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

Nucleoside Polyphosphates. IV.[1] A New Synthesis of Guanosine 5′-Phosphate

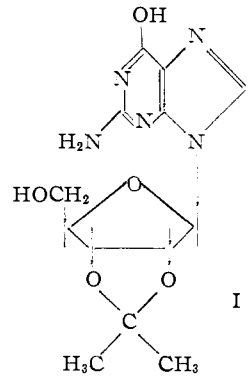
BY ROBERT WARNER CHAMBERS,[2] J. G. MOFFATT AND H. G. KHORANA

RECEIVED JANUARY 12, 1957

The previously recorded phosphorylation of 2′,3′-*O*-isopropylidene guanosine with phosphorus oxychloride in a mixture of pyridine and dimethylformamide has been re-examined. While some guanosine 5′-phosphate was produced, large amounts of the nucleoside always remained unreacted and phosphorus-containing side products, tentatively identified as guanosine 5′-di- and triphosphates, were formed. Guanosine 5′-phosphate was synthesized in a good (*ca.* 70%) yield by using tetra-*p*-nitrophenyl pyrophosphate as the phosphorylating agent. The steps were: 2′,3′-*O*-isopropylidene guanosine → 2′,3′-*O*-isopropylidene guanosine 5′-di-*p*-nitrophenyl phosphate $\xrightarrow{\bar{O}H}$ 2′,3′-*O*-isopropylidene guanosine 5′-mono-*p*-nitrophenyl phosphate $\xrightarrow[\bar{O}H]{\text{snake venom or}}$ 2′,3′-*O*-isopropylidene guanosine 5′-phosphate $\xrightarrow{H^+}$ guanosine 5′-phosphate. Some interesting properties of 2′,3′-*O*-isopropylidene guanosine 5′-di-*p*-nitrophenyl phosphate are recorded. An improved procedure for the preparation of 2′,3′-*O*-isopropylidene guanosine is described.

Nucleoside 5′-mono-, di- and triphosphates corresponding to all the four naturally occurring ribonucleosides were first demonstrated to be present in certain tissues by Potter and co-workers[3] and subsequent work reported from different laboratories has established the widespread occurrence[4] and biological importance[5] of these substances. In this Laboratory, attention has been devoted during the last several years to the development of methods for the chemical synthesis of nucleoside polyphosphates and related compounds. It was hoped that in addition to providing synthetic confirmation of structures, our studies might lead to new and improved procedures which would make some of the difficultly accessible members of this group readily available in a pure state. In continuing these studies,[6] we undertook the problems of the synthesis of guanosine 5′-mono-, di- and triphosphates. Chemical work in this series was anticipated to be particularly difficult, for the reasons which will become clear from the sequel,[7] and it was therefore considered that these problems would provide stringent tests for the synthetic methods which have been developed in this Laboratory. The present communication describes our work on the synthesis of guanosine 5′-phosphate (GMP) while the synthesis of the higher phosphates is reported in the following communication.[7] A brief account of a part of this work has already appeared.[8]

While highly satisfactory syntheses of a number of nucleoside 5′-phosphates[1,9,10c] have been recorded, previous attempts at the phosphorylation of guanosine derivatives[10] have met with only very limited success. In particular, the 5′-hydroxyl group in 2′,3′-*O*-isopropylidene guanosine (I) is



I

uniquely inert and resists phosphorylation[11] by the well-known phosphorylating agents, dibenzyl and diphenyl phosphorochloridates.[10] Michelson and Todd[10c] have, however, recorded the preparation of guanosine 5′-phosphate in 20% yield by using phosphorus oxychloride as the phosphorylating agent, and since at the time this work was undertaken this was the only applicable method,[12] we undertook a re-examination of this synthesis.

In the first experiments, Michelson and Todd's procedure was repeated exactly (addition of 1.1 equiv. of phosphorus oxychloride to a cold solution of 2′,3′-*O*-isopropylidene guanosine in a mixture of dimethylformamide and pyridine). Using paper chromatography, electrophoresis and ion-exchange analysis as analytical tools, two phosphorus-con-

(1) Paper III. R. H. Hall and H. G. Khorana, THIS JOURNAL, **77**, 1871 (1955).

(2) Life Insurance Medical Research Post-doctoral Fellow, 1954–1956.

(3) H. Schmitz, R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.*, **209**, 11 (1954).

(4) R. Bergkvist and A. Deutsch, *Acta Chem. Scand.*, **8**, 1880, 1889 (1954); H. Schmitz, *Naturwiss.*, **5**, 120 (1954); *Biochim. Z.*, **325**, 555 (1954); P. Ayengar, D. M. Gibson, C. H. L. Peng and D. R. Sanadi, *J. Biol. Chem.*, **218**, 521 (1956).

(5) Some selected references are: A. Munch-Petersen, H. M. Kalckar, E. Cutols and E. E. B. Smith, *Nature*, **172**, 1036 (1953); E. P. Kennedy and S. B. Weiss, THIS JOURNAL, **77**, 250 (1955); D. R. Sanadi, D. M. Gibson, P. Ayengar and M. Jacob, *J. Biol. Chem.*, **218**, 505 (1956); M. Grunberg-Manago, P. J. Ortiz and S. Ochoa, *Biochem. Biophys. Acta.*, **20**, 269 (1956); A. Kornberg, I. R. Lehman, M. J. Bessman and E. S. Simms, *ibid.*, **21**, 197 (1956).

(6) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954); R. H. Hall and H. G. Khorana, *ibid.*, **76**, 5056 (1954).

(7) R. W. Chambers and H. G. Khorana, *ibid.*, **79**, 3752 (1957).

(8) R. W. Chambers, J. G. Moffatt and H. G. Khorana, *ibid.*, **77**, 3416 (1955).

(9) J. Baddiley and A. R. Todd, *J. Chem. Soc.*, 648 (1947) and the references cited therein.

(10) (a) H. Bredereck and E. Berger, *Ber.*, **73**, 1124 (1940); (b) J. M. Gulland and G. I. Hobday, *J. Chem. Soc.*, 746 (1940); (c) A. M. Michelson and A. R. Todd, *ibid.*, 2476 (1949).

(11) It should be pointed out that 2′,3′-*O*-isopropylidene guanosine is highly insoluble in pyridine. In the procedure reported by Michelson and Todd,[10c] a mixture of dimethylformamide and pyridine was used in order to obtain a homogeneous reaction mixture. The situation may be complicated by the reaction of phosphorylating agents with dimethylformamide. See, *e.g.*, H. K. Hall, Jr., THIS JOURNAL, **78**, 2717 (1956).

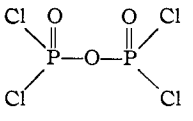
(12) The simple phosphorylating agent consisting of a mixture of phosphoric acid and phosphorus pentoxide which is satisfactory for the phosphorylation of the suitably protected pyrimidine nucleosides[1] was useless in the present case, 2′,3′-*O*-isopropylidene guanosine undergoing extensive degradation.

taining substances, in addition to guanosine 5′-phosphate, were detected as products. Further, about 50% of the starting material remained unreacted. The use of an approximately threefold excess of the phosphorylating agent caused no significant increase in the yield of the phosphorylated products. The results of the quantitative ion exchange analysis are recorded in the Experimental Section (Table II), the side products being designated as G-X and G-Y. As can be seen, GMP was formed in yields varying between 17 and 31%. However, the isolation of this desired substance was complicated by the presence of large amounts of inorganic phosphate and the yield of the pure product never exceeded 15% by this method of synthesis.

The phosphorus-containing side products (G-X and G-Y), both, had ultraviolet absorption spectra identical with that of guanosine 5′-phosphate and from their behavior on ion-exchange columns appeared to be polyphosphates. They were investigated more closely with the hope of obtaining some insight into the nature of the above phosphorylation reaction. G-X, which was present in amounts similar to those of GMP, was purified by ion-exchange chromatography followed by absorption and elution from charcoal. From its behavior on paper chromatograms, ionograms and an ion-exchange column, G-X was believed to be a "guanosine diphosphate." The evidence adduced below suggested that the product was guanosine 5′-diphosphate. (1) The ratio of guanosine (ultraviolet absorption) to labile phosphorus to total phosphorus was found to be 0.9:1.0:2.0. It may be mentioned that the location of the labile phosphoryl group on the amino group in the guanine ring was excluded by the ultraviolet absorption spectrum and the fact that the substance had survived the acidic treatment used to remove the isopropylidene group. (2) Crude snake venom degraded G-X to guanosine and inorganic phosphate. (3) The vicinal hydroxyl groups in the ribose moiety were intact as shown by the periodate–benzidine spray.

G-Y was only a very minor product and the availability of only a very small amount of the purified sample precluded a rigorous examination. However, experiments along the lines described above suggested that G-Y probably was guanosine 5′-triphosphate.

The formation of guanosine 5′-di- and triphosphates as side products suggests that linear phosphorylating agents of the type (II) are present in



II

the above reaction mixtures. (Inorganic pyro- and polyphosphates were also detected as reaction products.) These polyphosphorylating agents[13] could arise by the reaction of phosphorus oxychloride with traces of water present in the reaction mixture, although all possible precautions to exclude moisture were taken.[14]

The above experiments would appear to demonstrate conclusively the inertness of the 5′-hydroxyl group in I, since large amounts of the nucleoside always were recovered.[15] More recently, Hayes, Michelson and Todd[16] have also remarked on the inertness of the hydroxyl groups in 2′-deoxyguanosine derivatives. A possible explanation of this inertness is that the 5′-hydroxyl group is involved in hydrogen bonding with the 2-amino group in the guanine ring, a situation unique to this nucleoside.

The unsatisfactory results obtained above and the virtual failure of the other known phosphorylating agents led us to conclude that the phosphorylation of 2′,3′-*O*-isopropylidene guanosine (I) could be best accomplished with a powerful, monofunctional reagent which would not require basic catalysis. Tetra-*p*-nitrophenyl pyrophosphate, which is described in detail in the preceding communication,[17] was found to meet these requirements. The reagent was allowed to react with I in the presence of one equivalent of the free di-*p*-nitrophenyl hydrogen phosphate and, after a reaction period of 15 hours at 20°, 2′,3′-*O*-isopropylidene guanosine 5′-di-*p*-nitrophenyl phosphate (III) was isolated in a nearly quantitative yield. This successful phosphorylation in acidic medium would appear to support the belief that the 5′-hydroxyl group acquired "normal" reactivity when the 2-amino group in I was protonated.

As recorded in the preceding communication[17] the removal of one of the *p*-nitrophenyl groups from the neutral esters of the type III may be accomplished by very mild alkaline treatment; IV was thus obtained in a nearly quantitative yield, as estimated by spectrophotometric measurement of the liberated *p*-nitrophenol. The removal of the second *p*-nitrophenyl group was conveniently effected by digestion with crude rattlesnake venom, which is known to have a relatively unspecific and potent phosphodiesterase activity.[18]

(13) M. Viscontini and K. Ehrhardt, *Ang. Chem.*, **66**, 717 (1954).

(14) It also is possible that the polyphosphates are formed during decomposition of the excess of phosphorus oxychloride with aqueous pyridine (see Experimental).

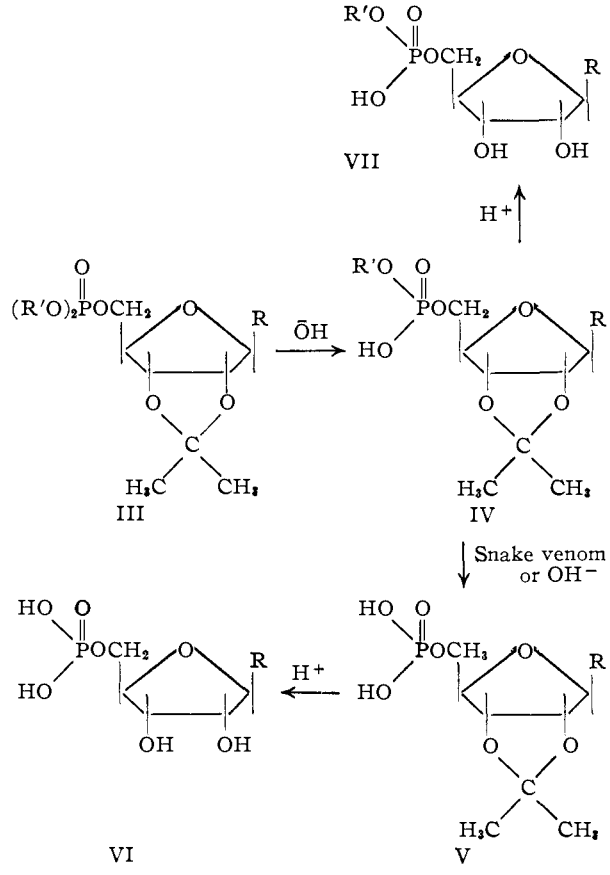
(15) It might be argued that the free nucleoside found in the reaction products arose from the breakdown of an acid-labile phosphorylated derivative, *e.g.*, N^2-phosphate, during the acidic removal of the isopropylidene group. Thus, Levene and Tipson (*J. Biol. Chem.*, **121**, 131 (1937)) have recorded the isolation of an adenosine diphosphate after treatment of 2′,3′-*O*-isopropylidene adenosine with 2.2 equivalents of phosphorus oxychloride. Since one of the two phosphate groups in the above product was acid-labile, Levene and Tipson formulated the substance as adenosine N^6 5′-diphosphate. If N-phosphorylation were also to occur in the guanosine series, then by using an excess of phosphorus oxychloride N^2,O5′-diphosphate would have been obtained, which after mild acidic treatment would have afforded guanosine 5′-phosphate. In other words, an increase in the amount of the phosphorylating agent should have resulted in an increase in the yield of guanosine 5′-phosphate. Actually, this was found not to be the case. Furthermore, when the phosphorylation mixture was examined by paper chromatography *before* any acidic treatment, a large proportion of isopropylidene guanosine was found to be present.

(16) D. H. Hayes, A. M. Michelson and A. R. Todd, *J. Chem. Soc.*, 808 (1955).

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

(18) For a recent review see G. Schmidt, "Enzymes Attacking Nucleic Acids," in "The Nucleic Acids," Vol. I, Academic Press, Inc., New York, N. Y., 1955, p. 585.

Treatment of the resulting V with dilute acid furnished guanosine 5′-phosphate which was isolated in yields of 65–75% based on isopropylidene guanosine. As was expected, the 5′-nucleotidase, which is also present in the crude snake venom, was inactive toward V, since the 3′-hydroxyl group[19] was protected by the isopropylidene

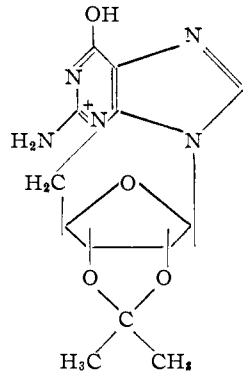


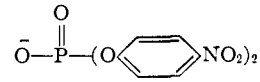
III → VII, R = guanine
III, IV and VII, R′ = *p*-nitrophenyl

group. However, it was fully active against guanosine 5′-phosphate (VI). Therefore, the removal of the protecting groups in the order IV → V → VI enabled the use of the crude snake venom.

The removal of the *p*-nitrophenyl group from IV also was accomplished by treatment with 1 *N* lithium hydroxide at 100° for about three hours. Some concomitant hydrolysis to inorganic phosphate also occurred under these conditions, but the product could be purified by fractional precipitation of the barium salt. The yield of guanosine 5′-phosphate prepared by alkaline hydrolysis was about 60%.

Two observations made during the course of the work on the neutral III may be mentioned. On heating its acetonitrile solution a new high-melting isomeric substance was produced. The product was concluded to be a quaternary ammonium salt of di-*p*-nitrophenyl phosphate, its formation being analogous to the quaternization reaction undergone by 2′,3′-*O*-isopropylidene adenosine 5′-*p*-toluenesulfonate.[20] While the structure VIII is tentatively advanced for this substance, the alternative possibility of cyclization with the 2-amino group in the guanine ring cannot be ignored.





VIII

Cyclization, instead of transesterification,[17] was also the preferred reaction when III was treated with sodium benzoxide in benzyl alcohol. Analogy for this reaction may again be found in the work of Brown, Todd and Varadarajan[21] who have recorded quaternization reactions of *p*-toluenesulfonic acid esters of uridine derivatives in methyl alcohol–ammonia.

Finally, the preparation of 2′,3′-*O*-isopropylidene guanosine, the starting material, by the published procedure[10c] was tedious and gave in our hands unsatisfactory yields. A simpler method of isolation which utilizes an ion-exchange resin for the removal of zinc ions is described in the Experimental Section. The procedure gave consistently 70–80% yields.

Experimental

Methods.—Paper chromatography was carried out with Whatman No. 1 paper, using mostly the descending technique. The solvent systems employed were: (1) *n*-butyl alcohol–acetic acid–water (4:1:5, v./v.)[22] (solvent I); (2) sopropyl alcohol–ammonia–water (7:1:2)[23] (solvent II); (3) isopropyl alcohol–1% ammonium sulfate solution (2:1, v./v.)[24] (solvent III); (4) pyridine–isoamyl alcohol–water (7:7:6, v./v.)[25] (solvent IV); (5) potassium dihydrogen phosphate solution (5%)–isoamyl alcohol[26] (solvent V); (6) isopropyl alcohol (75 ml.)–water (25 ml.)–trichloroacetic acid (5 g.)–ammonia (0.25 ml., sp. gr. 0.9)[27] (solvent VI); (7) isobutyric acid (100 ml.)–1 *N* ammonium hydroxide (60 ml.)–0.1 *M* ethylenediaminetetraacetic acid disodium salt[28] (1.6 ml.) (solvent VII). The R_f's of various compounds are given in Table I.

For paper electrophoresis, five inch strips of Whatman No. 3 MM paper were washed first with 2 *N* formic acid,[29] then with water and dried in air. The spots were applied and dried and the papers then sprayed with 0.02 *M* citrate buffer, *p*H 4.4, and then subjected to a potential of 12 volts/cm.

(19) The 5′-nucleotidase is inactive when the 3′-hydroxyl group is substituted. Thus, adenosine 3′,5′-diphosphate (A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.*, **186**, 557 (1950)) is not attacked by the snake venom.

(20) V. M. Clarke, A. R. Todd and J. Zussman, *J. Chem. Soc.*, 2952 (1951).

(21) D. M. Brown, A. R. Todd and S. Varadarajan, *ibid.*, 2388 (1956).

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

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

(24) N. Anand, V. M. Clarke, R. H. Hall and A. R. Todd, *ibid.*, 3665 (1950).

(25) P. Edman, *Ark. Kemi. Min. Geol.*, **22**, 35 (1946).

(26) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(27) J. P. Ebel, *Bull. soc. chim.* (France), 991 (1953).

(28) H. A. Krebs and R. Hems, *Biochim. Biophys. Acta*, **12**, 172 (1953).

(29) H. E. Wade and D. M. Morgan, *Biochem. J.*, **60**, 264 (1955).

TABLE I

PAPER CHROMATOGRAPHY OF GUANOSINE DERIVATIVES

Compound	R_f's in solvent systems I	II	III	IV
Guanine	0	0.24	0.30	0.85
Guanosine	0.18	.27	.44	.85
2',3'-*O*-Isopropylidene guanosine	.63	.60	.70	.70
2',3'-*O*-Isopropylidene guanosine 5'-di-*p*-nitrophenyl phosphate	.95	..	.95	..
2',3'-*O*-Isopropylidene guanosine 5'-mono-*p*-nitrophenyl phosphate	.48	.58	.68	.64
Guanosine 5'-mono-*p*-nitrophenyl phosphate	.15	.35	.50	.41
Guanosine 5'-phosphate	0	.03	.31	0
Di-*p*-nitrophenyl hydrogen phosphate	.73	.87	.85	.83
2',3'-*O*-Isopropylidene cycloguanosine (VIII)	.47	.47	.49	.46
p-Nitrophenol	.89	.69	.83	.91

for two hours. The apparatus employed was a modification of Paigen's design,[30] in that the starch troughs were replaced by a glass baking dish (13.5″ × 8.5″ × 2″) filled with carbon tetrachloride. The paper strips were immersed in this bath during the run.

Spots were located on paper chromatograms by their ultraviolet absorption and by spraying the chromatograms with the molybdate–perchloric acid spray[31] and subsequently irradiating with ultraviolet light.[32] Total phosphorus determination was carried out by King's method,[33] while inorganic phosphate was determined by the method of Lowry and Lopez.[34]

Preparation of 2',3'-*O*-Isopropylidene Guanosine.—Zinc chloride (56 g. of a freshly fused, anhydrous sample) was dissolved in anhydrous acetone (350 ml.). Twenty-two grams of guanosine (dried previously *in vacuo* at 100° over phosphorus pentoxide) was added and the mixture heated overnight under reflux with exclusion of moisture. The clear solution was then evaporated to a thick sirup and 8 *N* ammonium hydroxide was added until the initially formed precipitate redissolved and a clear solution resulted. Amberlite IR-120-ammonium form (100 ml.) was added and if a precipitate formed, it was dissolved by the gradual addition of concd. ammonia. The mixture was stirred for 15 minutes. The resin was then removed by filtration and washed twice with 50-ml. portions of very dilute ammonia. This whole process was repeated four times using 100–125 ml. portions of the wet resin each time. The total volume of the final filtrate was about 1000 ml. On evaporation of ammonia, either by keeping the solution in an open vessel for several days or by heating on a water-bath and then allowing the solution to cool, 2',3'-*O*-isopropylidene guanosine crystallized. It was collected by filtration, washed with water and dried in air to give 15.2 g. of a crystalline, pure sample. The mother liquor was concentrated and retreated twice with 100-ml. portions of IR-120-ammonium form resin. A further amount (4.2 g.) of pure material was thus obtained; total yield 19.4 g., m.p. 296° (resolidification) (reported[10a] m.p. 299° dec.). This material could be recrystallized from hot water with negligible loss. Paper chromatography in four solvent systems (Table I) showed a single strong spot; guanosine could not be detected.

2',3'-*O*-Isopropylidene Guanosine 5'-Di-*p*-nitrophenyl Phosphate (III).—Di-*p*-nitrophenyl phosphate[35] (4.2 g., *ca.* 12.4 mmoles) was dissolved in warm anhydrous dioxane (20 ml.) and the solution cooled to room temperature under running cold water with exclusion of moisture. Di-*p*-tolyl carbodiimide (1.10 g., 4.97 mmoles) was added, and the stoppered reaction flask was kept at room temperature for ten minutes. 2',3'-*O*-Isopropylidene guanosine[35] (1.0 g., 3.1 mmoles) was quickly added and the sealed flask stored overnight in a desiccator. Di-*p*-tolylurea (1.080 g., theory 1.190 g.) was then removed by filtration and washed three times with 3-ml. portions of dioxane. The filtrate and the washings were evaporated and the resulting oil shaken with a mixture of chloroform (30 ml.) and 1 *M* lithium acetate buffer (*p*H 6.5, 10 ml.). The chloroform layer was separated and further extracted three times with the same buffer. During the extractions a part of the product III separated as a white amorphous solid (1.106 g.) which was collected by filtration and washed thoroughly with water. The clear chloroform solution was also washed three times with water, evaporated to dryness and the resulting pale yellow resin dried to a constant weight (0.982 g.) under a high vacuum. The total yield of the neutral product III after accounting for 110 mg. of the residual di-*p*-tolylurea was thus 1.898 g. (95%). Paper chromatography in several solvent systems showed the presence of a single fast-travelling spot. This material was used directly in the subsequent steps. A small portion was crystallized[36] from acetonitrile and formed very pale yellow needles which underwent a sharp transition at 161–163° to an amorphous solid which decomposed at 263–264°. *Anal.* Calcd. for $C_{25}H_{24}N_7O_{12}P\cdot 1H_2O$: C, 45.28; H, 3.95; N, 14.80; P, 4.68. Found: C, 45.57; H, 4.45; N, 15.00; P, 4.80.

2',3'-*O*-Isopropylidene Guanosine 5'-Mono-*p*-nitrophenyl Phosphate (IV).—To a solution of 2',3'-*O*-isopropylidene guanosine 5'-di-*p*-nitrophenyl phosphate (III) (180 mg.) in acetone (6 ml.) was slowly added aqueous barium hydroxide (16 ml. of 0.12 *N*) and the total reaction mixture shaken for 30 minutes. A small quantity of unreacted neutral ester, which precipitated, was separated by filtration and retreated with acetone and barium hydroxide as above. The combined solutions were evaporated to dryness *in vacuo* and the residue taken up in water (5 ml.). The solution was neutralized to *p*H 5 with dilute acetic acid and *p*-nitrophenol was extracted with ether until an ether extract gave no yellow color with aqueous alkali. Barium ions were precipitated as barium sulfate by the addition of a stoichiometric amount of dilute sulfuric acid at 0°. The precipitate was removed by filtration and washed with cold water. The filtrate and the washing were lyophilized to give 2',3'-*O*-isopropylidene guanosine 5'-mono-*p*-nitrophenyl phosphate (IV) (155 mg., 85%) as an amorphous white solid which could not be crystallized either as the free acid or as its cyclohexylamine salt. *Anal.* Calcd. for $C_{19}H_{21}N_5O_{10}P\cdot 6H_2O$: P, 4.89. Found: P, 4.88. The ultraviolet absorption spectrum at *p*H 2 showed a maximum at 258 mμ (ϵ_{258} 15,100) with a shoulder at 276 mμ (ϵ_{276} 14,000).

Guanosine 5'-Mono-*p*-nitrophenyl Phosphate (VII).—2',3'-*O*-Isopropylidene guanosine 5'-di-*p*-nitrophenyl phosphate (180 mg.) was hydrolyzed with barium hydroxide in aqueous acetone as above. After extraction of the *p*-nitrophenol with ether, the aqueous solution was adjusted to *p*H 2.7 with acetic acid and held in a boiling water-bath for 1.5 hours. Barium was removed with sulfuric acid and the aqueous solution lyophilized to give guanosine 5'-mono-*p*-nitrophenyl phosphate (VII) as a fluffy white solid (145 mg., 90%) which could not be crystallized. *Anal.* Calcd. for $C_{16}H_{17}N_6O_{10}P.5H_2O$: P, 5.38. Found: P, 5.34.

Guanosine 5'-Phosphate.—2',3'-*O*-Isopropylidene guanosine (1.0 g.) was phosphorylated as described above to give the di-*p*-nitrophenyl phosphate ester III in 96% yield. This product was suspended in dioxane (25 ml.), lithium hydroxide (12 ml. of 1 *N*) was added and the suspension was shaken for ten minutes. More water (12 ml.) was added and the shaking was continued for 30 minutes. The yellow solution was neutralized with hydrochloric acid to *p*H 7–8, a little di-*p*-tolylurea was removed by filtration and the clear solution evaporated under reduced pressure. The residue was treated in either of the following two ways to remove the second *p*-nitrophenyl group.

(a) **Enzymatic Hydrolysis.**—The residue was dissolved in 25 ml. of tris[37]-buffer (0.1 *M*, *p*H 8.8) and filtered to remove a final trace of di-*p*-tolylurea. Magnesium acetate (25 ml. of 0.3 *M*) was added and the total volume brought to 150 ml. with the buffer solution. Crude rattlesnake

(30) K. Paigen, *Anal. Chem.*, **28**, 284 (1956).

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

(32) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

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

(34) O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).

(35) Dried at 110° *in vacuo* over phosphorus pentoxide.

(36) Prolonged heating must be avoided during crystallization since this results in the formation of the cyclonucleoside salt VIII (see below).

(37) Tris-(hydroxymethyl)-aminomethane.

venom[38] (62 mg.) was added and the solution was incubated at 37° for three hours.[39] The solution was cooled to room temperature, adjusted to *p*H 5 with glacial acetic acid and stirred for 20 minutes with 25 ml. of IR-120 ion-exchange resin (ammonium form) to remove magnesium ions. The resin was removed by filtration and washed thoroughly with water (the final volume of the filtrate was 260 ml.). Glacial acetic acid (28 ml.) was added and the solution adjusted to *p*H 2.7 (*p*H meter) by the dropwise addition of concentrated hydrochloric acid. The solution was heated in a boiling water-bath for 1 hour, cooled to room temperature and extracted seven times with 70-ml. portions of ether to remove *p*-nitrophenol. The aqueous solution was then evaporated to dryness under reduced pressure. The residue was taken up in water and adjusted to *p*H 8.5 with 4 *M* lithium hydroxide (total volume of solution *ca.* 25 ml.). Four ml. of 2 *M* barium acetate solution was added and the solution allowed to stand at room temperature for 1 hour. Barium phosphate was removed by centrifugation and the sediment washed with water. To the combined supernatants were added two volumes of 95% ethyl alcohol and the precipitation of the product was completed at −10° (1.5 hours). The barium salt of guanosine 5′-phosphate was collected by centrifugation, washed twice with cold 70% ethyl alcohol, then with 95% ethyl alcohol, acetone and finally ether. It was dried *in vacuo* over phosphorus pentoxide for several hours. The yield of this product which contained 8 moles of water of hydration varied between 1.263 and 1.377 g. (64–75%, based on 2′,3′-*O*-isopropylidene guanosine). *Anal.* Calcd. for $C_{10}H_{12}N_5O_8P\cdot Ba^{++}\cdot 8H_2O$ (mol. wt., 642.7): C, 18.69; H, 4.39; P, 4.82. Found in a reprecipitated sample: C, 18.59; H, 3.55; P, 4.67. A sample (117.48 mg.) of this material was dried overnight *in vacuo* at 100° over phosphorus pentoxide. The weight loss was 25.79 mg., corresponding to 7.8 moles of water. Paper chromatography in several solvent systems, paper electrophoresis (see above) and ion-exchange chromatography showed the sample to be homogeneous. R_f's in solvent systems not mentioned in Table I were as follows: solvent V, 0.63; solvent VI, 0.35; solvent VII, 0.28. Electrophoretic mobility under the conditions described above, 8.2 cm.

The above preparation has been carried out on three times the above scale and has given a 66% yield based on isopropylidene guanosine.

(b) **Alkaline Hydrolysis.**—The residue obtained above after mild alkaline hydrolysis of III was taken up in water (15 ml.), a final trace of di-*p*-tolylurea was removed by filtration and the solution evaporated again. The gummy residue was dissolved in 20 ml. of 1 *N* lithium hydroxide, the solution transferred to a polyethylene tube and heated in a boiling water-bath for 2.5 hours. The solution was cooled, adjusted to *p*H 2.7 with concentrated hydrochloric acid and heated in a boiling water-bath for 1.5 hours to remove the isopropylidene group. After cooling, the acidic solution was extracted repeatedly with ether until an extract gave no yellow color with alkali. The *p*H of the aqueous solution was then brought up to 8.5 with 4 *N* lithium hydroxide and the volume adjusted to *ca.* 25 ml. Four ml. of 2 *M* barium acetate was added and the precipitate (mixture of barium phosphate and some product) was collected by centrifugation and washed with water. Two volumes of ethyl alcohol were added to the combined supernatants and the precipitation was completed by storing at 0° for one hour. The white precipitate was collected by centrifugation, washed twice with 70% ethyl alcohol, once with 95% ethyl alcohol, then acetone and ether and dried at room temperature *in vacuo* over phosphorus pentoxide to give 1.00 g. of barium guanosine 5′-phosphate. Recovery of more product from the first precipitate was tedious, but could be accomplished as follows. The mixture of barium phosphate and guanosine 5′-phosphate was suspended in ice-water, cold hydrochloric acid was added until a clear solution resulted and the acidic solution was again neutralized to *p*H 8.5 with lithium hydroxide. The resulting precipitate was collected and again processed in the manner described above. Addition of two volumes of ethyl alcohol to the combined supernatants gave 163 mg. of an essentially pure product; total yield 1.163 g. (59%). Inorganic phosphate determination showed that this product contained 2.7% barium phosphate. One further reprecipitation gave a pure sample of barium guanosine-5′-phosphate (octahydrate); λ_{max} at *p*H 2, 256 mμ; ϵ_{max} 12,400; λ_{max} at *p*H 12, 260 mμ; ϵ_{max}, 12,100.

Quaternization of 2′,3′-*O*-Isopropylidene Guanosine 5′-Di-*p*-nitrophenyl Phosphate (III) to (VIII). (a) By Heating the Neutral Ester III.—2′,3′-*O*-Isopropylidene guanosine 5′-di-*p*-nitrophenyl phosphate (III) (45 mg.) was dissolved in acetonitrile (10 ml.) and heated under reflux for three hours. On cooling, very fine needles (30 mg.) separated and evaporation of the mother liquor gave more of the same material (10 mg., total yield 90%). This substance decomposed at 261–263°. *Anal.* Calcd. for $C_{25}H_{24}N_7O_{12}P$: C, 46.51; H, 3.75; N, 15.20. Found: C, 46.13; H, 4.10; N, 14.99.

Paper chromatography in four solvent systems (Table I) showed, as expected for the salt VIII, the presence of two spots, one corresponding to di-*p*-nitrophenyl phosphate and the other corresponding, presumably, to the cycloguanosine (R_f's in Table I). The ultraviolet absorption spectrum of the slower moving spot after elution from paper showed a single broad maximum (245–260 mμ) at *p*H 2. The spectra at *p*H 6.8 and 10.7 were essentially identical, there being strong end absorption (below 230 mμ) and a maximum at 266–267 mμ. Paper electrophoresis of VIII at *p*H 3.4 revealed the presence of the anion corresponding to di-*p*-nitrophenyl phosphate and a cation. Electrophoresis at *p*H 7.6, showed the presence of di-*p*-nitrophenyl phosphate ion and a second spot close to the origin and identical in behavior with guanosine, indicating the loss of a proton from the cation of cycloguanosine (VIII).

(b) Sodium benzoxide (40 mg. of sodium in 3 ml. of dry benzyl alcohol) was added to a solution of 2′,3′-*O*-isopropylidene guanosine 5′-di-*p*-nitrophenyl phosphate (300 mg.) in benzyl alcohol (2 ml.) and the resulting orange solution set aside for 15 minutes. Glacial acetic acid (0.15 ml.) was then added to the solution and benzyl alcohol removed under a high vacuum. The oily residue was taken up in 5% aqueous acetic acid, a trace of insoluble material removed by filtration and the solution extracted with ether until an extract gave no yellow color with alkali. Spectrophotometric analysis of the ethereal extracts showed that only 8 mg. of *p*-nitrophenol was present. Paper chromatography of the aqueous solution showed two major spots corresponding to the products obtained above in (a).

Phosphorylation of 2′,3′-*O*-Isopropylidene Guanosine with Phosphorus Oxychloride. (a) Using One Equivalent of Phosphorus Oxychloride.—The phosphorylation was carried out exactly as described by Michelson and Todd[10c] using 1.1 g. of 2′,3′-*O*-isopropylidene guanosine (one-fifth the scale used by the above authors). After the addition of water to the reaction mixture, the *p*H was brought to 9 with 4 *N* lithium hydroxide and the solvent was removed under reduced pressure. The residue was taken up in 50 ml. of 10% acetic acid and the *p*H of the solution adjusted to 2.7 (*p*H meter) by the addition of a little concentrated hydrochloric acid. The clear solution was heated for one hour in a boiling water-bath and then evaporated under reduced pressure. The white gel which resulted was dissolved in 30 ml. of water and the solution was lyophilized to a dry, light yellow powder (2.6 g.). This is designated as Product A.

(b) **Using 2.6 Equivalents of Phosphorus Oxychloride.**—Dry 2′,3′-*O*-isopropylidene guanosine (0.97 g., 3 mmoles) was dissolved in 15 ml. of freshly distilled dimethylformamide and 7 ml. of dry pyridine (distilled over calcium hydride) was added. The solution was cooled to −20° in an ice-salt bath and 0.72 ml. (7.8 mmoles) of freshly distilled phosphorus oxychloride was added in one batch, the temperature of the reaction mixture being maintained between −10 and −20°. Five-ml. aliquots were removed at intervals of 1 and 2 hours. These two aliquots and the remaining reaction mixture (after three hours reaction time) were worked up as described above in (a). These products of one, two and three hour reaction periods are designated as Products B, C and D, respectively.

Analysis of Products. (a) Paper Chromatography and Paper Electrophoresis.—Paper chromatography of Product A in solvents III and V showed the presence of five ultraviolet absorbing components. Three of these components were identified as guanine, guanosine and guanosine 5′-phosphate. The two other phosphorus-containing products corresponded in their R_f values to guanosine 5′-di- and tri-

(38) *Crotalus adamanteus*, Ross Allen's Reptile Farm, Silver Springs, Florida.

(39) Spectrophotometric estimation (440 mμ) of the liberated *p*-nitrophenol indicated 96% hydrolysis.

phosphates.[7] The ultraviolet spectra (obtained by eluting the spots with 0.01 *N* hydrochloric acid) of these products were identical with that of guanosine 5′-phosphate. Hydrolysis with 0.1 *N* hydrochloric acid at 100° for 10 minutes gave, in both cases, guanine.

Paper electrophoresis of Product A revealed the presence of six ultraviolet-absorbing components. Three of these components were identified as guanine, guanosine and guanosine 5′-phosphate. The other three spots had mobilities relative to guanosine 5′-phosphate of 0.6, 1.2 and 1.7, the higher mobilities corresponding to those of guanosine 5′-di- and triphosphate,[7] respectively.

(b) **Ion-exchange Analysis.**—The following standard procedure was employed for quantitative analysis of the products obtained above. A 15-mg. sample of the lyophilized material was dissolved and the solution, after adjusting the *p*H to 8.5 with 1 *N* ammonium hydroxide, was applied to the top of a Dowex 2-chloride form (200–325 mesh) column (3 cm. × 0.8 cm. diameter). The column was washed first with water and then with 0.003 *N* hydrochloric acid to remove guanine and guanosine. Elution was then carried out using the linear gradient technique.[7] Five-ml. fractions were collected, using an automatic fraction cutter, and the optical density of the individual fractions at 260 mμ was determined using a Beckman spectrophotometer, model DU. The two peaks which emerged from the column after guanosine 5′-phosphate (GMP) were designated as G-X and G-Y. These two peaks corresponded in their positions to guanosine 5′-di- and triphosphate,[7] respectively. The results of these analyses are shown in Table II.

TABLE II

ION-EXCHANGE ANALYSIS OF PRODUCTS OF PHOSPHORYLATION OF 2′,3′-*O*-ISOPROPYLIDENE GUANOSINE WITH PHOSPHORUS OXYCHLORIDE

Product	% of total topical density at 260 mμ				
	Guanine	Guanosine	Guanosine 5′-phosphate	G-X	G-Y
A	1	49	31	14	5
B	3	61	17	18	1
C	4	55	23	17	1
D	4	52	19	16	9

Isolation of G-X and G-Y.—One gram of the lyophilized solid (Product A, see above) was triturated twice with 15-ml. portions of methyl alcohol to remove most of the inorganic salt (lithium chloride). The residual solid was dissolved in 25 ml. of water, adjusted to *p*H 8.5 with 1 *N* ammonium hydroxide and applied to a column (4 cm. × 2.2 cm. diameter) of Dowex-2 chloride form. The procedure used was as described in the preparation of guanosine 5′-di- and triphosphates.[7] The following fractions were obtained: guanosine 5′-phosphate; with 0.003 *N* HCl + 0.015 *M* NaCl (1,100 ml., 3,077, O.D. units at 260 mμ); G-X, with 0.003 *N* HCl + 0.1 *M* NaCl (1,000 ml., 1,674, O.D. units); G-Y, with 0.003 *N* HCl + 0.2 *M* NaCl (700 ml., 504, O.D. units). G-X was isolated as the barium salt (78 mg.) as described for guanosine 5′-diphosphate.[7] Since this product was found to be contaminated by inorganic pyrophosphate, purification was carried out as follows. Forty milligrams of the barium salt was shaken with Dowex-50-sodium form until it dissolved. The resin was removed by filtration and washed with water. The total solution (10 ml.) of sodium G-X was adjusted to *p*H 2.7 and stirred with 100 mg. of Darco-G-60 charcoal[40] (95% of the optical density was absorbed). The charcoal was washed with 3 portions of 0.003 *N* HCl to remove the last traces of pyrophosphate and then with three 5-ml. portions of ethyl alcohol–ammonia–water[41] to elute G-X. The ammoniacal solution was evaporated under reduced pressure and the product was precipitated as its barium salt at *p*H 7 from its aqueous solution. One reprecipitation was carried out by dissolving the barium salt in 0.5 ml. of 0.5 *N* HCl at 0° and adding ethyl alcohol.

Characterization of G-X.—(1) The above sample was converted to the sodium salt as described above and examined by paper chromatography and paper electrophoresis. Only one ultraviolet absorbing spot, corresponding to guanosine 5′-diphosphate,[7] could be detected. (2) The guanosine (ultraviolet absorption) to labile phosphorus to total phosphorus was found to be 0.9:1.0:2.0. (3) Tris-buffer (*p*H 7.5) and rattlesnake venom[38] were added to a small portion of the sodium salt solution. The mixture was incubated for 6 hours at 37° and then subjected to chromatography in solvent system III. The original spot of G-X (R_f, 0.12) had completely disappeared and a new spot corresponding to an authentic sample of guanosine (R_f, 0.44) had appeared. (4) The periodate–benzidine spray[42] gave a positive test for vicinal hydroxyl groups in both standard guanosine 5′-diphosphate and G-X.

G-Y was isolated by dissolving the solid obtained by lyophilization of the last ion-exchange fraction in 40 ml. of water and absorbing the nucleotide component on 100 mg. of charcoal (Darco-G-60) in two 50-mg. portions. The charcoal was collected by filtration through Celite, washed as above, and the nucleotidic material then eluted with four 5-ml. portions of 5% aqueous pyridine. The barium salt was prepared in the usual manner. Paper chromatography and paper electrophoresis indicated the presence of a major component, resembling guanosine 5′-diphosphate in behavior and a minor component which behaved like guanosine 5′-triphosphate.[7] Phosphorus analysis gave a ratio of labile to total phosphorus of 2:4.6; theory for guanosine 5′-triphosphate, 2:3.

Acknowledgments.—We wish to thank the National Research Council and the National Cancer Institute of Canada for grants in aid of this work.

(40) D. Lipkin, P. T. Talbert and M. Cohn, THIS JOURNAL, **76**, 2871 (1954).

(41) E. Cabib, L. F. Leloir and C. E. Cardini, *J. Biol. Chem.*, **203**, 1055 (1953).

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

VANCOUVER 8, B.C., CANADA

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

Nucleoside Polyphosphates. V.[1] Syntheses of Guanosine 5′-Di- and Triphosphates

BY ROBERT WARNER CHAMBERS[2] AND H. G. KHORANA

RECEIVED JANUARY 12, 1957

The syntheses of guanosine 5′-di- and triphosphates by the reaction of guanosine 5′-phosphate and 85% phosphoric acid with dicyclohexyl carbodiimide are reported.

The condensation of an unprotected nucleoside 5′-monophosphate with orthophosphoric acid in the presence of a carbodiimide forms the basis of a simple method for the synthesis of nucleoside 5′-polyphosphates. The application of this method to the preparation of ADP, ATP,[3,4] UDP and

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

(2) Life Insurance Medical Research Fund Post-doctoral Fellow, 1954–1956.

(3) The following abbreviations are used: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; GMP, guanosine 5′-monophosphate; GPD, guanosine, 5′-diphosphate; GTP, guanosine 5′-triphosphate; DCC, dicyclohexyl carbodiimide.

(4) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954).