have found that deoxyuridylic acid is converted to thymine nucleotides by bacteria. It has been suggested recently[27] and partially supported by experimental evidence[26] that the methylation of pyrimidine derivatives may involve first an aldol condensation of the CH_2OH-THF compound with the pyrimidine derivative and then formation of the 5-CH_2OH-dihydro-pyrimidine compound. This is followed by removal of water, yielding the methylene derivative, and finally there is a shift of the double bond. These steps also could possibly involve ATP.

ADDENDUM:—Friedkin and Kornberg[25] and Friedkin[28] have shown that deoxyuridine-5'-phosphate is the substrate for methylation. They postulate a direct condensation of $HOCH_2$-THF with the deoxyuridylic acid, with the splitting out of water, and subsequent cleavage to yield the methyl group. Presumably this would lead to an oxidation of THF to dihydrofolic acid. The function of the reduced phosphopyridine nucleotide might then be to reconvert the latter to THF.

(27) R. L. Hamill, R. L. Herrmann, R. U. Byerrum and J. L. Fairley, *Biochim. Biophys. Acta*, **21**, 394 (1956).

(28) M. Friedkin, *Federation Proc.*, **16**, 183 (1957).

BERKELEY, CALIFORNIA

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

Carbodiimides. VII.[1] Tetra-*p*-nitrophenyl Pyrophosphate, a New Phosphorylating Agent

BY J. G. MOFFATT AND H. G. KHORANA

RECEIVED JANUARY 12, 1957

Tetra-*p*-nitrophenyl pyrophosphate, prepared *in situ* by the reaction of di-*p*-nitrophenyl hydrogen phosphate with di-*p*-tolyl carbodiimide in anhydrous dioxane, has been found to phosphorylate alcohols at room temperature without basic catalysis and, therefore, constitutes a powerful phosphorylating agent. A number of tertiary di-*p*-nitrophenyl phosphate esters were thus prepared in excellent yields. The nitrophenyl groups may be removed by hydrogenolysis in the presence of platinum or by alkaline treatment. Very mild alkaline hydrolysis results in the quantitative formation of alkyl mono-*p*-nitrophenyl hydrogen phosphates. Some other interesting properties of the neutral di-*p*-nitrophenyl phosphate esters have been recorded. A method for the large scale preparation of di-*p*-nitrophenyl hydrogen phosphate is detailed.

Methods for the synthesis of esters of phosphoric acid have been the subject of numerous investigations and several phosphorylating agents have been introduced. The reagents commonly employed fall broadly into two groups. To the first group belong phosphorus oxychloride[2] and certain of its monofunctional derivatives such as diphenyl,[3] dibenzyl[4] and di-*p*-nitrobenzyl[5] phosphorochloridates. Common features of the standard procedures employing these reagents are the mildness of conditions and the use of a basic catalyst, *e.g.*, pyridine, which often serves as the medium of reaction. The second group of phosphorylating agents embraces a number of anhydrides[6,7] such as polyphosphoric acid. Reagents of this type are usually employed in large excess and the phosphorylation reactions, which are carried out in the absence of a base, proceed under relatively drastic conditions.[8] Although lacking in elegance, reagents of the second type offer advantages of ready accessibility and simplicity in operation and have proved useful in certain cases.[9]

While the successful application of the existing methods of phosphorylation to a variety of synthetic problems has been recorded, inert hydroxyl functions are sometimes encountered whose phosphorylation in a satisfactory yield presents difficulties. One specific example is that of 2',3'-*O*-isopropylidene guanosine, the phosphorylation of which formed a part of our studies in the nucleotide field. In connection with this problem and our broader interest in developing new and improved methods for the synthesis of phosphate esters of biological interest it was considered desirable to devise a method of phosphorylation which would utilize a mono-functional, powerful and yet mild reagent and which would not require basic catalysis. The present communication describes a method which meets the above requirements and, therefore, combines the desirable features of the two aforementioned types of procedures. The application of this method to a satisfactory synthesis of guanosine 5'-phosphate is recorded in the following communication.[10]

A convenient and efficient method for the synthesis of the symmetrical esters of pyrophosphoric acid

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

(2) See *e.g.*, E. Fischer, *Ber.*, **47**, 3193 (1914); P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **106**, 113 (1934).

(3) (a) K. Zeile and H. Meyer, *Z. physiol. Chem.*, **256**, 131 (1938); (b) P. Brigl and H. Muller, *Ber.*, **72**, 2121 (1939); (c) H. Bredereck, E. Berger and J. Ehrenberg, *ibid.*, **73**, 269 (1940).

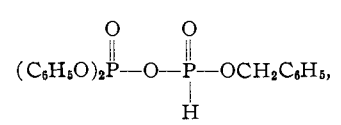
(4) F. R. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945) and subsequent papers.

(5) L. Zervas and I. Dilaris, THIS JOURNAL, **77**, 5354 (1955).

(6) Broadly speaking all phosphorylating agents are anhydride in character. The above classification serves to emphasize the different types of procedures used in phosphorylation reactions.

(7) For references to earlier literature see G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley and Sons, New York, N. Y., 1950.

(8) The reagent *O*-benzyl phosphorus *O,O*-dibenzylphosphoric anhydride



developed by N. S. Corby, G. W. Kenner and A. R. Todd, *J. Chem. Soc.*, 3669 (1952), for the preparation of mixed secondary phosphites is more akin to the phosphoric acid chlorides in that it is used under mild conditions in the presence of a tertiary base.

(9) See *e.g.*, A. N. Wilson and S. A. Harris, THIS JOURNAL, **73**, 4693 (1951); J. E. Seegmiller and B. L. Horecker, *J. Biol. Chem.*, **192**, 175 (1951); R. H. Hall and H. G. Khorana, THIS JOURNAL, **77**, 1871 (1955).

(10) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **79**, 3747 (1957).

(II) is now available[11] (equation 1), and our first aim in the present work was to utilize a suitable compound of the type II for the phosphorylation of an alcohol according to equation 2. Since the liberated acid I would be expected to react again with the carbodiimide reagent to form II, the over-all reaction would be represented by equation 3. The fully protected pyrophosphate we chose to study as a phosphorylating agent was tetra-*p*-nitrophenyl pyrophosphate[11] (II, R = *p*-nitrophenyl). The choice of this ester was influenced by certain observations recorded by Todd and co-workers in their studies of the reactivity of the various fully protected pyrophosphates[12] and by a consideration of the ease of the subsequent removal of the protecting groups from the resulting esters of the type III

$$2\underset{\text{I}}{(RO)_2P(O)OH} + R'N{=}C{=}NR' \longrightarrow \underset{\text{II}}{(RO)_2P(O)OP(O)(OR)_2} + R'NHCONHR' \quad (1)$$

$$\text{II} + R''OH \longrightarrow \underset{\text{III}}{(RO)_2P(O)OR''} + \underset{\text{I}}{(RO)_2P(O)OH} \quad (2)$$

$$\underset{\text{I}}{(RO)_2P(O)OH} + R'N{=}C{=}NR' + R''OH \longrightarrow \underset{\text{III}}{(RO)_2P(O)OR''} + R'NHCONHR' \quad (3)$$

In the initial experiments, therefore, a mixture of di-*p*-nitrophenyl hydrogen phosphate (1 equiv.) and methyl alcohol (1.5 equiv.) was brought into reaction at room temperature in anhydrous dioxane with dicyclohexyl or di-*p*-tolyl carbodiimide (1 equiv.). In these experiments, varying amounts of the desired di-*p*-nitrophenyl methyl phosphate[13] were formed but side reaction(s) occurred. Thus when dicyclohexyl carbodiimide was used, a highly crystalline side product could be isolated, which from its elemental analysis was concluded to be the di-*p*-nitrophenyl phosphoric acid salt of N,N'-dicyclohexyl-*O*-methylisourea (IV). This conclusion was confirmed by the isolation and elemental analysis of the basic isourea (IV) itself from the salt. The latter could, subsequently, be prepared in quantitative yield by the addition of dicyclohexyl carbodiimide to an anhydrous methyl alcoholic solution of di-*p*-nitrophenyl hydrogen phosphate. A similar acid-catalyzed addition[14] of methyl alcohol to di-*p*-tolyl carbodiimide would be expected to occur, although the analogous compound was not isolated.

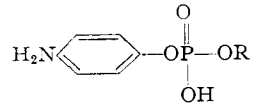
While the above experiments demonstrated the feasibility of the new approach, the side reaction observed precluded quantitative phosphorylation according to the scheme originally intended (equation 3) and it was therefore decided to add the alcohol to the preformed pyrophosphate, II (R = *p*-nitrophenyl), and to realize phosphorylation only according to equation 2 (the reaction mixture would thus contain no free carbodiimide). Because of the greater insolubility and consequent ease of removal of the resulting di-*p*-tolylurea, di-*p*-tolyl carbodiimide was preferred as the reagent.

Tetra-*p*-nitrophenyl pyrophosphate[15] was prepared *in situ* in anhydrous dioxane by the instantaneous reaction of di-*p*-tolyl carbodiimide with di-*p*-nitrophenyl hydrogen phosphate[11] and methyl alcohol was added. On working up (see Experimental) after a reaction period of some 15 hours, pure di-*p*-nitrophenyl methyl phosphate (III, R = *p*-nitrophenyl; R''' = CH_3) was obtained in over 90% yield (based on equation 2). The generality of the

$$\begin{matrix} C_6H_{11}NHC{=}NC_6H_{11} \\ | \\ OCH_3 \end{matrix} \qquad \text{IV}$$

technique was established by the preparation of a number of simple alkyl di-*p*-nitrophenyl phosphate esters. By the same method, the primary hydroxyl groups in 2',3'-*O*-isopropylidene uridine and 2',3'-*O*-isopropylidene guanosine[10] were phosphorylated to give 90–95% yields of the corresponding di-*p*-nitrophenyl phosphate esters. (The phosphorylation of 1,2-*O*-isopropylidene D-xylofuranose by this reagent has been recorded elsewhere.[16])

An important consideration in the use of a method of phosphorylation, clearly, is the ease and selectivity with which one and/or both of the protecting groups may be removed from the initial products of phosphorylation. In this respect, obviously, the new reagent is akin to the well-established reagent diphenyl phosphorochloridate,[3] and, by analogy, the *p*-nitrophenyl groups may be removed by the platinum-catalyzed hydrogenolysis of the di-*p*-nitrophenyl phosphate esters. However, the presence of an acid (*e.g.*, hydrochloric acid) is necessary for removing both the protecting groups since otherwise hydrogenolysis does not proceed beyond the formation of the mono-*p*-aminophenyl esters of the type V. *p*-Aminophenyl methyl hydrogen phosphate (V, R = CH_3) was thus isolated as the crystalline free acid. Examples of the mono alkyl di-hydrogen phosphates prepared by the hydrogenolytic removal of the *p*-nitrophenyl groups are given in the Experimental section.



V

(11) H. G. Khorana and A. R. Todd, *J. Chem. Soc.*, 2257 (1953).

(12) N. S. Corby, G. W. Kenner and A. R. Todd, *ibid.*, 1234 (1952). The reactivity (phosphorylating power) of symmetrical pyrophosphates of the type II is, as expected, a function of the acid strength of the parent acid.

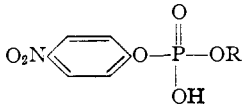
(13) J. A. A. Ketelaar and H. R. Gersmann, THIS JOURNAL, **72**, 5777 (1950).

(14) For base- and metal ion-catalyzed additions of alcohols to carbodiimides see H. G. Khorana, *Can. J. Chem.*, **32**, 231 (1954); E. Schmidt and F. Moosmüller, *Ann.*, **597**, 235 (1956).

(15) The isolation and storage of this substance, which is extremely susceptible to moisture, is unnecessary and is not recommended. Instead, it may be prepared, immediately prior to use, from the stable highly crystalline starting materials.

(16) J. G. Moffatt and H. G. Khorana, THIS JOURNAL, **79**, in press (1957).

A characteristic and frequently useful feature of the neutral di-*p*-nitrophenyl phosphate esters of the type III (R = *p*-nitrophenyl) is the extreme ease with which one of the *p*-nitrophenyl groups may be removed under mildly alkaline conditions. This hydrolytic reaction, which may be followed conveniently by paper chromatography or by measuring spectrophotometrically the liberation of *p*-nitrophenol, occurs rapidly at room temperature in 0.01 to 0.1 *N* alkali and results in the quantitative formation of alkyl *p*-nitrophenyl hydrogen phosphates of the type VI. A number of diesters of this type were prepared in a crystalline state (see Experimental). Heating esters of the type VI at 100°



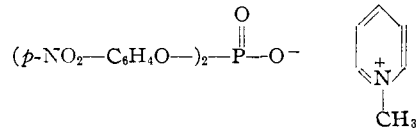
VI

in 1 *N* alkali for a few hours was necessary to remove the second *p*-nitrophenyl group. This alkaline hydrolysis[17] is useful as an alternative method for the removal of the protecting groups where platinum-catalyzed hydrogenolysis cannot be used (see for example the preparation of guanosine 5′-phosphate[10]).

Some other properties of the model substance di-*p*-nitrophenyl methyl phosphate may be briefly mentioned. As expected, in the presence of sodium benzoxide it underwent a rapid transesterification reaction to yield dibenzyl methyl phosphate. The latter substance was monodebenzylated with aqueous acetic acid[18] and the resulting benzyl methyl hydrogen phosphate was isolated as the crystalline cyclohexylammonium salt.

On heating di-*p*-nitrophenyl methyl phosphate in 50% acetic acid hydrolysis[19] occurred to give both di-*p*-nitrophenyl hydrogen phosphate and methyl *p*-nitrophenyl hydrogen phosphate; the proportions of the two products were, respectively, 77 and 23%. Hydrolysis in a mixture of 1 *N* hydrochloric acid and dioxane appeared to follow the same pattern.

An anhydrous pyridine solution of di-*p*-nitrophenyl methyl phosphate on standing for a few hours at room temperature deposited a highly crystalline material which from its elemental analysis, paper-chromatographic and paper-electrophoretic behavior was identified as *N*-methylpyridinium di-*p*-nitrophenyl phosphate (VII). This reaction is



VII

analogous to the quaternization reactions of neutral phosphate esters, especially, dibenzyl alkyl phosphates, which have been studied extensively by Todd and co-workers.[20] The great ease with which the reaction occurred in the present case, clearly, is a consequence of the strongly electron-withdrawing nitro groups since diphenyl *n*-propyl phosphate is apparently unaffected by refluxing in 4-methylmorpholine for three hours.[21]

Methods for the large scale preparation of di-*p*-nitrophenyl hydrogen phosphate have been examined in detail, since from the practical standpoint it was important that this starting material[22] in the described method of phosphorylation be readily available. The two step procedure involving (a) preparation of tri-*p*-nitrophenyl phosphate[23] from phosphorus oxychloride and (b) alkaline hydrolysis to the desired diester[24] (see Experimental) has given consistently an over-all yield of over 80% on a preparative scale. Initially, however, we prepared the diester by the reaction of phosphorus oxychloride with *p*-nitrophenol in pyridine according to Corby, Kenner and Todd.[12] The yields obtained in different runs varied and were rather unsatisfactory. Furthermore, in some runs the only crystalline product isolated was a low-melting (90–91°) substance. The nature of this interesting compound may be discussed in some detail. The substance, which was strongly acidic, was indistinguishable from authentic di-*p*-nitrophenyl hydrogen phosphate in its behavior on paper chromatograms developed in six solvent systems and, further, formed a crystalline cyclohexylammonium salt which was identical with that from di-*p*-nitrophenyl hydrogen phosphate (m.p., mixed m.p. and infrared spectrum). The possibility that the low-melting substance was a hydrate or an isomorphic form of di-*p*-nitrophenyl hydrogen phosphate was ruled out by the analytical data and the ultraviolet absorption spectrum. The latter, in fact, proved to be particularly informative; while it was practically identical with that of di-*p*-nitrophenyl hydrogen phosphate in the region 280–320 mμ, there was slightly increased absorption in the range 230–270 mμ. This evidence taken together with the observation that the low-melting substance was also encountered in the experiments in which aqueous pyridine was used for the hydrolysis of tri-*p*-nitrophenyl phosphate indicated that pyridine was present in the substance. Confirmatory evidence was provided by treating an aqueous solution of the 90–91° melting substance with an excess of Dowex-2-formate resin (removal of di-*p*-nitrophenyl phosphate ions) and determining the ultraviolet absorption spectrum of the clear supernatant. The "difference spectrum" was identical with that of pyridine. A quantitative determination of the pyridine content and of the neutralization equivalent, and the elemental analysis of the low-melting substance have led us to conclude that it represents a complex consisting of two molecules of di-*p*-nitrophenyl hydrogen phosphate and one molecule, each, of pyridine and water.

Finally, the preparation of the crystalline pyrophosphate VIII is recorded. This substance was prepared in order to test its use as a phosphorylat-

(17) The removal of phenyl groups from neutral phosphate esters by alkaline hydrolysis is well known. See *e.g.*, ref. 3c; and J. Baddiley and E. M. Thain, *J. Chem. Soc.*, 1610 (1953).

(18) J. Baddiley and A. R. Todd, *ibid.*, 648 (1947).

(19) For a recent detailed discussion of the hydrolysis of organic phosphates see P. W. C. Barnard, C. A. Bunton, D. R. Llewellyn, K. G. Oldham, B. L. Silver and C. A. Vernon, *Chemistry and Industry*, 760 (1955).

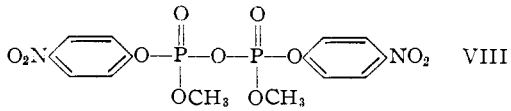
(20) J. Baddiley, V. M. Clark, J. J. Michalski and A. R. Todd, *J. Chem. Soc.*, 815 (1949).

(21) J. Lecocq and A. R. Todd, *ibid.*, 2381 (1954).

(22) Di-*p*-tolyl carbodiimide, the second starting material, may be readily prepared in quantity (ref. 14).

(23) M. Rapp, *Ann.*, **225**, 156 (1884).

(24) S. Yoshida, *J. Biochem.* (*Japan*), **34**, 23 (1941).



VIII

ing agent, in model experiments on the synthesis of unsymmetrical diesters of phosphoric acid. Preliminary experiments showed that basic catalysis was necessary for the phosphorylation of an alcohol by this pyrophosphate.

Experimental[25]

Paper chromatography was used extensively as an analytical tool. The two solvent systems which proved most useful were: *n*-butyl alcohol–acetic acid–water (4:1:5, v./v.)[26] (solvent A) and isopropyl alcohol–ammonia–water (7:1:2, v./v.)[27] (solvent B). In both solvent systems the R_f's of different types of esters followed the order: tertiary esters of phosphoric acid > diesters > monoesters. Some particularly relevant R_f's are mentioned in the text.

Sodium *p*-Nitrophenoxide.—Sodium hydroxide (110 ml. of 10 *N*) was added with stirring to a suspension of *p*-nitrophenol (150 g.) in boiling water (700 ml.). To the resulting clear solution was added a further volume (100 ml.) of 10 *N* alkali and the mixture rapidly cooled. The yellow sodium salt which separated was collected by filtration and washed three times with 50-ml. portions of ice-water. After drying thoroughly at 110° and pulverization the anhydrous salt was obtained as a red powder (165 g., 95%).

Tri-*p*-nitrophenyl Phosphate.—Rigorously dried, finely powdered sodium *p*-nitrophenoxide (112 g.) was added slowly to an anhydrous ethereal solution (400 ml.) of freshly distilled phosphorus oxychloride (16 ml.), with exclusion of moisture. The mixture was stirred mechanically at room temperature for 30 minutes and then refluxed for two hours. The resulting mixture of orange and white solids was collected by filtration and washed repeatedly with cold water (total volume, 1500 ml.) until the washings were colorless. The white crystalline residue of tri-*p*-nitrophenyl phosphate was dried *in vacuo* over phosphorus pentoxide. The yield was 76.6 g. (96%) and the m.p. 153–155° which was raised to 155–156° upon one recrystallization from ethyl acetate or acetone. The recrystallization was unnecessary for the next step.

Di-*p*-nitrophenyl Hydrogen Phosphate.—Tri-*p*-nitrophenyl phosphate (80 g.) was dissolved in warm dioxane (800 ml.) and the solution quickly cooled. To the solution were added 125 ml. of 4 *N* lithium hydroxide and sufficient water to give a homogeneous solution. The yellow solution was kept for 30 minutes and then neutralized by the portionwise addition of IR-120(H^+) resin. The resin was removed by filtration, washed with small portions of water and the combined filtrate evaporated to dryness under reduced pressure. The resulting sirup was dissolved in water (400 ml.) and the *p*H of the solution adjusted to 4 by the further addition of the acidic resin. The resin was removed and washed and the filtrate extracted repeatedly with ether until an extract produced no yellow color with aqueous alkali. The aqueous solution was then heated to 80° and strongly acidified by the slow addition (*ca.* 5 minutes) of concentrated hydrochloric acid.[28] The oil which initially separated solidified on cooling and scratching. The crystalline material was collected by filtration, dissolved in the minimum volume of boiling water and the solution kept at 0°. Di-*p*-nitrophenyl hydrogen phosphate (51 g.) separated as white needles and was collected by filtration. A further amount (2 g.) was recovered from the mother liquor by the addition of concentrated hydrochloric acid (2 ml.); total yield 53 g. (90%), m.p. after drying at 100° *in vacuo*, 175–175.5° (reported m.p. 176–178,°[12] 175°[29]).

Preparation of the Compound with M.P. 90–91°.—Tri-*p*-nitrophenyl phosphate (7 g.) was dissolved in dry, boiling pyridine (20 ml.) and an equal volume of water was added to the resulting colorless solution. The clear yellow solution now obtained was refluxed for 30 minutes. The solvent was then evaporated under reduced pressure and the oily residue taken up in water (100 ml.). The *p*H of the solution was adjusted to 5 with acetic acid and the resulting suspension was extracted with ether until an extract gave no yellow color with alkali. The clear aqueous solution was concentrated to 25 ml. and strongly acidified with concentrated hydrochloric acid. The light colored oil which separated, crystallized on trituration, was collected by filtration and recrystallized from ethyl acetate. The chunky crystals thus obtained (5.1 g.) had m.p. 90–91°, which remained unchanged upon further recrystallization. *Anal.* Calcd. for $C_{29}H_{23}N_5O_{16}P_2 \cdot H_2O$: C, 44.90; H, 3.24; N, 9.02; neut. equiv., 388. Found: C, 45.17; H, 3.27; N, 9.24; neut. equiv., 389.

Addition of excess cyclohexylamine to an acetonitrile solution of the above substance gave a salt which after recrystallization from ethyl alcohol melted at 188–188.5°. This material was identical (m.p., mixed m.p., and infrared spectrum) with an authentic sample of cyclohexylammonium di-*p*-nitrophenyl phosphate.

Forty grams of the substance with m.p. 90–91° was converted to the cyclohexylamine salt as described above and a hot suspension of the salt was treated with an excess of IR-120(H^+) ion-exchange resin. The strongly acidic solution was filtered from the resin and evaporated to give di-*p*-nitrophenyl hydrogen phosphate (35 g.) with m.p. 173–174° after crystallization from ethyl acetate.

Identification of Pyridine in the Compound with M.P. 90–91°.—The substance (5.81 mg.) with m.p. 90–91° and authentic di-*p*-nitrophenyl hydrogen phosphate (5.71 mg.) were each dissolved in 5-ml. portions of water and the solutions stirred with 1-ml. portions of freshly washed Dowex-2 (formate form) resin. The resin was removed and washed thoroughly with water until the optical density of the washings were zero. The two combined filtrates were diluted to 100 ml. with water and the ultraviolet absorption spectrum of the solution from the 91° melting substance was determined using the solution obtained from di-*p*-nitrophenyl phosphate as the blank. The spectrum was found to be identical with that of pyridine in 10^{-4} *M* formic acid solution. From the optical density of the above solution at 256 mμ and using a figure of 5400 as E_{max} for pyridine at this wave length, the pyridine content of the 91° melting substance could be calculated to be 10.07%. This corresponds to a monohydrate of a molecular compound of two moles of di-*p*-nitrophenyl phosphate and one mole of pyridine. This is in agreement with the elemental analysis recorded above.

Di-*p*-nitrophenyl Methyl Phosphate.—Di-*p*-nitrophenyl hydrogen phosphate (1 g., 2.94 mmoles) was dissolved in anhydrous dioxane (8 ml.) by warming with exclusion of moisture. The solution was cooled quickly to room temperature and di-*p*-tolyl carbodiimide (330 mg., 1.49 mmoles) was added. Di-*p*-tolylurea began to separate immediately and after ten minutes at room temperature anhydrous methyl alcohol (0.063 ml., 1.56 mmoles) was added. The stoppered flask was kept in a desiccator overnight and the solution was then filtered from di-*p*-tolylurea (335 mg., 94%), the urea being washed with small portions of dioxane. The combined filtrate and washings were evaporated to a sirup which was taken up in chloroform (10 ml.). The solution was extracted with water until the extracts were neutral. Crystalline di-*p*-nitrophenyl hydrogen phosphate (475 mg.) was recovered from the water extracts by acidification with concentrated hydrochloric acid. Evaporation of the chloroform solution gave a dry white residue which was crystallized from aqueous ethyl alcohol to give dinitrophenyl methyl phosphate[13] (490 mg., 90%) as white needles, m.p. 142–143°, identical with that of a sample prepared according to Ketelaar and Gersmann.[13] (No depression in m.p. on admixture of the two samples.) *Anal.* Calcd. for $C_{13}H_{11}N_2O_8P$: C, 44.08; H, 3.13; N, 7.90. Found: C, 44.25; H, 3.07; N, 7.98.

Benzyl di-*p*-nitrophenyl phosphate was prepared in 96% yield by the phosphorylation of benzyl alcohol; m.p. 101–102° after crystallization from carbon tetrachloride. *Anal.* Calcd. for $C_{19}H_{15}N_2O_8P$: C, 53.00; H, 3.51; N, 6.51. Found: C, 53.17; H, 3.51; N, 6.36.

Cyclohexyl di-*p*-nitrophenyl phosphate was similarly pre-

(25) Melting points are uncorrected. Analyses recorded were performed by Mr. W. Manser, Zürich, Switzerland.

(26) S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).

(27) R. Markham and J. D. Smith, *ibid.*, **52**, 552 (1952); D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 2040 (1953).

(28) The lithium salt may coprecipitate with the free acid if hydrochloric acid is added all at once or in the cold.

(29) J. M. A. Hoeflake, *Rec. trav. chim.*, **36**, 24 (1916).

pared in 85% yield; m.p. 81–82° after crystallization from alcohol. *Anal.* Calcd. for $C_{18}H_{19}N_2O_8P$: C, 51.19; H, 4.54; N, 6.64. Found: C, 51.17; H, 4.70; N, 6.75.

2',3'-*O*-isopropylidene Uridine 5'-Di-*p*-nitrophenyl Phosphate.—Di-*p*-nitrophenyl hydrogen phosphate (2.85 g.) was dissolved in anhydrous dioxane (15 ml.) by warming with exclusion of moisture. The solution was cooled to room temperature, di-*p*-tolyl carbodiimide (900 mg.) added and the mixture kept for ten minutes. Dry 2',3'-*O*-isopropylidene uridine[30] (1.0 g.) was added and the sealed reaction mixture kept overnight at room temperature. Di-*p*-tolylurea (880 mg.) was removed by filtration and washed with small portions of dioxane. The combined filtrate was evaporated to dryness, the residue taken up in chloroform (25 ml.) and the solution extracted with water until an extract was neutral. Evaporation of chloroform gave 2',3'-*O*-isopropylidene uridine 5'-di-*p*-nitrophenyl phosphate (1.916 g.) including the residual di-*p*-tolylurea (96 mg.). The material was used directly in the subsequent steps described later. A sample was crystallized from ethyl alcohol to give white needles with m.p. 118.5–120.5°. *Anal.* Calcd. for $C_{24}H_{23}N_4O_{13}P$: C, 47.53; H, 3.82; N, 9.24. Found: C, 47.34; H, 3.84; N, 9.03.

Methyl *p*-Nitrophenyl Hydrogen Phosphate.—To an acetonitrile (50 ml.) solution of di-*p*-nitrophenyl methyl phosphate (4.5 g.) was added 1 *N* aqueous lithium hydroxide solution (38 ml.) and the resulting yellow solution was kept for 30 minutes at room temperature. It was then partly evaporated to remove acetonitrile and the remaining aqueous solution adjusted to *p*H 5 with acetic acid. *p*-Nitrophenol was extracted with ether until an extract gave no yellow color with alkali. The residual solution was concentrated to 15 ml., and freed from lithium ions by passage through a 2.5 × 15 cm. column of IR-120(H^+) resin. The effluent and washings were evaporated to dryness under reduced pressure leaving free methyl *p*-nitrophenyl hydrogen phosphate as a colorless oil which crystallized on prolonged evacuation. The substance was recrystallized from a mixture of benzene and petroluem ether to give stout white needles (2.77 g., 94%) with m.p. 121–123°. One further recrystallization raised the m.p. to 123.5–124.5°. *Anal.* Calcd. for $C_7H_8NO_6P$: C, 36.09; H, 3.46; N, 6.01. Found: C, 36.27; H, 3.46; N, 5.91. The crystalline cyclohexylamine salt was prepared by adding an excess of cyclohexylamine to an acetonitrile–ether solution of the free acid. After recrystallization from benzene it melted at 150.5–151.0°. *Anal.* Calcd. for $C_{13}H_{21}N_2O_6P$: C, 47.00; H, 6.37; N, 8.44. Found: C, 47.18; H, 6.23; N, 8.44.

Other alkyl mono-*p*-nitrophenyl hydrogen phosphates prepared by the alkaline hydrolysis of the neutral esters are mentioned below.

Benzyl *p*-nitrophenyl hydrogen phosphate was prepared as the crystalline cyclohexylammonium salt in 90% yield; m.p. 168–169° after crystallization from benzene. *Anal.* Calcd. for $C_{19}H_{25}N_2O_6P$: C, 55.88; H, 6.17; N, 6.86. Found: C, 55.64; H, 6.17; N, 6.95.

Cyclohexyl *p*-nitrophenyl hydrogen phosphate was prepared as the cyclohexylammonium salt in 85% yield; m.p. 202–203° after crystallization from acetonitrile. *Anal.* Calcd. for $C_{18}H_{29}N_2O_6P$: C, 54.00; H, 7.30; N, 7.00. Found: C, 54.03; H, 7.50; N, 7.03.

***n*-Hexyl *p*-nitrophenyl hydrogen phosphate** also was prepared as the cyclohexylammonium salt in 85% yield; m.p. 109–110° after crystallization from acetonitrile. *Anal.* Calcd. for $C_{18}H_{21}N_2O_6P$: C, 53.90; H, 7.79; N, 6.97. Found: C, 54.08; H, 7.37; N, 7.00.

Uridine 5'-*p*-Nitrophenyl Hydrogen Phosphate.—To an acetonitrile (30 ml.) suspension of 2',3'-*O*-isopropylidene uridine 5'-di-*p*-nitrophenyl phosphate (1.22 g.) was added lithium hydroxide solution (6.5 ml.) and water (15 ml.), and the yellow mixture shaken for 30 minutes. Paper chromatography at this stage showed that removal of one of the nitrophenyl groups was complete; R_f's of 2',3'-*O*-isopropylidene uridine 5'-*p*-nitrophenyl hydrogen phosphate were: solvent A, 0.54; solvent B, 0.62. Most of the acetonitrile then was removed by evaporation under reduced pressure and the residual solution brought to *p*H 5 with acetic acid. The slight suspension of di-*p*-tolylurea was removed and the aqueous solution extracted with ether until an extract gave no yellow color with alkali. The *p*H of the solution was then adjusted to 2.7 by the addition of glacial acetic acid and the acidic solution heated in a boiling water-bath for 90 minutes. The solution was then evaporated to dryness, last traces of acetic acid being removed by co-evaporation with dioxane. The oily residue was dissolved in water and the solution passed slowly through a Dowex 50(H^+) column (10 cm. × 2 cm.), the column being washed thoroughly with water. The total acidic effluent was concentrated to about 5 ml. and the concentrate neutralized with aqueous barium hydroxide to *p*H 4.0. A trace of insoluble material was removed by centrifugation and the supernatant concentrated to an oil which solidified on trituration with ethyl alcohol. The amorphous barium salt (746 mg., 67%) was collected by centrifugation, washed with acetone and ether and dried under a vacuum at room temperature. Paper chromatography revealed a single strong spot; R_f's: solvent A, 0.25; solvent B, 0.45. *Anal.* Calcd. for $C_{18}H_{16}N_3O_{11}PBa^1/_2 \cdot 2H_2O$: C, 32.75; H, 3.66; N, 7.64. Found: C, 33.34; H, 3.75; N, 7.19.

A kinetic study of the degradation of this substance by snake venom diesterase has been carried out by Dr. C. A. Dekker and Miss M. L. Dirksen and will be reported by these authors elsewhere.

***p*-Aminophenyl Methyl Hydrogen Phosphate.**—A solution of di-*p*-nitrophenyl methyl phosphate (1 g.) in ethyl alcohol (200 ml.) was hydrogenated at room temperature in the presence of Adams platinum catalyst (200 mg.). After four hours, when hydrogen uptake (624 ml.) ceased, the catalyst was removed, washed with ethyl alcohol, and the combined filtrate evaporated to a colorless oil which was taken up in water (10 ml.). The aqueous solution was passed through a column (10 × 1 cm.) of IR-120(H^+) resin and the column washed until the effluent was neutral. The total effluent, which slowly turned brown in air, was evaporated to dryness under reduced pressure giving a brown solid (442 mg.), which was washed three times with ether. (Addition of cyclohexylamine to the ethereal extracts gave a small amount (27 mg.) of bis-cyclohexylammonium monomethyl phosphate). The ether-insoluble residue (m.p. 205–208°) was dissolved in hot aqueous ethyl alcohol, the solution decolorized with charcoal and allowed to cool. *p*-Aminophenyl methyl hydrogen phosphate (300 mg., 53%) was obtained as white needles of m.p. 212–213°. *Anal.* Calcd. for $C_7H_{10}NO_4P$: C, 41.62; H, 5.57; N, 6.90. Found: C, 41.62; H, 5.57; N, 6.93.

Benzyl Methyl Hydrogen Phosphate.—A freshly prepared solution of sodium benzoxide (from 200 mg. of sodium and 10 ml. of benzyl alcohol) was added to a benzyl alcohol (5 ml.) solution of di-*p*-nitrophenyl methyl phosphate (1 g.). The resulting orange solution was kept overnight, then filtered from some crystalline sodium *p*-nitrophenoxide which had separated, and evaporated under a high vacuum at 50°. The oily residue was dissolved in ether and the ethereal solution extracted repeatedly with 0.01 *N* alkali until the extracts were colorless. Evaporation of the ethereal solution gave an oil[31] which was treated with 35% acetic acid (30 ml.) at 100° for one hour. The acetic acid solution was then evaporated to dryness and the residue equilibrated with water (20 ml.) at *p*H 8. The aqueous solution was freed from the insoluble oil by extraction with ether. The clear aqueous solution was passed through a column (10 cm. × 1 cm.) of IR-120(H^+) resin, the column being washed with water until the effluent became neutral. The acidic solution was evaporated, the residual colorless oil (288 mg.) taken up in ether (5 ml.) and an excess of cyclohexylamine was added. On adding light petroleum and keeping the mixture at 0° cyclohexylammonium benzyl methyl phosphate (400 mg., 50%) crystallized; m.p. 99–101°, raised after one recrystallization from ether to 100–101.5°. *Anal.* Calcd. for $C_{14}H_{24}NO_4P \cdot 1H_2O$: C, 52.85; H, 8.21; N, 4.39. Found: C, 53.10; H, 8.14; N, 4.58.

Methyl Dihydrogen Phosphate from Di-*p*-nitrophenyl Methyl Phosphate: (1) By Alkaline Hydrolysis.—Aqueous lithium hydroxide (9 ml. of 1 *N*) was added to a dioxane (10 ml.) solution of di-*p*-nitrophenyl methyl phosphate (1 g.), and the yellow solution shaken for 30 minutes. The solution was neutralized with acetic acid and evaporated to a gum which was heated in 1 *N* lithium hydroxide (15 ml.) in a polyethylene tube at 100°. Paper chromatography in the solvent system butyl alcohol–acetic acid–water (4:1:5,

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

(31) The oil was very much in excess of the expected dibenzyl methyl phosphate and contained a large proportion of another substance, presumably dibenzyl ether.

v./v.) showed that methyl *p*-nitrophenyl hydrogen phosphate (R_f, 0.55) had disappeared completely after three hours, only two spots corresponding to *p*-nitrophenol (R_f, 0.93) and methyl dihydrogen phosphate (R_f, 0.11) being present. The *p*H of the alkaline solution was then adjusted to 5 with acetic acid and the solution extracted with ether until an extract gave no yellow color with alkali. The colorless aqueous solution was then passed through a column (15 cm. × 1.5 cm.) of IR-120(H^+) resin and the acidic effluent and washings were evaporated to dryness, the last traces of acetic acid being removed by repeated evaporation in presence of dioxane. The oily residue was dissolved in water (5 ml.), the solution neutralized to *p*H 8.5 with cyclohexylamine and then evaporated. The white solid residue was crystallized from ethyl alcohol giving bis-cyclohexylammonium methyl phosphate (790 mg., 90%) as colorless needles, which on heating decomposed at 195–198°. The sample was dried *in vacuo* at room temperature.[32] *Anal.* Calcd. for $C_{13}H_{31}N_2O_4P \cdot 1H_2O$: C, 47.54; H, 10.13; N, 8.53. Found: C, 47.53; H, 10.13; N, 8.64.

(2) **By Catalytic Hydrogenation.**—Di-*p*-nitrophenyl methyl phosphate (500 mg.) was hydrogenated in ethyl alcohol (200 ml.) containing one ml. of concentrated hydrochloric acid in the presence of Adams platinum catalyst (100 mg.). After four hours, when hydrogen uptake ceased, the catalyst was removed by filtration and the alcoholic solution evaporated. The white solid was dissolved in water (5 ml.) and the solution passed through a column (10 cm. × 1 cm.) of IR-120(H^+) resin. The effluent was evaporated to dryness and converted to the bis-cyclohexylammonium salt as described above. The product (345 mg., 78%) was identical with that obtained above by alkaline hydrolyses.

Benzyl dihydrogen phosphate[33] was prepared in 94% yield from benzyl di-*p*-nitrophenyl phosphate by alkaline hydrolysis as described above; m.p. 94–96° after crystallization from ether–petroleum ether (reported[33] m.p., sharp but varying between 85 and 113°, depending upon rate of heating). *Anal.* Calcd. for $C_7H_9O_4P$: C, 44.70; H, 4.82. Found: C, 44.84; H, 4.87. The bis-cyclohexylammonium salt prepared by the addition of excess of cyclohexylamine to an ethereal solution of the acid had m.p. 232–234° (Chase, *et al.*,[33] quote 233–235°). *Anal.* Calcd. for $C_{19}H_{35}N_2O_4P$: C, 59.02; H, 9.12; N, 7.25. Found: C, 58.91; H, 8.91; N, 6.96.

***n*-Hexyl dihydrogen phosphate** was prepared in 91% yield from the corresponding di-*p*-nitrophenyl ester by hydrogenolysis in the presence of acid and was characterized as the bis-cyclohexylammonium salt. M.p. of the salt was 195–196° after crystallization from ethyl alcohol. *Anal.* Calcd. for $C_{18}H_{41}N_2O_4P$: C, 56.81; H, 10.86; N, 7.36. Found: C, 56.77; H, 11.01; N, 6.95.

Bis-cyclohexylammonium cyclohexyl phosphate also was prepared in 85% yield by hydrogenolysis of the corresponding ester; m.p. 218–219° after crystallization from a mixture of benzene and ethyl alcohol. *Anal.* Calcd. for $C_{18}H_{39}N_2O_4P$: C, 57.15; H, 10.38; N, 7.40. Found: C, 57.31; H, 10.38; N, 7.46.

N-Methylpyridinium Di-*p*-nitrophenyl Phosphate.—An anhydrous pyridine (5 ml.) solution of di-*p*-nitrophenyl methyl phosphate (1 g.) was kept at room temperature with exclusion of moisture. The highly crystalline N-methylpyridinium di-*p*-nitrophenyl phosphate which separated was collected by filtration after 18 hours; yield 1.06 g. Evaporation of the mother liquor afforded a further amount (100 mg.) of the same product; m.p. 134–135°, unchanged after recrystallization from ethyl acetate–ethyl alcohol. *Anal.* Calcd. for $C_{18}H_{16}N_3O_8P$: C, 49.89; H, 3.72; N, 9.70. Found: C, 49.87; H, 3.74; N, 9.72.

Paper chromatography and paper electrophoresis (*p*H 4) of the crystalline compound showed two ultraviolet absorbing spots; one, corresponding to di-*p*-nitrophenyl phosphate ion, and the second corresponding to N-methylpyridinium ion (identical in electrophoretic mobility and ultraviolet absorption spectrum to this ion prepared from dimethyl sulfate and pyridine in ether).

Acidic Hydrolysis of Di-*p*-nitrophenyl Methyl Phosphate.—To an acetic acid (2 ml.) solution of di-*p*-nitrophenyl methyl phosphate (40 mg.), maintained at 100°, slowly was added two ml. of water so as to maintain a homogeneous solution. Duplicate five microliter aliquots were removed at intervals and applied to Whatman No. 1 paper. Paper chromatography in solvent A showed the formation of spots corresponding to di-*p*-nitrophenyl hydrogen phosphate (R_f, 0.73) and methyl *p*-nitrophenyl hydrogen phosphate (R_f, 0.55). The percentages of the two products were ascertained by elution of the spots and determination of the optical density at 285 mμ. These were found to be: di-*p*-nitrophenyl hydrogen phosphate, about 77%; methyl *p*-nitrophenyl hydrogen phosphate, about 23%. The amounts of these products reached a maximum after three hours and then slowly decreased, with the concomitant formation of mono-*p*-nitrophenyl and monomethyl dihydrogen phosphates.

O-Methyl-N,N′-dicyclohexylisourea.[14]—Dicyclohexyl carbodiimide (310 mg.) was added to an anhydrous methyl alcoholic solution (5 ml.) of di-*p*-nitrophenyl hydrogen phosphate (500 mg.) and the solution kept at room temperature for four hours. The solvent was then evaporated and the residue crystallized from dioxane to give the di-*p*-nitrophenyl phosphate salt of *O*-methyl-N,N′-dicyclohexylisourea (IV) (710 mg., 84%) as colorless needles of m.p. 153–153.5°. *Anal.* Calcd. for $C_{26}H_{35}N_4O_9P$: C, 54.00; H, 6.10; N, 9.70. Found: C, 54.20; H, 6.30; N, 9.91.

A warm aqueous ethyl alcoholic solution of the salt (500 mg.) was well stirred with freshly washed IRA 400($O\bar{H}$) resin. The resin was removed by filtration, washed twice with ethyl alcohol and the total filtrate evaporated to dryness. The residual colorless oil was distilled in a short path apparatus to give O-methyl-N,N′-dicyclohexylisourea (185 mg., 90%) as a colorless mobile liquid; b.p. 90° (0.1 mm.). *Anal.* Calcd. for $C_{14}H_{26}N_2O$: C, 70.45; H, 11.00. Found: C, 70.10; H, 11.08.

P^1P^2-Di-*p*-nitrophenyl P^1P^2-Dimethyl Pyrophosphate (VIII).—To a solution of methyl *p*-nitrophenyl hydrogen phosphate (466 mg.) in anhydrous acetonitrile (5 ml.) was added di-*p*-tolyl carbodiimide (240 mg.) and the mixture, which deposited di-*p*-tolylurea immediately, was kept at room temperature for one hour. The urea was then removed by filtration in a dry box and the filtrate evaporated to an oil. It was suspended in ether (5 ml.) and acetonitrile gradually added until a clear solution resulted. On keeping at 0°, the solution deposited the pyrophosphate VIII as colorless stout crystals (350 mg., 78%); m.p. 81–83°, unchanged on recrystallization from the same solvents. *Anal.* Calcd. for $C_{14}H_{14}N_2O_{11}P_2$: C, 37.50; H, 3.15; N, 6.25. Found: C, 37.31; H, 3.21; N, 6.35.

Acknowledgment.—This work has been carried out under Defence Research Board of Canada, grant number 2020-23, Project D52-20-20-23.

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(32) Drying at higher temperatures results in some loss of cyclohexylamine. All the bis-cyclohexylammonium salts reported herein were therefore dried only at room temperature.

(33) B. H. Chase, G. W. Kenner, A. R. Todd and R. F. Webb, *J. Chem. Soc.*, 1371 (1956).