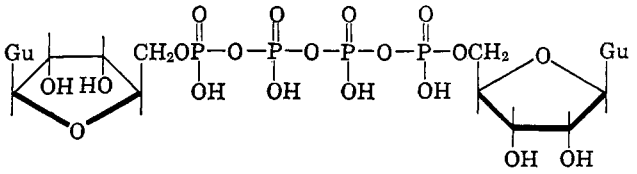
Dismutation Reactions of Nucleoside Polyphosphates. V. Syntheses of P^1,P^4-Di(guanosine-5′) Tetraphosphate and P^1,P^3-Di(guanosine-5′) Triphosphate[1]

A. Adam[2] and J. G. Moffatt

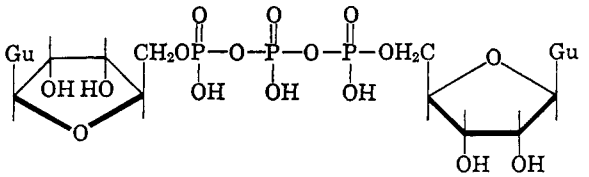
Contribution No. 35 from the Syntex Institute of Molecular Biology, Stanford Industrial Park, Palo Alto, California. *Received October 23, 1965*

Abstract: Chemical syntheses of two unusual, naturally occurring guanosine nucleotides, P^1,P^3-di(guanosine-5′) triphosphate and P^1,P^4-di(guanosine-5′) tetraphosphate, are reported. Condensation of guanosine 5′-phosphoromorpholidate and tributylammonium pyrophosphate (2:1) in pyridine–dimethylformamide gave both these compounds in a single reaction. In addition, two alternative syntheses of P^1,P^3-di(guanosine-5′) triphosphate are reported through condensation of guanosine 5′-phosphoromorpholidate with orthophosphate and through condensation of P^1-(guanosine-5′) P^2-(4-morpholine) pyrophosphate with guanosine 5′-phosphate. Characterization of the various products was accomplished by analytical and enzymatic methods.

In 1963 Finamore and Warner[3] found brine shrimp eggs to be a rich source of guanosine nucleotides. In particular, the unusual nucleotide P^1,P^4-di(guanosine-5′) tetraphosphate (I) was present in amounts up to 0.05% of the dry weight of the eggs. More recently, the related compound P^1,P^3-di(guanosine-5′) triphosphate (II) has also been isolated and characterized from brine shrimp eggs,[4] and both I and II have been isolated from Daphnea eggs.[4a] An enzyme specific for the hydrolysis of I to guanosine 5′-phosphate (GMP)[5] and guanosine 5′-triphosphate (GTP) has been isolated,[6] but as yet there is no indication as to the biological functions of these unusual compounds.



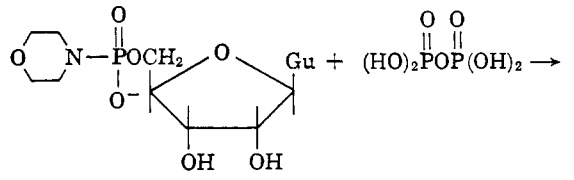
I



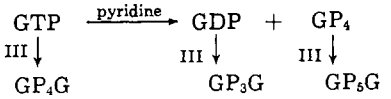
II

We have recently isolated and characterized a homologous series of α,ω-di(adenosine-5′) polyphosphates as minor products arising during dismutation of adenosine 5′-triphosphate in anhydrous pyridine.[7] In addition, we have described the synthesis of these compounds through a direct condensation of adenosine 5′-phosphoromorpholidate with tributylammonium pyrophosphate in anhydrous pyridine.[8] A direct extension of this general reaction to the synthesis of the naturally occurring diguanosine polyphosphates I and II has now been accomplished and is the subject of this paper.

The formation of both I and II in a single reaction proceeds by way of an initial condensation of guanosine 5′-phosphoromorpholidate (III) with pyrophosphate to form guanosine 5′-triphosphate (GTP).[5] In a pyridine-containing solvent, however, GTP undergoes partial dismutation to a mixture of GDP, GTP, and GP_4,[7] each of which is capable of reaction with excess III, giving the dinucleoside polyphosphates II, I, and GP_5G, respectively. Since dinucleoside polyphosphates are not susceptible to dismutation,[8] these products tend to accumulate in the reaction.



III



Unlike the adenosine analog previously studied,[8] the 4-morpholine-N,N′-dicyclohexylcarboxamidine salt of guanosine 5′-phosphoromorpholidate[9] has very limited solubility in anhydrous pyridine. The condensation of 2 equiv of III with 1 of tributylammonium pyrophosphate was accordingly carried out in a mixture of pyridine and dimethylformamide (2:1). An initial homogeneous solution resulted, but a gelatinous precipitate slowly separated and, after 4 days at room temperature, the reaction products were separated by ion-exchange chromatography on a column of DEAE cellulose (HCO_3^-). The elution pattern is shown in

(1) For part IV see W. E. Wehrli and J. G. Moffatt, *J. Am. Chem. Soc.*, 87, 3760 (1965).
(2) On leave from the Centre National de la Recherche Scientifique, Gif-sur-Yvette, France, and holder of a NATO Fellowship.
(3) F. J. Finamore and A. H. Warner, *J. Biol. Chem.*, 238, 344 (1963).
(4) (a) Personal communication from Dr. M. Smith and T. G. Oikawa of the Fisheries Research Board of Canada, Vancouver, B. C.; (b) A. H. Warner and F. J. Finamore, *Federation Proc.*, 24, Part I, 669 (1965).
(5) The abbreviations GMP, GDP, GTP, and GP_4 refer, respectively, to guanosine 5′-mono-, di-, tri-, and tetraphosphate. Similarly, GP_2G, GP_3G, and GP_4G refer to P^1,P^2-di(guanosine-5′) diphosphate, P^1,P^3-di(guanosine-5′) triphosphate (II), and P^1,P^4-di(guanosine-5′) tetraphosphate (I), respectively.
(6) A. H. Warner and F. J. Finamore, *Biochemistry*, 4, 1568 (1965).
(7) D. L. M. Verheyden, W. E. Wehrli, and J. G. Moffatt, *J. Am. Chem. Soc.*, 87, 2257 (1965).
(8) J. R. Reiss and J. G. Moffatt, *J. Org. Chem.*, 30, 3381 (1965).
(9) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 649 (1961).

Table I. Identification of the Peaks in Figure 1

Peak no.	% of total OD	P:guanosine ratios			Structure
		Total P	Labile P	Guanosine	
I	1.2	...	...	1.00	Guanosine
II	11.4	...	...	...	GMP-morpholidate
III[a]	2.3	1.01	0.0	1.00	IV
IV	4.5	0.96	0.0	1.00	GMP
V[a]	7.7	0.99	0.0	1.00	GP_2G
VI	6.3	2.02	1.04	1.00	GDP
VIIa[b]	24.1	2.95	1.99	1.00	GTP
b	9.4	1.49	0.51	1.00	GP_3G
VIIIa[b]	1.1	...	...	...	GTP
b	2.5	3.92	2.94	1.00	GP_4
c	19.4	2.03	1.05	1.00	GP_4G
IX	0.8	Mixture of products; not further studied			

[a] Purified from some inorganic phosphates by preparative paper chromatography. [b] Rechromatographed on a column of DEAE cellulose acetate, pH 5).

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Figure 1, and the identification of the various peaks is summarized in Table I.

The large peaks VII and VIII were found to contain more than one compound and were accordingly rechromatographed on columns of DEAE cellulose (AcO^-) at pH 5, giving the subfractions indicated in Table I. Characterization of the various compounds was accomplished through a combination of chromatographic, analytical, and enzymatic methods. Thus, the simple guanosine 5′-polyphosphates (GMP, GDP, GTP, and GP_4) were readily identified by chromatographic comparison with authentic markers in several solvent systems and by their stepwise degradation (ultimately to guanosine) by *E. coli* alkaline phosphatase.[7,8] The dinucleoside polyphosphates GP_2G, GP_3G, and GP_4G, on the other hand, possess no terminal, monosubstituted phosphate group and are, accordingly, resistant to the action of this enzyme. The extent of such degradations can either be qualitatively assessed by paper chromatographic examination or quantitatively measured by determination of the orthophosphate released.[10] Further confirmation of the structures came from incubation of the various compounds with purified snake venom phosphodiesterase. With this enzyme the nucleoside 5′-polyphosphates were cleaved, at variable rates, between the α and β phosphate groups, giving guanosine 5′-phosphate and an inorganic phosphate or polyphosphate. Treatment of GP_4, for example, gave GMP and tripolyphosphate, both of which could be identified chromatographically. As in the adenosine series,[7,11] GDP proved to be a much poorer substrate for venom diesterase than its higher homologs. The diguanosine polyphosphates proved to be good substrates for this enzyme and were rapidly cleaved to GMP and a guanosine 5′-polyphosphate. The latter compounds were then, as above, more slowly split to GMP and an inorganic phosphate. This method has been applied by Finamore and Warner[3] during characterization of naturally occurring GP_4G. Thus

$$GPPPPG \xrightarrow[\text{diesterase}]{\text{venom}} GP + PPPG \xrightarrow{\text{slower}} GP + PP$$

Both synthetic GP_3G and GP_4G were chromatographically indistinguishable from samples of the naturally occurring materials kindly provided by Dr. Michael Smith of the Fisheries Research Board of Canada.

In general, the products obtained in this reaction are qualitatively similar to those previously reported in the adenosine series. The isolated yields of GP_3G and

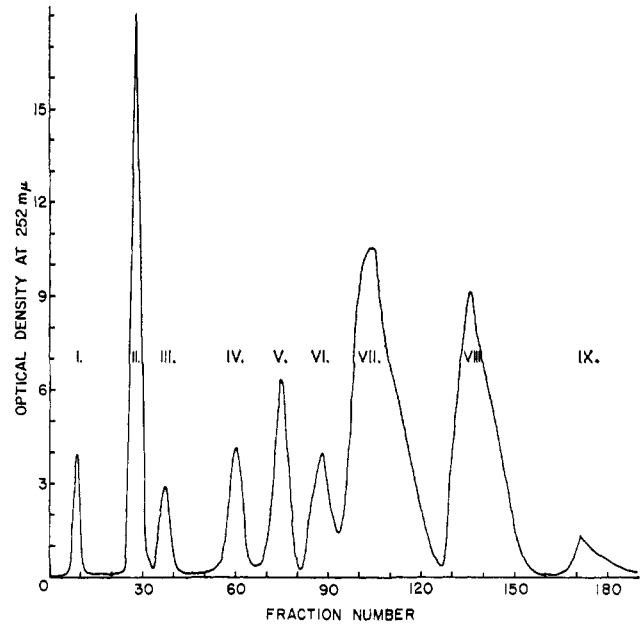
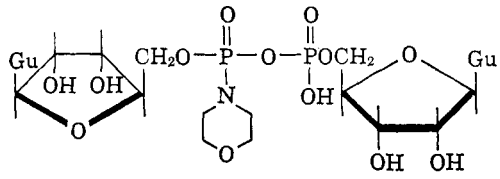


Figure 1. Ion-exchange chromatography of the products from the reaction of GMP-morpholidate and pyrophosphate (2:1) (see Experimental Section for details of the chromatography and identification of the peaks).

GP_4G (9.4 and 19.4%, respectively) are somewhat lower than those for the analogous adenosine derivatives, however. This is probably a consequence of the very limited solubility of the initially formed GTP which tends to precipitate out before it can react with the excess morpholidate. The reaction is, however, sufficiently simple as to make both GP_3G and GP_4G readily available for further study. Only one unusual product was isolated. Thus, the single ultraviolet-absorbing product in peak III, which contained equimolar amounts of guanosine and phosphate, was found to move just slightly faster than GMP on paper chromatograms in solvent II. On paper electrophoresis at either pH 5 or pH 7.5, however, it showed only one-

(10) O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).
(11) W. E. Razzell, *Methods Enzymol.*, **6**, 236 (1963)

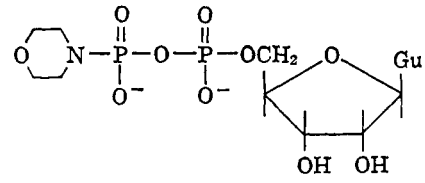
half the mobility of the singly charged marker GMP-morpholidate. The compound was resistant to *E. coli* alkaline phosphatase but was rapidly converted to GMP and GMP-morpholidate by venom phosphodiesterase. These data are compatible with structure IV. The analogous adenosine compound has been identified as the major product arising from the reaction of AMP-morpholidate in pyridine with strong acids,[9] and Hamer[12] has recently obtained a similar derivative by heating solutions of free acid N-cyclohexyl methyl phosphoramidate in inert solvents. In view of the previously observed need for acid catalysis in the formation of such substances, it is difficult to explain the formation of IV in the present reaction.



IV

Two other routes specifically to GP_3G have also been investigated. Thus the direct condensation of 2 equiv of GMP-morpholidate (III) with 1 equiv of tributylammonium orthophosphate was studied in mixtures of pyridine and dimethylformamide (1:1) and in pyridine:dimethyl sulfoxide (1:1). Ion-exchange chromatography of both reactions gave patterns qualitatively similar to that in Figure 1, except that peak VIII (mainly GP_4G) was greatly reduced and amounted to only 1–3% of the total. Rechromatography at pH 5 of peak VII from the pyridine–dimethylformamide reaction showed it to consist of GP_3G and GTP in a ratio of 4:1, the over-all isolated yield of GP_3G being only 11%. No improvement of this yield was achieved using pyridine–dimethyl sulfoxide as solvent, and these reactions show little advantage over that using tributylammonium pyrophosphate which simultaneously gives both GP_3G and GP_4G.

It has previously been shown[13] that the terminal phosphate in adenosine 5′-diphosphate can be selectively activated through formation of its phosphoromorpholidate derivative. We have now applied this reaction in the guanosine series. Guanosine 5′-diphosphate was synthesized in 77% yield by condensation of guanosine 5′-phosphoromorpholidate (III) with tributylammonium orthophosphate in anhydrous dimethyl sulfoxide. The use of dimethyl sulfoxide as the solvent during syntheses of nucleoside diphosphates thus shows distinct advantages over the use of pyridine, particularly when dealing with the highly insoluble guanosine series. A similar, and even more pronounced, advantage has been demonstrated during syntheses of nucleoside 5′-triphosphates[14] where inhibition of dismutation of the product is essential. The guanosine 5′-diphosphate was then allowed to react with morpholine and dicyclohexylcarbodiimide, giving P^1-(guanosine-5′) P^2-(4-morpholine) pyrophosphate (V, GDP-morpholidate) in 63% yield. The only other ultraviolet-absorbing product was GMP-morpholidate (III) which was recovered in 21% yield. The structure of V was confirmed by elemental analysis and by its complete resistance to *E. coli* alkaline phosphatase which showed the morpholine residue to be exclusively located on the β phosphate. Venom phosphodiesterase smoothly hydrolyzed V to GMP.



V

The reaction of V with 4 molar equiv of tributylammonium guanosine 5′-phosphate was carried out in anhydrous dimethyl sulfoxide, and after 4 days the products were separated by ion-exchange chromatography. In addition to unreacted V and GMP, some GDP and a 23% yield of GP_3G were obtained. It is entirely to be expected that, as in the adenosine series, GTP could be efficiently converted into GTP-morpholidate which could be condensed with GMP to provide a selective synthesis of GP_4G. In view of the availability of the latter compound as described earlier in this paper, however, this approach has not been investigated.

Throughout this work extensive use has been made of thin-layer ion-exchange chromatography on glass plates covered with microcrystalline cellulose[15] impregnated with polyethylenimine. This slight modification of the method of Randerath[16] has proved to be extremely valuable since very sharp, well-separated spots can be obtained within 2 hr while comparable separation by paper chromatography required several days. A compilation of the relative mobilities of the various compounds by paper chromatography, paper electrophoresis, and thin-layer ion exchange is to be found in Table II.

Table II. Chromatographic and Electrophoretic Mobilities

Compound	R_{GMP}: Paper chromatography, solvent I	R_{GMP}: Thin-layer ion exchange	R_{GMP}: Paper electrophoresis, pH 3.8
GMP	1.0	1.00	1.00
GDP	0.63	0.43	1.71
GTP	0.44	0.12	2.32
GP_4	0.25	0.05	2.55
GP_2G	0.36	0.85	1.27
GP_3G	0.32	0.50	1.64
GP_4G	0.21	0.20	1.82
GMP-morpholidate	...[a]	1.65	1.00
GDP-morpholidate	...[a]	1.71	1.70
GP_2G-morpholidate (IV)	1.10	1.12	0.53

[a] Partially decomposes in this solvent.

Experimental Section

General Methods. Paper chromatography was carried out by the descending technique on Schleicher and Schuell No. 589 orange ribbon paper using the following systems: solvent I, isobutyric acid–1 *M* ammonium hydroxide–0.1 *M* tetrasodium ethylene-

(12) N. K. Hamer, *J. Chem. Soc.*, 46 (1965).
(13) W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *J. Am. Chem. Soc.*, **87**, 2265 (1965).
(14) J. G. Moffatt, *Can. J. Chem.*, **42**, 599 (1964).
(15) Avicel, obtained from the FMC Corporation, Newark, Del.
(16) K. Randerath and E. Randerath, *J. Chromatog.*, **16**, 111 (1964).

diaminetetraacetic acid (100:60:1.6); solvent II, 1-propanol–28% ammonium hydroxide–water (6:3:1). Paper electrophoresis was carried out on the same type of paper impregnated with either 0.1 *M* ammonium acetate buffer, pH 3.8, or 0.05 *M* ammonium bicarbonate, pH 7.6. A potential difference of 1500 v was generally used for 30–45 min. Thin-layer ion-exchange plates were prepared by spreading a briefly homogenized mixture of Avicel[15] (25 g) dialyzed polyethylenimine[17] (25 ml of a 3% aqueous solution), water (45 ml), and methanol (50 ml) on clean 10 × 20 cm glass plates with a DeSaga applicator.[18] After drying overnight at room temperature, the plates were marked according to Randerath[16] and developed with 1.0 *M* sodium chloride. Phosphorus-containing compounds were visualized on chromatograms using the molybdate spray of Hanes and Isherwood[19] followed by ultraviolet irradiation.[20] Total phosphorus analyses were obtained by the method of King[21] and acid-labile phosphorus by the same method except that digestion with perchloric acid was replaced by treatment with 1 *N* hydrochloric acid at 100° for 8 min. Ultraviolet measurements were made on Zeiss PMQ-II and Cary Model 15 spectrophotometers. Elemental analyses other than for phosphorus were obtained by A. Bernhardt, Mulheim, Germany. All evaporations were carried out at a pressure of roughly 1 mm and a bath temperature of 30°.

Enzyme Degradations. (A). *E. coli* alkaline phosphatase purified by ion-exchange chromatography was obtained from the Worthington Biochemical Corp., Freehold, N. J., and was made up to a concentration of 100 μg/ml in 0.05 *M* Tris buffer, pH 9.

(B). Phosphodiesterase-I from *Crotalus adamanteus* was obtained from the Worthington Biochemical Corp., and was made up to a concentration of 500 μg/ml in 0.05 *M* Tris buffer. This preparation showed negligible 5′-nucleotidase activity.

Guanosine 5′-Phosphoromorpholidate (III). The 4-morpholine-N,N′-dicyclohexylcarboxamidine salt of guanosine 5′-phosphoromorpholidate was prepared in 93% yield as the tetrahydrate as previously reported.[9]

Condensation of Guanosine 5′-Phosphoromorpholidate and Pyrophosphate. 4-Morpholine-N,N′-dicyclohexylcarboxamidinium guanosine 5′-phosphoromorpholidate (800 mg, 1 mmole) was dissolved in anhydrous dimethylformamide[22] (5 ml). After addition of anhydrous pyridine (10 ml) the mixture was evaporated to dryness. This procedure was repeated three times. Separately, tetrakis(tributylammonium) pyrophosphate (0.5 mmole) was prepared as previously described[14] and rendered anhydrous by three evaporations *in vacuo* with 10-ml portions of anhydrous pyridine. The pyrophosphate was then added to the morpholidate in 15 ml of anhydrous pyridine, and the mixture was clarified by the addition of anhydrous dimethylformamide (7.5 ml). On storage at room temperature a gelatinous precipitate started to separate within several hours and after 4 days the solvent was evaporated *in vacuo*. Traces of residual pyridine were removed by several evaporations of the residue with water, and the final aqueous solution was applied to a 2.2 × 45 cm column of DEAE cellulose (HCO_3^-). Elution was effected with a linear gradient of 6 l. of triethylammonium bicarbonate (0.005 to 0.5 *M*) and 25-ml fractions were collected (see Figure 1). The various pooled peaks (Table I) were carefully evaporated to dryness *in vacuo* and freed from residual bicarbonate by four evaporations of the residues with 25-ml portions of methanol. The final residues were dissolved in methanol (5 ml), and a 1 *M* solution of sodium iodide in acetone (100% excess relative to the phosphate anions present) was added, followed by acetone (25 ml). The resulting white precipitates were washed three times with acetone and dried *in vacuo*.

Peaks I, II, IV, and VI were homogeneous and shown analytically (Table I) and chromatographically to be guanosine, GMP-morpholidate, GMP, and GDP, respectively. Peaks III and V were contaminated by small amounts of inorganic phosphates and 500 ODU (252 mμ) portions were purified by preparative paper chromatography in solvent I. The single ultraviolet-absorbing band in each case was eluted with water and characterized analytically (Table I) and enzymatically. Incubation of either material (5 ODU at 252 mμ) with 10 μl of *E. coli* alkaline phosphatase and 5 μl of 1 *M* Tris buffer, pH 9, for 2 hr at 37° led to no degradation. Identical treatment of the substances with 10 μl of phosphodiesterase-I converted the product from peak III into GMP and GMP-morpholidate, and that from peak V into GMP alone. These enzymatic tests together with analytical and chromatographic results identify peaks III and V as compound IV and GP_2G, respectively.

Peaks VII and VIII contained several ultraviolet-absorbing products and were rechromatographed on 2.3 × 33 cm columns of DEAE cellulose (acetate) using linear gradients of 5 l. of ammonium acetate (pH 5) from 0.05 to 0.5 *M*. Rechromatography of peak VII (4000 ODU) separated it cleanly into two peaks, the first (72%) being GTP and the second (28%), GP_3G. The second peak was diluted threefold with water, adjusted to pH 7.5 with ammonia, and passed through a 2 × 5 cm column of DEAE cellulose (HCO_3^-). After a thorough water wash the nucleotide (1120 ODU at 252 mμ) was eluted with 100 ml of 0.5 *M* triethylammonium bicarbonate and isolated as its sodium salt with sodium iodide in acetone as described above. It showed a ratio of total phosphorus:acid labile phosphate:guanosine of 1.49:0.51:1.00 (GP_3G requires 1.50:0.50:1.00). Incubation of 5 ODU of GP_3G with 10 μl of *E. coli* alkaline phosphatase at pH 9 overnight led to no change, while incubation with 10 μl of phosphodiesterase-I gave a mixture of GMP and GDP after 30 mn. Even after 3 hr complete conversion of the GDP to GMP had not resulted.

Rechromatography of peak VIII (3000 ODU at 252 mμ) on DEAE cellulose (acetate) as above gave 5% GTP, 11% GP_4 (total P:labile P:guanosine = 3.92:2.94:1.00), and 84% GP_4G. The latter was diluted four times with water, adjusted to pH 7.5 with ammonia, and desalted by adsorption on a small column of DEAE cellulose (HCO_3^-) and elution with 0.5 *M* triethylammonium bicarbonate as above. Isolation as the sodium salt in the usual way gave 85 mg of a white, chromatographically homogeneous powder which showed a ratio of total P:labile P:guanosine of 2.03:1.05:1.00 (GP_4G requires 2.00:1.00:1.00).

The material (5 ODU) was completely resistant to *E. coli* alkaline phosphatase (10 μl) for 3 hr but was degraded to a mixture of GMP and GTP by treatment with 10 μl of phosphodiesterase-I at pH 9 for 15 min and to GMP and pyrophosphate after 30 min.

The Reaction of GMP-Morpholidate and Orthophosphate (2:1). The 4-morpholine-N,N′-dicyclohexylcarboxamidine salt of guanosine 5′-phosphoromorpholidate (800 mg, 1 mmole) was dried by several evaporations with pyridine and dimethylformamide as above. Separately, 0.5 mmole of bis(tributylammonium) orthophosphate was dried by three evaporations with anhydrous pyridine and the two components were mixed in 30 ml of pyridine–dimethylformamide (2:1). After 4 days at room temperature the solvent was carefully evaporated *in vacuo* and the residue was chromatographed on a 2.2 × 45 cm column of DEAE cellulose (HCO_3^-) using a linear gradient of 6 l. of triethylammonium bicarbonate. Eight peaks were obtained as in the reaction with pyrophosphate above and contained 2.5% guanosine, 34% GMP-morpholidate, 3.5% IV, 8.5% GMP, 9% GP_2G, and 27% GDP in addition to a very small amount (1.5%) of peak VIII (GP_4G + GP_4) and 14% of peak VII. The pooled peak VII was evaporated to dryness and rechromatographed on a 2.2 × 25 cm column of DEAE cellulose (acetate) using a linear gradient of ammonium acetate (4 l. from 0.05 to 0.45 *M*). Two peaks were obtained, desalted, and isolated as the sodium salts as above. The first (20%) was GTP, while the second (80% of peak VII and 11% over-all) was GP_3G. The yield was 25 mg of a product chromatographically, analytically, and enzymatically identical with that described from the pyrophosphate reaction.

Guanosine 5′-Diphosphate. The 4-morpholine-N,N′-dicyclohexylcarboxamidine salt of guanosine 5′-phosphoromorpholidate (2 mmoles) was carefully dried by three evaporations with pyridine–dimethylformamide (10 ml) as above, and then evaporated twice with 10-ml portions of benzene. Separately, bis(tributylammonium) orthophosphate (10 mmoles) was dried by four evaporations with pyridine and two with benzene. The two dried residues were then dissolved in rigorously anhydrous dimethyl sulfoxide (15 ml) and stored at room temperature for 4 days. The mixture was then diluted with water and directly chromatographed on a 3.2 × 30 cm column of DEAE cellulose (HCO_3^-) using a linear gradient of 6 l. of triethylammonium bicarbonate (0.005 to 0.30 *M*). The resulting first peak contained unreacted GMP-morpholidate and a little GMP (3000 ODU at 252 mμ total) together with excess orthophosphate. The second peak contained 21,000 ODU (77%) of chromatographically homogeneous GDP which was evaporated to dryness and freed from bicarbonate by four evaporations with

(17) Chemirad Corporation, East Brunswick, N. J.
(18) Brinkmann Instruments, Menlo Park, Calif.
(19) C. A. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).
(20) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).
(21) E. J. King, *Biochem. J.*, **26**, 292 (1932).
(22) Dimethylformamide was dried by distillation under reduced pressure and storage over Linde Molecular Sieve Type 4A from the Linde Co., Los Angeles, Calif.

methanol. The ratio of total P:acid labile P:guanosine was 1.92:0.94:1.00, and the compound was chromatographically identical with an authentic sample.

P^1-(Guanosine-5′) P^2-(4-Morpholine) Pyrophosphate (V). The GDP directly obtained above (1.5 mmoles) was passed through a column containing 25 ml of Dowex-50 (morpholine form) resin, and the eluates were concentrated to a volume of 20 ml. Morpholine (0.52 ml, 6 mmoles) and *t*-butyl alcohol (20 ml) were added and the mixture was gently refluxed while a solution of dicyclohexylcarbodiimide (2.15 g, 10.5 mmoles) in *t*-butyl alcohol (30 ml) was added dropwise over 4 hr. After a further hour under reflux, the solvent was evaporated to roughly half its volume and the aqueous residue was extracted twice with ether with an intermediate filtration of dicyclohexylurea. The aqueous layer was then applied to a 2.2 × 42 cm column of DEAE cellulose (HCO_3^-), and the column was eluted with a linear gradient of triethylammonium bicarbonate (4 l. from 0.005 to 0.20 *M*). Two peaks resulted; the first (3600 ODU at 252 mμ, 18%) was GMP-morpholidate, while the second (10,800 ODU 53%) was GDP-morpholidate. Some of the early fractions in the second peak were contaminated by a little orthophosphate and these were separately rechromatographed as above. The chromatographically homogeneous GDP-morpholidate was evaporated to dryness and freed from residual bicarbonate by four evaporations with 25-ml portions of methanol. The residue was dissolved in methanol (10 ml) together with free base 4-morpholine-N,N′-dicyclohexylcarboxamidine[9] (600 mg), and the solution was evaporated to dryness. The residue was dissolved in methanol (5 ml), and dry ether (25 ml) was added. The resulting precipitate was washed several times with ether and dried *in vacuo*, giving the di(4-morpholine-N,N′-dicyclohexylcarboxamidine) salt of P^1-(guanosine-5′) P^2-(4-morpholine) pyrophosphate (V) as the tetrahydrate.

Anal. Calcd for $C_{48}H_{84}N_{12}O_{13}P_2 \cdot 4H_2O$: C, 49.21; H, 7.91; N, 14.35; P, 5.28; total P:guanosine, 2.00:1.00. Found: C, 49.07; H, 7.79; N, 14.24; P, 5.23; total P:guanosine, 1.95:1.00.[23]

Condensation of GDP-Morpholidate with GMP. GDP-morpholidate (V, 0.1 mmole) was carefully dried by three evaporations with 10-ml portions of anhydrous pyridine, followed by two evaporations with benzene. Separately tributylammonium guanosine 5′-phosphate (0.4 mmole) was dried by evaporations with pyridine and benzene as above. The two were then combined in anhydrous dimethyl sulfoxide and stored at room temperature for 4 days. Water (25 ml) was then added and the mixture was directly applied to a 2 × 35 cm column of DEAE cellulose (HCO_3^-). After a thorough water wash the column was eluted with a linear gradient of 3.5 l. of triethylammonium bicarbonate (0.005 to 0.5 *M*). Three main peaks were obtained. The first (4600 ODU) was unreacted GDP-morpholidate and excess GMX, the second (665 ODU) was GDP, and the third (640 ODU 23%) was GP_3G. The latter was isolated as its sodium salt in the usual way and was indistinguishable from GP_3G obtained by the other routes above.

(23) Determination of acid labile phosphate was complicated by precipitation of the 4-morpholine-N,N′-dicyclohexylcarboxamidine. The triethylamine salt of GDP-morpholidate directly obtained from the ion-exchange column showed total P:labile P:guanosine = 1.95:0.98:1.00. The guanosine content had to be determined from the ultraviolet absorption at 280 mμ in view of some slight absorption by the carboxamidine salt at the λ_{max} of 252 mμ.

Communications to the Editor

Organic Nitrenes in Single Crystals. Observation of Hyperfine Structure in the Electron Spin Resonance[1]

Sir:

Photolysis of organic azides may be considered to proceed with loss of molecular nitrogen and formation of a monovalent nitrogen radical known as a nitrene. Numerous chemical reactions have been rationalized in terms of this description.[2] Direct physical evidence has been obtained for the existence of alkyl[3] and aryl nitrenes[4] as well as a dinitrene.[5] This was accomplished by observation of the electron spin resonance spectra of their ground-state triplets at −269 and −196°. The nitrenes were generated photochemically in dilute frozen glassy solutions.

In this communication we report the observation by means of esr of triplet nitrenes in single crystals. Also, for the first time, hyperfine structure in nitrenes has been observed. Furthermore, single-crystal nitrenes display unusual thermal stability. As representative materials, *p*-fluorobenzenesulfonyl azide,[6] triphenyltin azide,[7] cyanuric triazide (2,4,6-triazido-*s*-triazine),[8] phenyl azide, and methanesulfonyl azide[9] were photolyzed.

Single crystals of *p*-fluorobenzenesulfonyl azide, triphenyltin azide, and cyanuric triazide were photolyzed at −160° with a medium-pressure mercury arc and the esr spectra were determined using a Varian X-band spectrometer or a Strand Labs K-band spectrometer. Figure 1 shows the spectrum of *p*-fluorobenzenesulfonyl nitrene at a perpendicular orientation of the magnetic field with respect to the sulfur–nitrogen bond axis. Three nitrogen nuclear hyperfine interactions split the electronic line into three equal lines with spacing of about 18 gauss. The single-crystal spectrum exhibited the proper orientational variation for a triplet $S = 1$ nitrene spectrum at 9.2 (and 24.2) kMc/sec. Also the resolved hyperfine interactions showed characteristic intensity variations with orientation in the magnetic field. The zero-field splitting parameters determined for *p*-fluorobenzenesulfonyl nitrene are $D = 1.555$, $E = <0.005$ cm^{-1}. The observation of hyperfine structure agrees with the model assumed by Smolinsky, *et al.*[3]

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(2) For reviews see L. Horner and A. Christmann, *Angew. Chem. Intern. Ed. Engl.*, **2**, 599 (1963); R. A. Abramovitch and B. A. Davis, *Chem. Rev.*, **64**, 149 (1964).

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(6) This compound was prepared by treatment of *p*-fluorobenzenesulfonyl chloride with sodium azide in ethanol.

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