

cose?) to citrate. Such conversions should cause a lowering of the pH in the vicinity of the affected bone cells, an observation reported by Cretin<sup>31</sup> some years ago.

It has been known<sup>32</sup> that the circulating fluids are supersaturated with respect to bone mineral and that some such localized production of acid or surface-acting ion such as citrate or carbonate is needed to account for the normal levels of circulating calcium and phosphate.<sup>9,32</sup> It is, perhaps, surprising, however, that the effectiveness of citrate

(31) A. Cretin, *Presse med.*, **69**, 1240 (1951).

(32) W. F. Neuman and M. W. Neuman, "The Chemical Dynamics of Bone Mineral," University of Chicago Press, Chicago, Ill., 1958.

in solubilizing bone mineral is not ascribable to its chelation of calcium ion.<sup>9</sup>

Thus, it is not clear how the hormone increases citric (and others?) acid production but it is clear how variations in acid production in bone regulates the solubility equilibria involving bone mineral and the extra cellular fluids.

**Acknowledgments.**—The authors are indebted to Dr. Arthur Dutton for his help in the statistical analyses of the data and to Dr. Victor DiStefano for placing at our disposal surgical equipment and expert advice.

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## Carbodiimides. VIII.<sup>1</sup> Observations on the Reactions of Carbodiimides with Acids and Some New Applications in the Synthesis of Phosphoric Acid Esters

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The reactions of a variety of acids (in particular, mono- and diesters of phosphoric acid) with a group of carbodiimides in the presence of tertiary bases have been studied. The carbodiimides chosen were dicyclohexyl-, di-*p*-tolyl- and di-*t*-butylcarbodiimides and the bases used were pyridine and tri-*n*-butylamine. The mechanism of reactions of carbodiimides with acids is discussed in the light of these findings. The formation of diesters of phosphoric acid by the reaction of a monoalkyl phosphate with dicyclohexylcarbodiimide in the presence of an alcohol has been examined and it has been demonstrated that this synthesis can occur by two discrete mechanisms. The present studies have resulted in new and improved procedures for the synthesis of ribonucleoside-2',3' cyclic phosphates, diesters of pyrophosphoric acid, nucleoside-5' monoalkyl phosphates and simple diesters of phosphoric acid.

Carbodiimides have proved to be valuable synthetic reagents and, by their use, new methods for the synthesis of esters of ortho- and pyrophosphoric acids,<sup>1,2</sup> nucleotide coenzymes<sup>3</sup> and related compounds,<sup>4</sup> cyclic phosphates,<sup>5</sup> polynucleotides,<sup>6</sup> nucleoside-5' phosphoramidates<sup>7</sup> and peptides<sup>8</sup> have been developed. These applications all involve the reaction of acids with carbodiimides. In general, reaction occurs rapidly with a variety of acids<sup>9</sup> and, in the absence of competitive reactants such as amino compounds, leads to the formation of anhydrides. Carboxylic acids can, however, also yield

the corresponding N-acylureas. The substituents on the carbodiimide molecule, the acid and the solvent have been found to influence the course of these reactions.<sup>9</sup> While general schemes for the mechanism of carbodiimide reactions have been suggested,<sup>9</sup> observations made recently in this Laboratory on the effects of tertiary bases on the reactions of phosphate esters with carbodiimides<sup>3d</sup> stimulated us to examine somewhat more closely the factors influencing these reactions. This study, in turn, has led to further synthetic applications in the phosphate field. In the present communication, the results of experiments performed primarily to throw light on the reaction mechanisms are presented and discussed first while the synthetic applications are described at the end.

### Results

In the present work, dicyclohexyl-, di-*p*-tolyl- and di-*t*-butylcarbodiimides have been studied. Dicyclohexylcarbodiimide has been used in most of the synthetic work involving carbodiimides and is therefore regarded as a standard. Di-*p*-tolylcarbodiimide was chosen as a representative of carbodiimides containing electron-withdrawing substituents, while the reactions of di-*t*-butylcarbodiimide might give an indication of steric effects in the reactions of carbodiimides. Pyridine and tri-*n*-butylamine were chosen as organic bases of widely differing strengths ( $pK_a$ 's in water, 5.19 and 10.89,<sup>10</sup> respectively). Reactions of mono- and diesters of phosphoric acid and of acetic acid were studied and

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

(2) (a) H. G. Khorana, *Can. J. Chem.*, **32**, 227 (1954); (b) H. G. Khorana and A. R. Todd, *J. Chem. Soc.*, 2257 (1953).

(3) See for example (a) H. G. Khorana, *THIS JOURNAL*, **76**, 3517 (1954); (b) E. P. Kennedy, *J. Biol. Chem.*, **222**, 185 (1956); (c) N. A. Hughes, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 3733 (1957); (d) M. Smith and H. G. Khorana, *THIS JOURNAL*, **80**, 1141 (1958).

(4) These include mixed anhydrides derived from nucleoside-5' phosphates and (a) sulfuric acid, P. Reichard and N. R. Ringertz, *THIS JOURNAL*, **79**, 2025 (1957); (b) carboxylic acids, P. T. Talbert and F. M. Huennekens, *ibid.*, **78**, 4671 (1956); (c) amino acids, P. Berg, *Federation Proc.*, **16**, 152 (1957).

(5) (a) C. A. Dekker and H. G. Khorana, *THIS JOURNAL*, **76**, 3522 (1954); (b) G. M. Tener and H. G. Khorana, *ibid.*, **77**, 5349 (1955); (c) H. G. Khorana, G. M. Tener, R. S. Wright and J. G. Moffatt, *ibid.*, **79**, 430 (1957); (d) T. Ukita, K. Nagasawa and M. Irie, *Pharm. Bull. (Tokyo)*, **5**, 121 (1957).

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

(7) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 4240 (1957); R. W. Chambers and J. G. Moffatt, *ibid.*, **80**, 3752 (1958).

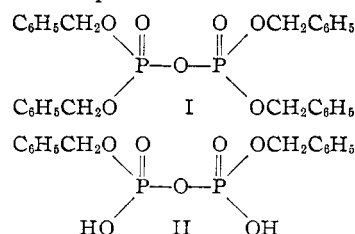
(8) J. C. Sheehan and G. P. Hess, *ibid.*, **77**, 1067 (1955); H. G. Khorana, *Chemistry & Industry*, 1087 (1955).

(9) H. G. Khorana, *Chem. Revs.*, **53**, 145 (1953).

(10) P. Damsgaard-Sorensen and A. Unmack, *Z. physik. Chem.*, **A172**, 389 (1935).

observations on the reactions of *p*-toluenesulfonic and trifluoroacetic acids also are included. The types of reactions studied were (a) anhydride formation and (b) ester formation, both intramolecular (cyclic phosphate formation) and intermolecular.

**Pyrophosphate Formation.**—The formation of pyrophosphates from dibenzyl hydrogen phosphate and monobenzyl dihydrogen phosphate was studied in chloroform solution in the presence of an appropriate amount of tertiary amine and carbodiimide. The progress of the reaction was followed by (a) the disappearance of the strong infrared absorption band at 2100–2145  $\text{cm}^{-1}$  which is characteristic of carbodiimides<sup>9,11</sup> and (b) the appearance of bands characteristic of the pyrophosphate. (These were at 955  $\text{cm}^{-1}$  for tetrabenzyl pyrophosphate<sup>12</sup> (I) and at 1240  $\text{cm}^{-1}$  for  $\text{P}^1, \text{P}^2$ -dibenzyl pyrophosphate (II).) Strictly quantitative analysis of the reaction mixtures was not possible because of the presence of overlapping bands and considerable background absorption.



The results of the experiments with dibenzyl hydrogen phosphate are shown in Table I. As can be seen, the results using dicyclohexylcarbodiimide are in agreement with those reported earlier.<sup>2b</sup> With di-*p*-tolylcarbodiimide, anhydride formation was barely detectable using the pyridinium salt of the acid.<sup>13</sup> No reaction with di-*t*-butylcarbodiimide was detected in the presence of either pyridine or tri-*n*-butylamine.<sup>13</sup>

TABLE I

REACTION OF DIBENZYL HYDROGEN PHOSPHATE (0.2 MOLAR) WITH CARBODIIMIDES (0.1 MOLAR) IN CHLOROFORM AT 20°, IN THE PRESENCE OF ORGANIC BASE (0.2 MOLAR)<sup>a</sup>

Carbodiimide	Pyridine	Tri- <i>n</i> -butylamine
Dicyclohexyl-	Complete in 1 hr.	No reacn. detectable after 72 hr.
Di- <i>p</i> -tolyl-	Trace of anhydride in 72 hr.	No reacn. detectable after 72 hr.
Di- <i>t</i> -butyl-	No reacn. detectable after 72 hr.	No reacn. detectable after 72 hr.

<sup>a</sup> Spectra were run 1, 5, 10, 24, 48 and 72 hours after the start of the reaction. A complete spectrum was scanned in 10 minutes.

The results of parallel experiments with monobenzyl dihydrogen phosphate are shown in Table II. Notable features of these results are: (a) with

(11) G. D. Meakins and R. J. Moss, *J. Chem. Soc.*, 993 (1957).

(12) The band at 955  $\text{cm}^{-1}$  in the spectrum of I can be assigned to the asymmetric P–O–P vibration of the pyrophosphate (L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," John Wiley and Sons Inc., New York, N. Y., 1954, p. 262). No assignment has been made for the band at 1240  $\text{cm}^{-1}$  in the spectrum of II.

(13) The free acid reacts practically instantaneously with di-*p*-tolylcarbodiimide.<sup>2b</sup> Reaction also occurs between the free acid and di-*t*-butylcarbodiimide to form the corresponding pyrophosphate within five hours (unpublished experiments).

dicyclohexylcarbodiimide the reaction proceeded to completion using both pyridinium and tri-*n*-butylammonium salts, but was much slower with the latter; (b) with di-*p*-tolylcarbodiimide the reaction rate was about the same with either base; and (c) no reaction was discernible with di-*t*-butylcarbodiimide in the presence of tri-*n*-butylamine, although in the presence of pyridine the reaction of this carbodiimide proceeded at about the same rate as that of the aromatic carbodiimide.

TABLE II

REACTION OF MONOBENZYL DIHYDROGEN PHOSPHATE (0.2 MOLAR) WITH CARBODIIMIDES (0.1 MOLAR) IN CHLOROFORM AT 20° IN THE PRESENCE OF ORGANIC BASE (0.4 MOLAR)<sup>a</sup>

Carbodiimide	Pyridine	Tri- <i>n</i> -butylamine
Dicyclohexyl-	Complete in 1 hr.	Complete in 72 hr.
Di- <i>p</i> -tolyl-	Complete in 72 hr.	Complete in 72 hr.
Di- <i>t</i> -butyl-	Complete in 72 hr.	No reacn. detectable after 72 hr.

<sup>a</sup> Spectra were run 1, 5, 10, 24, 48 and 72 hours after the start of the reaction. A complete spectrum was scanned in 10 minutes.

**Acetic Acid and Carbodiimides.**—The reactions were followed by the disappearance of the carbodiimide band and the appearance of the bands due to acetic anhydride (at 1824, 1748 and 1115  $\text{cm}^{-1}$ )<sup>14</sup> and, in the case of di-*p*-tolylcarbodiimide, the bands due to *N*-acetyl-*N,N'*-di-*p*-tolylurea (at 1595, 1310 and 1165  $\text{cm}^{-1}$ ). The results which are given in Table III show that the reactions using tri-*n*-butylamine were slower than those using free acid with all the three carbodiimides. The product in the case of dicyclohexyl- and di-*t*-butylcarbodiimides was acetic anhydride.<sup>15</sup> With di-*p*-tolylcarbodiimide, the reaction of the free acid gave mostly the corresponding *N*-acetylurea but a trace of acetic anhydride was detected in the infrared spectrum. In the presence of tri-*n*-butylamine no anhydride was detected.

TABLE III

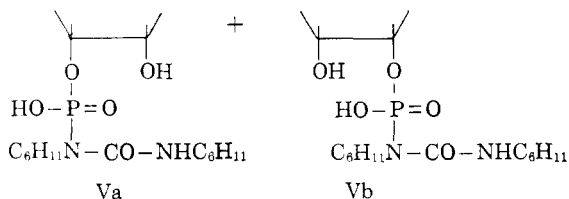
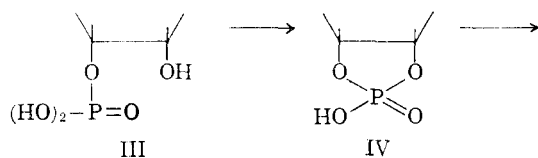
REACTION OF ACETIC ACID (0.2 MOLAR) WITH CARBODIIMIDES (0.1 MOLAR) IN CARBON TETRACHLORIDE AT 20°, AS FREE ACID AND IN THE PRESENCE OF TRI-*n*-BUTYLAMINE (0.2 MOLAR)

Carbodiimide	Free acid	Tri- <i>n</i> -butylamine
Dicyclohexyl-	Complete in 0.5 hr.	Complete in 1 hr.
Di- <i>p</i> -tolyl-	About 20% complete in 0.5 hr., 100% in under 18 hr.	Complete in 44 hr.
Di- <i>t</i> -butyl-	About 80% complete in 44 hr.	No reacn. detectable in 68 hr.

**Cyclic Phosphate and Phosphorylurea Formation.**—The reaction of dicyclohexylcarbodiimide with a monoalkyl phosphate (III) bearing a *cis*-vicinal hydroxyl group has been shown to form first the five-membered cyclic phosphate IV and then the phosphorylureas Va and Vb.<sup>5b</sup> This reaction

(14) L. J. Bellamy, ref. 12, p. 110.

(15) In the experiment with dicyclohexylcarbodiimide in the presence of tri-*n*-butylamine, there seemed to be a slow reduction in the intensity of the anhydride bands and, simultaneously, an increase in the acetic acid band (1705  $\text{cm}^{-1}$ ). This probably indicates that some base-catalyzed hydrolysis of the anhydride occurs in the presence of the strong base.



sequence (monoester  $\rightarrow$  diester  $\rightarrow$  N-phosphorylurea) appeared to be particularly suitable for the present purpose of comparing reactivities and, especially in view of the convenience with which it may be followed paper chromatographically, it was studied using uridine-2'(3') monophosphate.<sup>5b</sup> The results are shown in Table IV. It will be seen that

TABLE IV

REACTION OF URIDINE-2'(3') PHOSPHATE (0.031 MOLAR) WITH CARBODIIMIDES (0.31 MOLAR) IN PYRIDINE CONTAINING WATER (7.0% v./v.) AND, WHERE INDICATED, TRI-*n*-BUTYLAMINE (0.08 MOLAR) AT 20°

Carbodiimide	Time, hr.	Products, %
		III IV V
Dicyclohexyl-	1	0 54 46
	5.5	0 33 67
	18.5	0 35 65 <sup>a</sup>
	1	77 23 0
	5.5	28 72 0
+ tri- <i>n</i> -butylamine	18.5	0 100 0
	210	0 100 0
Di- <i>p</i> -tolyl-	1.5	85 15 0
	5.5	64 36 0
	18	44 56 0
	44	38 <sup>b</sup> 62 0
	1.5	72 28 0
+ tri- <i>n</i> -butylamine	5.5	37 63 0
	18	6 94 0
	44	0 100 0
Di- <i>t</i> -butyl-	1	73 27 0
	5.5	13 87 0
	18.5	3 <sup>b</sup> 97 0
	1	100 0 0
	5.5	100 0 0
+ tri- <i>n</i> -butylamine	18.5	78 22 0
	210	0 100 0

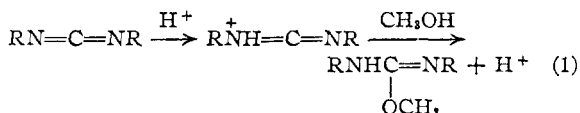
<sup>a</sup> The N-phosphorylurea slowly decomposes to cyclic phosphate and dicyclohexylurea.<sup>5a</sup> <sup>b</sup> These reactions did not go to completion since the cyclic phosphate is hydrolyzed slowly in aqueous pyridine.

there is good over-all agreement between these results and those obtained above in parallel experiments on the formation of pyrophosphates. Thus, the reaction of the cyclic diester with dicyclohexylcarbodiimide in aqueous pyridine to form N-phosphorylurea was completely inhibited by tri-*n*-butylamine and the cyclic phosphate was the only product of the reactions of the other carbodiimides even in the absence of tri-*n*-butylamine. Further, the latter base slowed down the reaction with di-

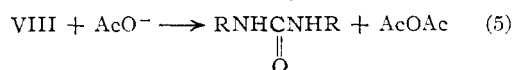
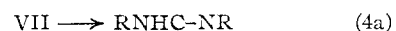
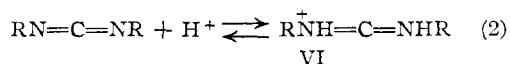
cyclohexyl- and di-*t*-butylcarbodiimide. This is again in accord with the above findings but the interesting observation was that the reaction was distinctly faster with di-*p*-tolylcarbodiimide using the strong tertiary base than in the presence of pyridine alone.

### Discussion

**General Mechanism of Reaction of Acids with Carbodiimides.**—In common with other classes of compounds containing twinned double bonds (isocyanates, ketenes, allenes, etc.), carbodiimides readily undergo 1,2-addition reactions. An example is the acid-catalyzed addition of methanol to yield O-methylisoureas<sup>1</sup> (eq. 1)



The reactions of acids are also believed to involve initially a 1:2-addition reaction as depicted below for acetic acid (eq. 2 and 3), although the adducts of the type VII have so far not been isolated. Subsequent reaction of VII leads to the formation of either N-acylureas (reaction 4a), or, more commonly, the acid anhydride and the simple N,N'-disubstituted ureas (eq. 4b and 5). The steps leading to anhydride formation will be analogous for the sulfonic and phosphoric acids.



While the data obtained in the present work are insufficient for precise analysis in terms of the individual steps postulated above, they permit a discussion of the reactions of acids with carbodiimides in much greater detail than has hitherto been possible.

**The Effect of the Base and the Reacting Acid.**—Acids differing widely in strength (*e.g.*, carboxylic acids and sulfonic acids) react rapidly with carbodiimides in *neutral* solvents. Under these conditions the equilibria 2 and 4b should be well on the side of the protonated carbodiimide VI and adduct VIII. The addition of a tertiary base to a reaction between a carbodiimide and an acid will reduce the concentration of the protonated species VI and VIII, and will thereby reduce the rate of reaction. Although the results of the present experiments using tertiary bases are best appreciated together with a consideration of the acids used, it is, nevertheless, easy to demonstrate this effect of

added base. Thus acetic acid reacts, with the three carbodiimides examined, much faster as the free acid than in the presence of tri-*n*-butylamine. Also, while pyridinium dibenzyl phosphate reacts readily with dicyclohexylcarbodiimide to form tetrabenzyl pyrophosphate and pyridinium uridine-2',3' cyclic phosphate reacted to give the N-phosphorylureas (V), neither of these reactions occurred when tri-*n*-butylamine was added.

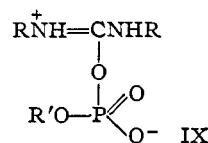
The study of the reactions of dicyclohexyl- and di-*p*-tolylcarbodiimide with diesters of phosphoric acid in the presence of bases further illustrates the role of the protonated carbodiimide and adduct in these reactions. While pyridinium salts of diesters of phosphoric acid reacted readily with dicyclohexylcarbodiimide, hardly any reaction occurred under these conditions with di-*p*-tolylcarbodiimide. Since the concentration of dibenzyl phosphate anion should be the same in both cases, the lack of reaction with the aromatic carbodiimide is attributed to the equilibria 2 and 4b being further over to the left than for dicyclohexylcarbodiimide.

The rates of reactions of acids with carbodiimides also depend very much on the nucleophilicity of their anions. This is shown by experiments carried out with different acids in the presence of the same base. In the first series of experiments using pyridine, while carboxylic acids, monoalkyl and dialkyl esters of phosphoric acid reacted readily with dicyclohexylcarbodiimide, *p*-toluenesulfonic acid was completely inert. Since the concentrations of protonated carbodiimide in the case of pyridinium dibenzyl phosphate and pyridinium *p*-toluenesulfonate would be expected to be very similar, the lack of reaction with the sulfonic acid must be attributed to the poor nucleophilicity of the sulfonate anion. In fact, both *p*-toluenesulfonic anhydride (see Experimental) and *p*-toluenesulfonyl chloride<sup>16</sup> react with dicyclohexylurea in pyridine to give excellent yields of dicyclohexylcarbodiimide. This result indicates that with anhydrides derived from very strong acids such as sulfonic acids all the anhydride-forming steps in the general mechanism formulated above can be reversed so as to give the stable acid anions.

In the experiments carried out with dicyclohexylcarbodiimide in the presence of tri-*n*-butylamine, reactions occurred again with carboxylic acids,<sup>17</sup> phosphoric acid and its monoalkyl esters. In contrast, no reaction was observed with diesters, such as dibenzyl hydrogen phosphate. This result is, analogously, attributed to the poor nucleophilicity of dibenzyl phosphate anion, relative to the anions of carboxylic acids and monoalkyl esters of phosphoric acid.

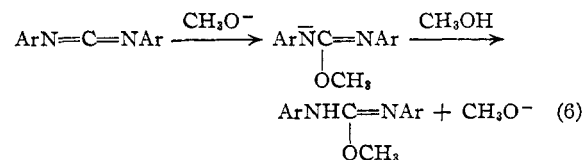
It may, however, be added that no dicyclohexylcarbodiimide was formed when tetrabenzyl pyrophosphate was treated with dicyclohexylurea in the presence of tri-*n*-butylamine. The reversal of the reaction thus seems to be limited to the anhydrides of very strong acids.

The reactions of monoalkyl esters of phosphoric acid with carbodiimides deserve further consideration. In the presence of pyridine, their greater reactivity relative to that of the dialkyl phosphates may be a consequence of several effects. Firstly, monoalkyl esters are weaker acids than the corresponding diesters<sup>18</sup> and their monoionized anions would be expected to be better nucleophiles. Secondly, the adducts IX derived from monoalkyl esters and a carbodiimide may have enhanced reactivity because of their zwitterionic character. Thirdly, the second dissociation of a monoalkyl ester of phosphoric acid is that of a weak acid and the



dionized anions would therefore be more powerful nucleophiles than the monoionized anions. In pyridine the former may be present in an appreciable concentration and may in fact be the species involved in the reaction of the monoalkyl esters. The reactions of trialkylammonium salts are of particular interest in this connection. In contrast with the virtually total inhibition by tri-*n*-butylamine of the reaction of diesters of phosphoric acid, the effect of the strong base on the reactions of monoesters is not so marked. Thus, monobenzyl dihydrogen phosphate still reacts quite readily with dicyclohexylcarbodiimide to give P<sup>1</sup>,P<sup>2</sup>-dibenzyl pyrophosphate (II). Similarly, the formation of uridine-2',3' cyclic phosphate from uridine-2'(3') phosphate using dicyclohexylcarbodiimide is only about four to six times slower when tri-*n*-butylamine is present than when aqueous pyridine alone is used (Table IV). These results are attributed to the increased concentration of the dionized ions in the presence of tri-*n*-butylamine. This effect compensates largely for the adverse effect of the strong base on the equilibria 2 and 4b above.

The formation of uridine-2',3' cyclic phosphate from uridine-2'(3') phosphate with di-*p*-tolylcarbodiimide is distinctly faster in the presence of tri-*n*-butylamine than in pyridine alone. Also, P<sup>1</sup>,P<sup>2</sup>-dibenzyl pyrophosphate is formed at about the same rate in the presence of either pyridine or tri-*n*-butylamine. This high reactivity of the monoalkyl esters with aromatic carbodiimides may be due to another factor. It is known that aromatic carbodiimides undergo base-catalyzed addition reactions (e.g., eq. 6) with special ease<sup>19</sup> and it is possible that the first step in the reaction of the monoalkyl phos-



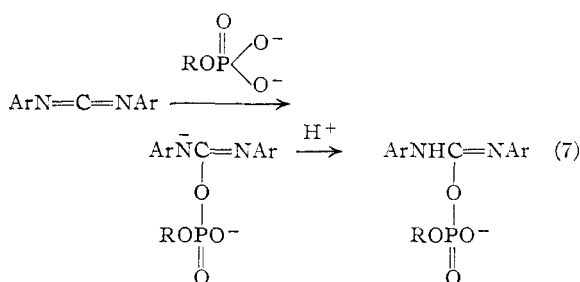
(16) G. Amlard and R. Heymes, *Bull. soc. chim. (France)*, 1360 (1956).

(17) Cf. the formation of amide bonds from carboxylic acids and amino acid esters (ref. 8). The use of tri-*n*-butylamine in these syntheses also has been recorded; R. A. Boissonas, St. Guttman, J. P. Waller and P. A. Jaquenoud, *Experientia*, **12**, 446 (1956).

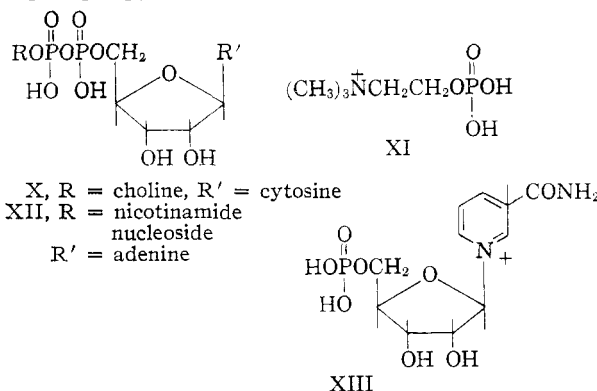
(18) W. D. Kumler and J. J. Eiler, *THIS JOURNAL*, **65**, 2355 (1943).

(19) While di-*p*-tolylcarbodiimide reacts exothermically with alcohols in the presence of sodium alkoxides, dicyclohexylcarbodiimide is inert.<sup>20</sup> Metal ion-catalyzed addition of alcohols to aliphatic carbodiimides has been reported recently; E. Schmidt and F. Moosmüller, *Ann.*, **597**, 235 (1956).

phate esters with aromatic carbodiimides may analogously be the anionic attack by the diionized ester on unprotonated carbodiimide (eq. 7).



**Preferential Formation of Unsymmetrical Pyrophosphates in the Carbodiimide Reaction.**—When two different phosphoric acid esters are together treated with a carbodiimide, a reaction which has been used widely for the synthesis of unsymmetrical nucleoside pyrophosphates, a mixture of the two possible symmetrical and the unsymmetrical pyrophosphate would be expected to be formed. In the reported synthesis<sup>3b</sup> of cytidine diphosphate choline (X) from cytidine-5' phosphate and choline phosphate (XI), the symmetrical P<sup>1</sup>,P<sup>2</sup>-dicholine pyrophosphate was not detected and the desired unsymmetrical pyrophosphate was obtained in an unusually good yield. Similarly, in the formation of diphosphopyridine nucleotide<sup>3c</sup> (XII) from nicotin-



amide nucleotide (XIII) and adenosine-5' phosphate, only a very small amount of the symmetrical P<sup>1</sup>,P<sup>2</sup>-dinicotinamide nucleoside-5' pyrophosphate was formed. Although the ionization constants of choline phosphate and nicotinamide nucleotide do not appear to have been determined, it is very reasonable to assume that these dipolar substances (XI and XIII) are stronger acids<sup>20</sup> than cytidine-5' and adenosine-5' phosphate, respectively, and therefore that the above results probably can be attributed to the difference in the acidities of the two reacting components in each of the above syntheses. However, the exact manner in which the difference in the acid strengths controls the products deserves further comment. In this connection, the view has been expressed recently by Kenner<sup>21</sup> that the 1,2-adduct "can be formed only from an undissociated

(20) Cf. the difference in the ionization constants of  $\alpha$ -amino acids and simple carboxylic acids; E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., A.C.S. Monograph Series, 1943, p. 135.

(21) G. W. Kenner in "Phosphoric Esters and Related Compounds," Chemical Society (London), 1957, p. 99.

acid" and that the reaction between carbodiimide and the stronger acid (e.g., nicotinamide nucleotide) "would be inhibited by the lack of undissociated acid." We do not subscribe to this view which is in direct contrast with the general mechanism proposed and discussed above—namely that dissociated acids are the reacting species.

We advance the following explanation for the results obtained, for example, in the reaction of cytidine-5' phosphate and choline phosphate. The anion of the stronger acid, choline phosphate, is a poorer nucleophile than the cytidine-5' phosphoric acid anion and, consequently, does not compete effectively with the latter for protonated carbodiimide. Thus, the adduct which is formed preferentially is that from cytidine-5' phosphate and the carbodiimide. In contrast, the subsequent step (eq. 5) resulting in the formation of the anhydride (pyrophosphate) is, presumably, relatively insensitive to the nucleophilicity of the acid anion and, therefore, both cytidine diphosphate choline and dicytidine-5' pyrophosphate are formed. Support for these postulates is derived from the experiments on the synthesis of nucleoside-5' monomethyl phosphate esters<sup>22</sup> from nucleoside-5' phosphates and dicyclohexylcarbodiimide in the presence of an excess of methyl alcohol (see below for discussion of diester synthesis). In this synthesis, which is considered to involve the formation of the adduct IX and the attack on the phosphorus atom in the latter by the alcohol, excellent yields are obtained in the presence of only a moderate excess of dicyclohexylcarbodiimide. These results are again interpreted to mean that the weakly nucleophilic alcohol cannot compete effectively with the phosphate anion for the protonated carbodiimide and, therefore, the adduct IX is formed in preference to the O-methylisourea (eq. 1). In contrast, the alcohol *does* compete effectively for the protonated form of the adduct IX and thus the ester is formed exclusively instead of the pyrophosphate.

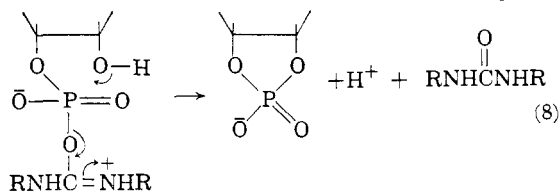
**N-Acylurea Formation.**—The formation of N-acylureas from carboxylic acids and carbodiimides is considered to involve an O  $\rightarrow$  N migration of the acyl group in the 1,2-adduct (VII) (eq. 4a). Although this rearrangement is probably the most common route to the formation of N-acylureas, an alternative mechanism for their production, namely, by the subsequent acylation of the initially formed disubstituted urea by the acid anhydride, was discovered in experiments with trifluoroacetic acid and di-*t*-butylcarbodiimide. The two substances reacted rapidly in ether at room temperature to form di-*t*-butylurea. Over prolonged periods, however, the urea reacted further to form an excellent yield of N-trifluoroacetyl-N,N'-di-*t*-butylurea. The latter product was also formed when di-*t*-butylurea was treated with trifluoroacetic anhydride. Analogously, it is considered possible that, in the experiments in which increased yields of N-acylureas are obtained from dicyclohexylcarbodiimide and carboxylic acids using pyridine and high temperature, the base could catalyze the acylation of the initially formed dicyclohexylurea.<sup>23</sup>

(22) The experiments on the synthesis of nucleoside-5' phosphoramidates (ref. 6) may be interpreted analogously.

(23) The formation of N-acylureas by the reaction of dicyclohexyl

**The Reactivity of Di-*t*-butylcarbodiimide.**—The differences in reactivity of dicyclohexyl- and di-*p*-tolylcarbodiimide have been ascribed in the foregoing discussion to the electron-withdrawing power of the aromatic rings. While the general behavior of di-*t*-butylcarbodiimide paralleled that of dicyclohexylcarbodiimide, it was much less reactive than the latter. Since *t*-butylamine and cyclohexylamine, from which the two carbodiimides are derived, have about the same base strength ( $pK_a$ 's, 10.45 and 10.64,<sup>24</sup> respectively) it does not seem likely that the lower reactivity of di-*t*-butylcarbodiimide is due to a lesser concentration of its protonated form. It is suggested that the poor reactivity of di-*t*-butylcarbodiimide is the result of a steric hindrance by a *t*-butyl group to attack by an anion on the central carbon atom of the protonated carbodiimide. Hindrance of this type can be considered to be analogous to that observed in the acid-catalyzed esterification of carboxylic acids.<sup>25</sup>

**Mechanisms of the Formation of Diesters of Phosphoric Acid Using Carbodiimides.**—The reaction of a monoalkylester of phosphoric acid with a carbodiimide results ordinarily in the formation of the corresponding symmetrical pyrophosphate. Partly aqueous media may be used in these reactions since water<sup>3a</sup> (or a hydroxyl group, which may also be present in reactions involving nucleoside-5' phosphates) is a poor nucleophile relative to a phosphate anion and, therefore, the pyrophosphate formation can still proceed. However, when the above reaction is carried out with a monoalkyl ester bearing a hydroxyl function suitably placed for an *intramolecular reaction*, the weakly nucleophilic hydroxyl group is able to compete effectively with the attack by another phosphate ion, and the cyclic diester results.<sup>5</sup> Formation of *diesters* by this



mechanism (eq. 8), which involves "alcoholysis" of the protonated 1,2-adduct, may also be realized *intermolecularly* by treating a *monoester* with a carbodiimide and a large excess of an alcohol. Thus 0.1 molar solutions of phosphoric acid or monoalkyl dihydrogen phosphates in methyl alcohol gave exclusively dimethyl and alkyl methyl hydrogen phosphates, respectively.

Diesters of phosphoric acid may also be prepared from monoesters by reaction with dicyclohexylcarbodiimide in the presence of *stoichiometric amounts of hydroxylic compounds*.<sup>6</sup> In this type of reaction, it has been concluded<sup>6b</sup> that the symmetrical pyrophosphate corresponding to the monoester is probably formed initially and that it reacts further with the carbodiimide *under anhydrous conditions* to form an activated intermediate which serves as the phos-

carbodiimide with acid anhydrides in refluxing dimethylformamide has been reported; G. Schulz and K. Fiedler, *Chem. Ber.* **89**, 2681 (1956).

(24) N. F. Hall and M. R. Sprinkle, *THIS JOURNAL*, **54**, 3469 (1932).

(25) M. S. Newman, in "Steric Effects in Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., pp. 204-211.

phorylating agent. Diester synthesis by this second mechanism has been found to be inhibited by the presence of tri-*n*-butylamine. Thus, while the reaction of uridine-5' or thymidine-5' phosphate<sup>6c</sup> with dicyclohexylcarbodiimide in anhydrous pyridine at room temperature gave oligonucleotides, under these conditions in the presence of tri-*n*-butylamine, the corresponding dinucleoside pyrophosphates were the sole products. In contrast, the diester synthesis in the presence of an excess of alcohol (first mechanism above) was relatively unaffected by tri-*n*-butylamine. That the synthesis by the first mechanism did not involve the symmetrical pyrophosphates as intermediates was shown by the experiments in which tri-*n*-butylammonium uridine-5' phosphate and diuridine-5' pyrophosphates were separately treated under identical conditions with dicyclohexylcarbodiimide in an excess of methyl alcohol. While the former reacted to form the methyl ester quantitatively in about four days, the latter was completely unaffected during this time.

**Synthetic Applications of the Present Work.**—In the past, syntheses of nucleoside pyrophosphates and nucleotide coenzymes using dicyclohexylcarbodiimide have been carried out in pyridine-water mixture.<sup>3a-c</sup> Homogeneous solutions were generally not obtained and, further, because of the presence of water, very large quantities of the carbodiimide were necessary. The use of tri-*n*-alkylammonium salts enhances the solubility of the nucleotides and related compounds in anhydrous organic solvents and they can be converted to pyrophosphates in better yield and with much smaller amounts of the carbodiimide reagent. An improved and general procedure, which is based on these observations, already has been described for the synthesis of ribo- and deoxyribonucleoside-5' triphosphates.<sup>3d</sup> Similar improvements should result generally in the synthesis of pyrophosphates by the carbodiimide method. It may also be noted that the pyrophosphates are the stable ultimate products in the presence of trialkylamines at room temperature, and do not undergo the subsequent reactions which occur when anhydrous pyridine alone is present.<sup>6c</sup>

Cyclic phosphates are of fundamental interest in connection with the chemistry of many biologically important phosphate esters and, specifically, the ribonucleoside-2',3' cyclic phosphates are important in studies of the ribonucleic acids.<sup>5a-b</sup> The present results show that the reaction of ribonucleoside-2'(3') phosphates with dicyclohexylcarbodiimide in the presence of trialkylamine results in the quantitative formation of the corresponding nucleoside-2',3' cyclic phosphates, the subsequent reaction of the cyclic phosphates to form N-phosphorylureas being inhibited. These observations lead to a highly improved procedure for the preparation of these cyclic phosphates. In practice, in order to facilitate isolation in high yield, the trialkylamine may be replaced by ammonia.<sup>25a</sup> Using the standard procedure described in the Experimental section,

(25a) Recently Dr. Shugar has kindly informed us that they have also prepared ribonucleoside 2',3' cyclic phosphates by treating ammonia salts of the mononucleotides with dicyclohexyl carbodiimide (D. Shugar and K. L. Wierzchowski, *Bull. Acad. Polon. Sci.*, in press).

ribonucleoside-2',3' cyclic phosphates were prepared in yields of over 90%. The procedure should be applicable to the preparation of other cyclic phosphate esters.

Finally, the observations recorded above on the quantitative formation of diesters from monoalkylesters or phosphoric acid by reaction with dicyclohexylcarbodiimide in the presence of an excess of alcohol lead to new and convenient procedures for the synthesis of symmetrical and mixed diesters of phosphoric acid. The preparation of dimethyl hydrogen phosphate and the methyl esters of adenosine-5' and uridine-5' phosphates by this method are recorded. In these syntheses tri-*n*-alkylammonium salts of phosphoric acid or its monoalkyl esters can be used when the free acids are insoluble in the alcohol used, as in the reaction of orthophosphoric acid and benzyl alcohol.

### Experimental

**General.**—Paper electrophoresis was carried out at either pH 7.5 (0.05 *M* phosphate buffer) or pH 4.0 (0.1 *M* ammonium acetate buffer). For paper chromatography the following solvent systems were used: solvent I, isopropyl alcohol-ammonia (sp. gr. 0.9)—water (7:1:2); solvent II, *n*-butyl alcohol-acetic acid—water (5:2:3); solvent III, ethyl alcohol—0.5 *M* ammonium acetate buffer (pH 3.8) (5:2). Ultraviolet absorption measurements were made with a Cary, model 14, spectrophotometer, and infrared spectra determined using a Perkin-Elmer, model 21, spectrophotometer (NaCl prism). Pyridine was dried over calcium hydride. Phosphorus analyses were carried out by the method of King.<sup>26</sup>

**Monobenzyl Dihydrogen Phosphate.**<sup>27</sup>—Dibenzyl hydrogen phosphate<sup>28</sup> (4 g.) was dissolved in 2-ethoxyethanol saturated with lithium chloride (50 ml.) and the solution heated under reflux for 10 minutes, during which time a white solid separated. The mixture was cooled, acidified with 1 *N* sulfuric acid (50 ml.) and extracted five times with ether. The ethereal solution was evaporated to dryness, dissolved in ethyl alcohol (20 ml.), treated with concentrated ammonium hydroxide (5 ml.) and the crude ammonium monobenzyl phosphate (2.48 g.) precipitated by the addition of ether (100 ml.). The salt was dissolved in water and passed through a column of Amberlite IR-120(H<sup>+</sup>) resin. The column was washed with water until the effluent was neutral and the total acidic solution was evaporated to dryness *in vacuo*. The residue was recrystallized from chloroform containing a little methyl alcohol. Monobenzyl dihydrogen phosphate (1.5 g., 60%) formed chunky white crystals which melted at 92–93°,<sup>1</sup> resolidified and then remelted at 270–290° with decomposition.

**P<sup>1</sup>,P<sup>2</sup>-Dibenzyl Pyrophosphate.**—Monobenzyl dihydrogen phosphate (188 mg., 1 mmole) in dioxane (4 ml.) containing triethylamine (0.3 ml.) and a few drops of acetonitrile was treated with dicyclohexylcarbodiimide (309 mg., 1.5 mmoles) at 20°. After 4 days P<sup>1</sup>,P<sup>2</sup>-dibenzylpyrophosphate was the only phosphorus-containing product (*R<sub>f</sub>* in solvent I, 0.72). The solvent was removed by evaporation and water (15 ml.) added. After filtration to remove dicyclohexylurea, the aqueous solution was extracted with ether (×3) and passed through a cooled column of Amberlite IR-120(H<sup>+</sup>) resin into an excess of cyclohexylamine. The solid obtained after evaporation of the solvent was dissolved in methanol (5 ml.) and crystallized by addition of acetone. The yield of crystalline dicyclohexylammonium P<sup>1</sup>,P<sup>2</sup>-dibenzyl pyrophosphate (m.p., 209–211°) was 200 mg. (75%). *Anal.* Calcd. for C<sub>26</sub>H<sub>42</sub>N<sub>2</sub>O<sub>7</sub>P<sub>2</sub>: P, 11.11. Found: P, 11.03.

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

(27) This is a modification of the method of M. Miyano, *This Journal*, **77**, 3524 (1955).

(28) Sodium dibenzyl phosphate was prepared by the method of Clark and Todd [*J. Chem. Soc.*, 2023 (1950)]. The addition of hydrochloric acid to a concentrated aqueous solution of the crude sodium salt resulted in the crystallization of the free acid which was then recrystallized from water.

**Reactions of Monobenzyl Dihydrogen and Dibenzyl Hydrogen Phosphates with Carbodiimides.**—Dicyclohexylcarbodiimide was a commercial sample. Di-*p*-tolylcarbodiimide was prepared as previously described.<sup>29</sup> Di-*t*-butylcarbodiimide was prepared by the method of Schmidt, *et al.*<sup>29</sup> The following is the general procedure used for the reactions of carbodiimides with dibenzyl hydrogen phosphate. The acid (0.1 mmole), pyridine or tri-*n*-butylamine (0.1 mmole) and the carbodiimide (0.05 mmole) were dissolved in alcohol-free chloroform (total volume 0.50 ml.) and the solution kept in a sealed centrifuge tube (1-ml. capacity) at 20°. Reactions with monobenzyl dihydrogen phosphate were set up similarly except that 0.2 mmole of the base (pyridine or tri-*n*-butylamine) was used. Before recording the infrared spectra, the solutions were clarified by centrifugation. The results are recorded in Tables I and II.

The procedure used for following the reactions of acetic acid with carbodiimides was identical. The results are recorded in Table III.

**Reactions of Uridine-2'(3') Phosphate with Carbodiimides. General Procedure.**—Uridine-2'(3') phosphate (0.031 mmole), the carbodiimide (0.31 mmole) and water (0.05 ml.) were made up to a total volume of 0.8 ml. with pyridine. In a parallel experiment 0.02 ml. (0.08 mmole) of tri-*n*-butylamine was added to the reaction mixture. After suitable intervals aliquots (about 0.1 ml.) were removed, diluted with water (about 0.5 ml.) and extracted with petroleum ether to remove the carbodiimide. The aqueous solutions were examined by paper chromatography in solvent I. The spots were located by using an ultraviolet lamp and eluted with 3 ml. of 0.01 *N* hydrochloric acid. The optical densities of the solutions were determined at 262 mμ. The results are recorded in Table IV.

**N-Acetyl-N,N'-di-*p*-tolylurea.**—A solution of acetic acid (0.3 g.) and di-*p*-tolylcarbodiimide (1.1 g.) in tetrahydrofuran (5 ml.) was kept at room temperature for three hours. The clear solution then was evaporated *in vacuo* to give a solid residue which was evidently a mixture of di-*p*-tolylurea and the desired N-acetylurea. Fractional crystallization from ether afforded the N-acetylurea with m.p. 139°.

*Anal.* Calcd. for C<sub>17</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub>: C, 72.38; H, 6.42. Found: C, 72.45; H, 6.22.

**The Reaction of Trifluoroacetic Acid with Di-*t*-butylcarbodiimide.**—Di-*t*-butylcarbodiimide (620 mg., 4 mmoles) was dissolved in dry ether (2 ml.). Freshly distilled trifluoroacetic acid (440 mg., 3.9 mmoles) then was added and the resulting clear solution was stored at 20° with exclusion of moisture. The white needles which separated from the reaction mixture within a few minutes<sup>30</sup> disappeared gradually and were replaced by large chunky crystals after a few days. A small amount of petroleum ether was added slowly to the reaction mixture and after a total of four days the crystalline product was collected by filtration and washed with a small amount of chilled ether giving N-trifluoroacetyl-N,N'-di-*t*-butylurea (653 mg., 62%) with m.p. 116–117°.

*Anal.* Calcd. for C<sub>11</sub>H<sub>20</sub>F<sub>3</sub>N<sub>2</sub>O<sub>2</sub>: C, 49.10; H, 7.48; N, 10.40. Found: C, 49.71; H, 7.61; N, 10.79.

In a separate experiment a solution of trifluoroacetic anhydride (1.06 g.) and di-*t*-butylurea (0.865 g.) in anhydrous ether (5 ml.) was kept overnight at room temperature. Evaporation and fractional crystallization of the residue gave the acylurea described above.

**Reaction of *p*-Toluenesulfonic Anhydride with Dicyclohexylurea.**—Dicyclohexylurea (896 mg., 4.0 mmoles) was dissolved in anhydrous pyridine (25 ml.) by warming. *p*-Toluenesulfonic anhydride<sup>31</sup> (1.304 g., 4.0 mmoles) was then added and the mixture kept at 20° for one hour, during which time white needles (presumably pyridinium *p*-toluenesulfonate) separated. The mixture now was poured into ice-water and extracted with pentane. Unreacted dicyclohexylurea (103 mg., m.p. 220–225°) separated at the interface. The pentane solution was washed with water, dried over magnesium sulfate, and evaporated to dryness giving dicyclohexylcarbodiimide (660 mg., 80%) as an oil which crystallized on standing (m.p. 25–30°). The infrared spectrum of this material was identical with that of the authentic carbodiimide.

(29) E. Schmidt, M. Seefelder, J. G. Jenner, W. Striewsky and H. Martius, *Ann.*, **571**, 83 (1951).

(30) When the reaction mixture was worked up at this stage the crystalline product was di-*t*-butylurea, m.p. 240° (subl.).

(31) H. G. Khorana, *Can. J. Chem.*, **31**, 585 (1953).



**Treatment of Tetrabenzyl Pyrophosphate with Dicyclohexylurea in the Presence of Tri-*n*-butylamine.**—A solution of the pyrophosphate<sup>2b</sup> (54 mg., 0.1 mmole) and tri-*n*-butylamine (0.05 ml., 0.2 mmole) in freshly purified chloroform (2 ml.) was shaken with dicyclohexylurea (45 mg., 0.2 mmole) at 20°. The infrared spectrum of the solution after 24 hr. showed that no reaction had occurred.

**Reaction of Uridine-5' Phosphate with Dicyclohexylcarbodiimide.**—(a) Pyridinium uridine-5' phosphate<sup>32</sup> (0.125 mmole) was dried by repeated evaporation with anhydrous pyridine under reduced pressure. The resulting gum was dissolved in anhydrous pyridine (5 ml.) and treated with dicyclohexylcarbodiimide (130 mg., 0.625 mmole) at room temperature in a sealed flask. The progress of the reaction was followed by paper chromatography in solvents I and II. After 12 days the nucleotidic material was isolated in water. Analysis on a small Ecteola<sup>33</sup> cellulose column as described by Tener, *et al.*,<sup>36</sup> showed that oligonucleotides up to the hexanucleotide were present.<sup>34</sup>

(b) The nucleotide (0.1 mmole) was dried as in (a) and treated with dicyclohexylcarbodiimide (15.5 mg., 0.075 mmole) in anhydrous pyridine (5 ml.) for 24 hr. at 20°. Paper chromatography in solvent II showed that some unreacted uridine-5' phosphate was present. A further amount of dicyclohexylcarbodiimide (0.05 mmole) was added and after five hours paper chromatography (solvent II) and paper electrophoresis at pH 7.5 and 4.5 showed that the sole product was P<sup>1</sup>,P<sup>2</sup>-diuridine-5' pyrophosphate. (This product gave as expected a positive periodate benzidine test.)<sup>35</sup>

Subsequent addition of dicyclohexylcarbodiimide (0.5 mmole) to the main reaction mixture gave after three days products essentially identical with those obtained in (a).

**Reaction of Tri-*n*-butylammonium Uridine-5' Phosphate with Dicyclohexylcarbodiimide.**—The nucleotide (0.125 mmole) and tri-*n*-butylamine (0.06 ml., 0.25 mmole) were dried as above and the solid residue was dissolved in dry pyridine (5 ml.). Dicyclohexylcarbodiimide (129 mg., 0.625 mmole) was added and the reaction mixture kept at 20° in a stoppered flask. Chromatography in solvents I and II showed that conversion to P<sup>1</sup>,P<sup>2</sup>-diuridine-5' pyrophosphate was complete in 48 hours. No subsequent reaction occurred at 20° on further keeping the reaction mixture for 12 days, although dicyclohexylcarbodiimide was present (effervescence with oxalic acid).<sup>36</sup>

**Uridine-5' Methyl Phosphate.**—Dicyclohexylcarbodiimide (2.0 mmoles) was added to a solution of pyridinium uridine-5' phosphate (0.4 mmole) in anhydrous methyl alcohol (100 ml.) and the mixture kept at room temperature. Paper chromatography in solvent I showed that the starting material (*R<sub>f</sub>* 0.12) had completely disappeared after five days, the sole product being an ultraviolet absorbing spot with *R<sub>f</sub>* 0.33. The solvent was evaporated under reduced pressure and the residual gum shaken with a mixture of ether and water. The aqueous layer was passed through a column of Amberlite IR-120 (H<sup>+</sup>) resin into an excess of ammonium hydroxide. The solution was evaporated to dryness and the residue dissolved in a small volume of methyl alcohol. Ammonium uridine-5' methyl phosphate was precipitated by the addition of ether and dried in a vacuum overnight at room temperature (yield 120 mg., 85%).

*Anal.* Calcd. for C<sub>10</sub>H<sub>18</sub>N<sub>3</sub>O<sub>8</sub>P: P, 8.72. Found: P, 8.69.

The product had an ultraviolet absorption spectrum identical with that of the 5'-phosphate, gave a positive periodate test<sup>35</sup> and had the same electrophoretic mobility at pH 4.5 and 7.5. Oxidation with sodium periodate and then alkaline treatment<sup>37</sup> gave monomethyl phosphate as the only phosphorus-containing product.

The formation of uridine-5' methyl phosphate occurred at about the same rate (4–6 days for completion of reaction) both when the free nucleotide and its tri-*n*-butylammonium salt were used under the above conditions.

(32) R. H. Hall and H. G. Khorana, *THIS JOURNAL*, **76**, 5056 (1954).

(33) E. A. Peterson and H. A. Sober, *ibid.*, **78**, 751 (1956).

(34) Experiments on the polymerization of ribonucleotides will be reported in detail separately.

(35) M. Viscontini, D. Hoch and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).

(36) F. Zetzsch and A. Fredrich, *Ber.*, **72B**, 363 (1939).

(37) P. R. Whitfield, *Biochem. J.*, **58**, 390 (1954); D. M. Brown, M. Fried and A. R. Todd, *J. Chem. Soc.*, 2206 (1955).

**Treatment of Bis-(tri-*n*-butylammonium) P<sup>1</sup>,P<sup>2</sup>-Diuridine-5' Pyrophosphate with Dicyclohexylcarbodiimide in Methyl Alcohol.**—A solution of the pyrophosphate (0.05 mmole) in methyl alcohol (12.5 ml.) was treated with dicyclohexylcarbodiimide (0.25 mmole) at 20°. After three days, although carbodiimide was still present the only product detectable on chromatography in solvents I and II was unchanged starting material.

**Adenosine-5' Methyl Phosphate.**—Adenosine-5' phosphate (1 mmole of monohydrate) was dissolved in methanol (250 ml.) containing tri-*n*-butylamine (2 mmoles) and dicyclohexylcarbodiimide (5 mmoles). After three days at room temperature the solvent was evaporated and the residue dissolved in water containing sodium hydroxide (2.2 mmoles). The solution was filtered, extracted with ether, concentrated to a small volume and passed through a column (1 × 7 cm.) of Amberlite IR-120 (NH<sub>4</sub><sup>+</sup>) resin. After washing the resin well with water the effluent was evaporated to dryness, the residue dissolved in methanol (5 ml.) and precipitated with acetone (100 ml.). The white precipitate was washed with acetone, then with ether and dried over P<sub>2</sub>O<sub>5</sub> at 100° *in vacuo* for two hours. The resulting ammonium adenosine-5' methyl phosphate monohydrate weighed 353 mg. (90%) and was chromatographically and electrophoretically homogeneous.

*Anal.* Calcd. for C<sub>11</sub>H<sub>19</sub>N<sub>5</sub>O<sub>7</sub>P·H<sub>2</sub>O: P, 7.81; Ad/P, 1.00. Found: P, 7.82; Ad/P, 1.01.

**Reaction of Orthophosphoric Acid with Dicyclohexylcarbodiimide in Methyl Alcohol.**—A solution of anhydrous orthophosphoric acid<sup>38</sup> (0.1 mmole) and tri-*n*-butylamine (0.05 ml., 0.2 mmole) in dry methyl alcohol (25 ml.) was treated with dicyclohexylcarbodiimide (206 mg., 1 mmole) at 20°. Paper chromatography in solvent I after five hours showed the presence of unchanged phosphoric acid, mono-methyl dihydrogen phosphate and a trace of dimethyl hydrogen phosphate. After 24 hr. these three compounds were present in about equal amounts and after a total of four days conversion to dimethyl hydrogen phosphate was complete.

The above experiment was repeated using increasingly concentrated solutions (0.02, 0.1 and 1 molar solutions of orthophosphoric acid in methyl alcohol). Dimethyl phosphate was again the only product using the first two concentrations; however, with the molar solution products other than the diester were formed. In the next preparative run a 0.1 molar solution therefore was used.

**Isolation of Dimethyl Hydrogen Phosphate.**—Anhydrous phosphoric acid (2.0 mmoles) in methyl alcohol (20 ml.) was treated with dicyclohexylcarbodiimide (5 mmoles) at 20° for four days. The solvent was then removed under reduced pressure and the residue extracted with water. The aqueous solution was passed through an Amberlite IR-120(H<sup>+</sup>) resin column and the acidic effluent and washings were neutralized with ammonia. The solution then was evaporated *in vacuo* leaving a gum which crystallized upon trituration with acetone. The yield of crystalline ammonium dimethyl phosphate was 176 mg. (62%). *Anal.* Calcd. for C<sub>2</sub>H<sub>10</sub>NO<sub>4</sub>P: P, 21.7. Found: P, 21.5.

**Reaction of Orthophosphoric Acid with Dicyclohexylcarbodiimide in Benzyl Alcohol.**—A solution of anhydrous orthophosphoric acid (0.1 mmole) and tri-*n*-butylamine (0.2 mmole) in benzyl alcohol (25 ml.) was treated with dicyclohexylcarbodiimide (1.0 mmole) at 20°. Aliquots (1 ml.) were removed at suitable intervals and treated with oxalic acid to decompose excess carbodiimide. After removal of the solvent by evaporation at 0.1 mm., the product was examined chromatographically in solvent I. Conversion to dibenzyl hydrogen phosphate *via* the monobenzyl ester was complete in 48 hr.

**Preparation of Ribonucleoside-2',3' Cyclic Phosphates.**—Adenosine-2'(3') phosphate (1 mmole) was dissolved in 2.5 ml. of 2 *N* ammonium hydroxide. Formamide (2.5 ml.) then was added and next a solution of dicyclohexylcarbodiimide (5 mmoles) in *t*-butyl alcohol (6 ml.). The homogeneous solution which resulted on gentle warming was heated under reflux for 2.5 hr. (Paper electrophoresis at pH 7.5 after 1.5 hr. showed that conversion to the cyclic phosphate was virtually complete.) The solution now was cooled to room temperature and the *t*-butyl alcohol evaporated under reduced pressure. Water (20 ml.) was added to the residual formamide solution and the mixture ex-

(38) Fluka AG Chemische Fabrik, Buchs, Switz.



tracted three times with ether. (Dicyclohexylurea which separated was removed by filtration after the first extraction.) The aqueous solution was evaporated first under reduced pressure and then under suction with an oil-pump. The residue was dissolved in acetone (30 ml.) and a solution of barium iodide (1 mmole) in acetone (5 ml.) was added. The resulting white precipitate was collected by centrifugation and washed four times with 30-ml. portions of acetone. The washed precipitate was dried in a vacuum for one hour at 100° to give barium adenosine-2',3' cyclic phosphate (504 mg., 99%)<sup>39</sup> as the hexahydrate. This material was homogeneous as shown by paper chromatography (solvents I-III) and paper electrophoresis at pH 7.5. The ratio of phosphorus to adenosine was 1.02.

(39) As estimated spectrophotometrically.

Uridine-2',3' cyclic phosphate was prepared by the same procedure in 92% yield as the barium salt. Guanosine-2',3' cyclic phosphate was obtained as the mixed ammonium and dicyclohexylguanidinium salts in a yield of 89%, based on ultraviolet absorption of the isolated product. In this case the product was precipitated from the residual formamide upon addition of acetone alone.

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[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL]

## Studies on Polynucleotides. I. A New and General Method for the Chemical Synthesis of the C<sub>5'</sub>-C<sub>3'</sub> Internucleotidic Linkage. Syntheses of Deoxyribo-dinucleotides<sup>1</sup>

BY P. T. GILHAM AND H. G. KHORANA

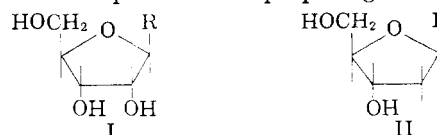
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A new method has been developed for the specific synthesis of the naturally-occurring (C<sub>5'</sub>-C<sub>3'</sub>) internucleotidic linkage; it involves reaction of a suitably protected deoxynucleotide with a second protected deoxy-nucleoside or -nucleotide in the presence of dicyclohexylcarbodiimide or *p*-toluenesulfonyl chloride. By this approach the three dinucleoside phosphates VIIa, VIIb and VIIc have been prepared in good yield. Procedures are described for the synthesis of deoxyribo-dinucleotides bearing 5'- or 3'-phosphoryl end-groups; these are illustrated by the synthesis of the two isomeric dithymidine dinucleotides (XII and XIV), and a mixed dinucleotide (XVI) containing the nucleosides, deoxyadenosine and thymidine. The results of enzymic and acidic degradative experiments are recorded and these provide additional characterization of the synthetic compounds. Some general observations on the scope and mechanism of this method of "phosphodiester" synthesis also are included.

Recent work in different laboratories has led to the clarification of the nature of the internucleotidic linkage in the nucleic acids.<sup>2</sup> Thus, both ribo- and deoxyribo-nucleic acids may be regarded as consisting of polynucleotide chains in which the individual nucleosides are joined together by C<sub>5'</sub>-C<sub>3'</sub> phosphodiester bonds. However, relatively little progress has so far been made in the fields of organic synthesis and fine structural (end-group and sequential) analysis of polynucleotides. Recognition of the vital biological functions of the nucleic acids and continuing intensive research in these areas further emphasize the need for a complementary attack on the problems of their macromolecular chemistry. Attention is therefore being devoted in this Laboratory to the various aspects of such problems, and the chemical synthesis of polynucleotides forms, in the initial phase, a major part of our studies. The preparation of synthetic polynucleotides of well-defined structure and covering a wide range of size is most desirable; not only will they provide more information regarding the structures and properties of the nucleic acids, but also their availability will undoubtedly offer new opportunities for chemical, physico-chemical and biochemical studies in the polynucleotide field. Thus, for example, by use of these synthetic polynucleotides we are currently in-

vestigating chemical and enzymic<sup>3</sup> methods for the structural analysis of the nucleic acids.

The main problems associated with the specific synthesis of the naturally-occurring (C<sub>5'</sub>-C<sub>3'</sub>) internucleotidic linkage are (a) the preparation of suitably protected mononucleosides containing only the desired hydroxyl group (on C<sub>3'</sub> or C<sub>5'</sub>) exposed for a phosphorylation reaction, the protecting groups being such that they can be removed finally under conditions which do not cause disruption of other parts of the molecule, and (b) the creation of the phosphodiester bond, either by stepwise phosphorylation of two protected nucleosides or by adequate activation of the phosphoryl group of a preformed mononucleotide derivative for reaction with the appropriate hydroxyl function of a second nucleoside or nucleotide. In the ribonucleoside (I) series the problems of preparing suitably pro-



R = purine or pyrimidine

tected intermediates are, in some ways, complicated by the presence of the *cis*- $\alpha$ -glycol system. Studies on the deoxyribonucleosides II, however, are free from this complication and, although the chemistry of these compounds has been much less explored it is possible to exploit the difference in reactivities

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(2) For a recent review see D. M. Brown and A. R. Todd in E. Chargaff and J. N. Davidson, "The Nucleic Acids," Academic Press, Inc., New York, N. Y., 1955, p. 409.

(3) H. G. Khorana, G. M. Tener, W. E. Razzell and R. Markham, *Fed. Proc.*, **17**, 253 (1958); W. E. Razzell and H. G. Khorana, *THIS JOURNAL*, **80**, 1770 (1958).