465. *Studies on Phosphorylation. Part XI.* The Reaction between Carbodi-imides and Acid Esters of Phosphoric Acid. A New Method for the Preparation of Pyrophosphates.*

By H. G. Khorana and A. R. Todd.

Di-*p*-tolyl- and di*cyclo*hexyl-carbodi-imides react with mono- and di-esters of phosphoric acid at room temperature to yield the corresponding symmetrical di- and tetra-esters of pyrophosphoric acid. Reaction is almost instantaneous and the yields well-nigh quantitative.

In Part X * of this series, methods for the preparation of tetra-esters of pyrophosphoric acid were discussed, and their production from diesters of phosphoric acid by means of exchange reactions was described. An important feature of this new method was that it did not involve the use of phosphorochloridates (chlorophosphonates), which are sometimes difficultly accessible in the nucleotide field (cf. Mason and Todd, Part VIII, *J.*, 1951, 2267). Since it was desirable to have available as wide a range of methods for polyphosphate preparation as possible, in view of our researches directed towards the synthesis of nucleotide coenzymes, further investigations were undertaken.

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* Part X, *J.*, 1952, 1234.

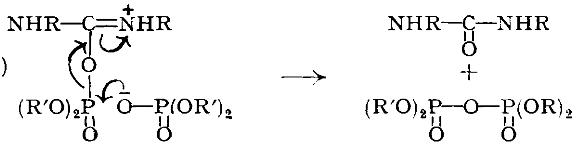
The reactions of carbodi-imides with carboxylic acids have been extensively studied by Zetzsche and his collaborators (*Ber.*, **1938**, **71**, 1088, and subsequent papers). In general two reactions were observed, (*a*) the formation of *N*-acylureas and (*b*) the formation of acid anhydrides and ureas :

$$(R'\cdot CO)_2O + CO(NHR)_2 \xleftarrow{b} \underset{\text{(I)}}{R\cdot N{:}C{:}N\cdot R} + R'\cdot CO_2H \xrightarrow{a} R'\cdot CO\cdot NR\cdot CO\cdot NHR$$

As a rule, aromatic carbodi-imides reacted by route (*a*), whereas aliphatic carbodi-imides gave mainly the anhydrides [reaction (*b*)] or mixtures of the products expected from reactions (*a*) and (*b*). As far as we are aware the reaction of carbodi-imides with acids other than carboxylic acids, and hydrochloric and hydrocyanic acids, has not been studied, but it might be expected that, particularly if dialkyl carbodi-imides were used, anhydride formation would occur. Dibenzyl hydrogen phosphate was therefore treated with di*cyclo*hexylcarbodi-imide (I; $R = C_6H_{11}$) at room temperature in a variety of inert solvents. In every case separation of *NN'*-di*cyclo*hexylurea was almost instantaneous, and from the solution tetrabenzyl pyrophosphate was isolated in excellent yield. Similar results were obtained with di-*p*-tolylcarbodi-imide. The reaction appears to be general, since tetraphenyl pyrophosphate and tetra-*p*-nitrophenyl pyrophosphate could be prepared similarly. When di*cyclo*hexylcarbodi-imide was used there was some difficulty in isolating tetra-*p*-nitrophenyl pyrophosphate owing to the similar solubility of the substituted urea, but its formation was demonstrated by reaction with *cyclo*hexylamine. By using di-*p*-tolylcarbodi-imide in dioxan this difficulty was overcome : the sparingly soluble *NN'*-di-*p*-tolylurea separated and from the residual solution tetra-*p*-nitrophenyl pyrophosphate was readily isolated.

A detailed discussion of the mechanism of the reactions of carbodi-imides with acids of various types is reserved until further studies can be reported, but it may be observed that the first step in pyrophosphate production is almost certainly the formation of a cation of type (II), which is further attacked by a second phosphate anion to yield the urea and pyrophosphate. The rate of formation of the intermediate (II) will be a function of the strength of the acid employed, and the observed well-nigh instantaneous formation of pyrophosphate from diesters of phosphoric acid is in accord with this mechanism. This contrasts with the factors governing the production of pyrophosphates by the exchange reactions discussed in Part X (*loc. cit.*) : in them the anhydride of a diester of phosphoric acid is always converted into a less reactive anhydride. Thus the very reactive tetra-*p*-nitrophenyl pyrophosphate, which is readily prepared by the carbodi-imide method, could not be prepared by exchange reactions using triethylammonium di-*p*-nitrophenyl phosphate and either tetraphenyl pyrophosphate or trifluoroacetic anhydride (Part X; *loc. cit.*).

(II)



It also follows that in the attack on the intermediate (II) leading to pyrophosphate formation, the anions of the acid used would be much more effective than neutral water molecules, *i.e.*, that it should be possible to observe formation of the anhydride even in presence of moderate amounts of water. Zetzsche and his colleagues (*locc. cit.*), indeed, observed that reactions involving carbodi-imides were largely unaffected by traces of water. Since, in the nucleotide field, where we hope to use this method of pyrophosphate preparation, it would frequently be advantageous (on solubility grounds) to carry out the reaction in media containing water, a series of experiments has been carried out in which the reaction between dibenzyl hydrogen phosphate (2 mols.) and di*cyclo*hexylcarbodi-imide (1 mol.) was studied in methyl cyanide containing various amounts of water. Although the yield of tetrabenzyl pyrophosphate decreased with increasing water content (see

Experimental), it is worthy of note that even when a large excess (100 mols.) of water was present the yield was still of the order 50%. It thus appears that essentially complete formation of pyrophosphate could be attained in presence of moderate amounts of water provided a substantial excess of the carbodi-imide were used.

Many of the nucleotide coenzymes (*e.g.*, cozymase) are di-esters of pyrophosphoric acid and, although their synthesis might well be effected by first preparing a tetra-ester and subsequently removing two of the unwanted ester groups, it was clearly of interest to see whether the carbodi-imide method would offer a direct route to the production of diesters. Accordingly phenyl dihydrogen phosphate was brought into reaction with di*cyclo*hexylcarbodi-imide in the usual way; *s*-diphenyl dihydrogen pyrophosphate, PhO·PO(OH)·O·PO(OH)·OPh, was isolated in good yield and characterised as its bis*cyclo*hexylammonium salt.

EXPERIMENTAL

Tetrabenzyl Pyrophosphate.—Di*cyclo*hexylcarbodi-imide (227 mg., 1·1 mols; Schmidt, Hitzler, and Lahde, *Ber.*, 1938, **71**, 1933) in dry ether (1 c.c.) was added to dibenzyl hydrogen phosphate (556 mg., 2 mols.) in dry ether (5 c.c.). *NN'*-Di*cyclo*hexylurea began to separate almost at once, and after 30 min. was filtered off. The colourless filtrate was evaporated and the residue recrystallised from chloroform–light petroleum (b. p. 40—60°), giving tetrabenzyl pyrophosphate (90%), m. p. 61—62° alone or mixed with an authentic sample (m. p. 61—62°).

The same reaction was also carried out with benzene or methyl cyanide in place of ether, the yields being 87% and 82% respectively. Dimethylformamide was less convenient since separation of the urea was incomplete until the solution was evaporated and stirred with ether. With ether as solvent and di*cyclo*hexylcarbodi-imide replaced by di-*p*-tolylcarbodi-imide (Zetzsche and Nerger, *Ber.*, 1940, **73**, 467), the yield of tetrabenzyl pyrophosphate was 93%.

Effect of Tertiary Bases on Formation of Tetrabenzyl Pyrophosphate.—To a solution of dibenzyl hydrogen phosphate (556 mg., 2 mols.) in benzene (3 c.c.) pyridine (0·16 c.c., *ca.* 2 mols.) was added, followed by di*cyclo*hexylcarbodi-imide (227 mg., 1·1 mols.). Di*cyclo*hexylurea was filtered off after 15 min. and the filtrate evaporated. The residual oil solidified when shaken with ice-water, yielding directly practically pure tetrabenzyl pyrophosphate (93%).

When triethylamine was used in place of pyridine no di*cyclo*hexylurea separated.

Effect of Water on the Formation of Tetrabenzyl Pyrophosphate.—Di*cyclo*hexylcarbodi-imide (120 mg.) in dry methyl cyanide (10 c.c.) was mixed rapidly with a solution of dibenzyl hydrogen phosphate (278 mg.) in methyl cyanide (10 c.c.), to which had been added various amounts of water; an apparatus similar to that described by Grewe and Nolte (*Annalen*, 1951, **575**, 11) was used. The amount of unchanged acid was estimated immediately by dilution with water and titration with alkali (phenolphthalein). The results are given in the following table (10·46 c.c. of alkali were equivalent to 278 mg. of dibenzyl hydrogen phosphate) :

Water added (c.c.)	Nil	1	2	4
Alkali used (c.c.)	0·94	5·34	7·68	9·6
Yield of tetrabenzyl pyrophosphate (%)	91	49	26·6	8·3

Tetraphenyl Pyrophosphate.—When ethereal di*cyclo*hexylcarbodi-imide (230 mg., 1·1 mols.) was added to anhydrous diphenyl hydrogen phosphate (500 mg., 2 mols.) in ether, quantitative separation of di*cyclo*hexylurea occurred almost at once. Filtration, followed by evaporation of the filtrate, gave tetraphenyl pyrophosphate as a viscous oil; the product was assayed by reaction with excess of *cyclo*hexylamine (Corby, Kenner, and Todd, Part X), giving diphenyl *N*-*cyclo*hexylphosphoramidate (92%) and *cyclo*hexylammonium diphenyl phosphate (96%).

*Tetra-*p*-nitrophenyl Pyrophosphate.*—Ether could not be employed as solvent owing to the very low solubility of di-*p*-nitrophenyl hydrogen phosphate and, although somewhat better, nitromethane, acetonitrile, and chloroform did not give satisfactory results. In preliminary experiments with di*cyclo*hexylcarbodi-imide and dioxan, however, it was clear that the desired pyrophosphate was formed, since treatment of the reaction mixture with *cyclo*hexylamine yielded di-*p*-nitrophenyl *N*-*cyclo*hexylphosphoramidate; the following method was finally adopted.

Di-*p*-nitrophenyl hydrogen phosphate (2·33 g.) was dissolved in a minimum of warm anhydrous dioxan (*ca.* 15 c.c.). The solution was rapidly cooled, and di-*p*-tolylcarbodi-imide (1·54 g.) added. *NN'*-Di-*p*-tolylurea (700 mg.) separated at once, and was removed by rapid filtration. The filtrate was now evaporated *in vacuo*, giving an oil which was dissolved in a small amount of dry benzene. Gradual addition of light petroleum (b. p. 40—60°) at intervals during 24 hr. completed crystallisation of the crude product, which was dissolved in warm dioxan (7—8 c.c.). After filtration from a small amount of *NN'*-di-*p*-tolylurea the solution was set aside. The *tetra*-p-*nitrophenyl pyrophosphate* which separated (1·2 g.) was recrystallised from dioxan, forming stout, almost colourless prisms. Heated in a vacuum the pure substance, which is very susceptible to moisture, melted at 126—127°, then re-solidified and melted at 146—148° (Found: C, 44·0; H, 2·8; N, 8·3. $C_{24}H_{16}O_{15}N_4P_2$ requires C, 43·6; H, 2·4; N, 8·5%).

Treated with *cyclo*hexylamine in warm methyl cyanide the product yielded di-*p*-nitrophenyl *N*-*cyclo*hexylphosphoramidate (93%; m. p. and mixed m. p. 174—176°) and *cyclo*hexylammonium di-*p*-nitrophenyl phosphate (91%).

s-*Diphenyl Dihydrogen Pyrophosphate*.—Ethereal solutions of phenyl dihydrogen phosphate (174 mg., 1 mol.) and di*cyclo*hexylcarbodi-imide (226 mg., 1·1 mols.) were rapidly mixed, with shaking. The oil which separated almost immediately was converted into a crystalline powder by prolonged trituration, and was then collected and washed with ether. The powder was extracted successively with portions (2—3 c.c.) of cold water until the extracts were no longer acidic. To the combined extracts (*ca.* 10 c.c.), aqueous *cyclo*hexylamine was added to pH 8, and the solution set aside at 0°. s-*Bis*cyclo*hexylammonium diphenyl pyrophosphate* which separated crystallised from water as colourless needles, m. p. 250—255° (62—76%) (Found: C, 55·0; H, 7·3; N, 5·4. $C_{24}H_{38}O_7N_2P_2$ requires C, 54·6; H, 7·3; N, 5·3%). Electrometric titration of the salt with acid and alkali showed the absence of secondary phosphoryl dissociation in the pH range 4·5—8·5; the back-titration curve from pH 10, however, showed a kink at pH *ca.* 7·5, presumably due to a small amount of hydrolysis.

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