphatic aldehydes in that transfer

(1) A preliminary report of portions of this work has been published: S. A. Buckler and V. P. Wystrach, THIS JOURNAL, **80**, 6454 (1958).

(2) J. Messinger and C. Engels, *Ber.*, **21**, 326, 2919 (1888).

(3) A. Hoffman, THIS JOURNAL, **43**, 1684 (1921).

(4) W. A. Reeves, F. F. Flynn and J. D. Guthrie, *ibid.*, **77**, 3923 (1955).

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(6) M. A. De Girard, *Ann. chim.*, [6] **2**, 11 (1884).

(7) S. A. Buckler and M. Epstein, THIS JOURNAL, **82**, 2076 (1960).

(8) S. A. Buckler, *ibid.*, **82**, 4215 (1960).