ml). The precipitate was collected by centrifugation and washed with ether three times. The dried precipitate was coevaporated with anhydrous pyridine three times in the presence of pyridinium Dowex 50−X2 (1 g). The residue was allowed to react with DCC (7 mmol) in pyridine (6 ml) under the exclusion of moisture for 4 days at 20°. Aqueous pyridine (50%, 6 ml) was added and the solution was extracted with *n*-hexane. After overnight treatment at room temperature, the aqueous pyridine solution was filtered to remove the urea. The filtrate and washings were evaporated with pyridine and the residue was dissolved in a mixture of pyridine (5 ml) and acetic acid (5 ml). Isoamyl nitrite (1.33 ml) was added and the mixture was kept at 20° for 4 hr. Volatile materials were evaporated and the residue was dissolved in pyridine (5 ml). The nucleotides were precipitated in a mixture of ether (300 ml) and *n*-hexane (100 ml) and collected by centrifugation. The precipitate was dissolved in pyridine and 95% ethyl alcohol (100 ml) and applied to a column (2.7 × 50 cm) of TEAE-cellulose preequilibrated with 95% ethyl alcohol. Elution was carried out using a linear salt gradient of triethylammonium acetate (pH 6.5) in 95% ethyl alcohol (2 l. of 0.05 *M* salt in the mixing vessel and an equal volume of 0.25 *M* salt in the reservoir). The dinucleotide was eluted at salt concentration of 0.18–0.2 *M*. The center of the peak contained 3500 OD_{304} units, 0.2 mmol (28% yield). Treatment with methanolic ammonia gave MMTr-CpCp and the removal of monomethoxytrityl group yielded C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*. The R_f values of these compounds are shown in Table II. Pooled fractions were evaporated and triethylammonium acetate was removed either by precipitation of the anhydrous pyridine solution of the protected nucleotide with ether or extraction of the product with *n*-butyl alcohol from the aqueous buffer solution. Spectral properties of the protected dinucleotide (VI) were λ_{max} 230 (sh), 262, and 304 nm, λ_{min} 249 and 290 nm, and $\epsilon_{304}/\epsilon_{280}$ = 0.81 in ethyl alcohol. Those of CpCp were $\lambda_{max}^{H_2O}$ 265 nm, $\lambda_{min}^{H_2O}$ 235 nm, $\epsilon_{250}/\epsilon_{260}$ = 0.62 in water, and $\lambda_{max}^{pH\ 1}$ 273 nm, $\lambda_{min}^{pH\ 1}$ 237 nm, $\epsilon_{280}/\epsilon_{260}$ = 1.1 at pH 1.

Trinucleotide MMTr-C^{Bz}-OBz-*p*-C^{Bz}-OBz-*p*-A^{Bz}-OBz-*p* (VIII). The pyridinium salt of the dinucleotide VI (3250 OD_{304} units, 0.185 mmol) was allowed to react with pyridinium N,2′-O-dibenzoyladenosine 3′-phosphoranilidate (3110 OD_{280} units) and DCC (2 mmol) in the presence of pyridinium Dowex 50−X2 (0.2 g) in pyridine (1.5 ml) for 6 days at 20°. The aqueous pyridine treatment was as above. The anhydrous mixture was treated with isoamyl nitrite (0.87 ml) in a mixture of pyridine (4 ml) and acetic acid (4 ml) at 20° for 4 hr. After evaporation of the volatile materials the residue was dissolved in pyridine (1.5 ml) and precipitated in ether (100 ml) and *n*-hexane (30 ml). The trinucleotide VIII was isolated by column chromatography using TEAE-cellulose (acetate). The elution conditions and the pattern are shown in Figure 2. The identification of peaks is given in Table I. Peak IV contained almost pure trinucleotide VIII (1090 OD_{304} units, 0.051 mmol). The yield was 28%. The purity of the compound was checked by paper chromatography and electrophoresis with and without protecting groups. The R_f values are given in Table II. Debenzoylation of the trinucleotide yielded MMTr-CpCpAp and a trace of trityl-negative side product. Detritylation of the product (VIII) gave a single spot in paper chromatography and electrophoresis. The completely deprotected trinucleotide CpCpAp (3 OD_{260} units) was hydrolyzed with pancreatic RNase. The ratio of cytidine phosphate and adenosine 3′-phosphate was found to be 2.07:1.00 as estimated spectrophotometrically after paper chromatography in solvent C. Spectral properties of the protected trinucleotide (VIII) were λ_{max} 264, 280 (sh), and 300 (sh) nm, λ_{min} 250 nm, and $\epsilon_{304}/\epsilon_{280}$ = 0.50 in ethyl alcohol. CpCpAp had $\lambda_{max}^{H_2O}$ 264 nm, $\lambda_{min}^{H_2O}$ 234 nm, $\epsilon_{280}/\epsilon_{260}$ = 0.58 in water, and $\lambda_{max}^{pH\ 1}$ 269 nm, $\lambda_{min}^{pH\ 1}$ 236 nm, $\epsilon_{280}/\epsilon_{260}$ = 0.93 at pH 1.

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Studies on Transfer Ribonucleic Acids and Related Compounds. II. A Method for Synthesis of Protected Ribooligonucleotides Using a Ribonuclease

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Abstract: A protected trinucleotide 3′-phosphate, 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidylyl-(3′–5′)-N,2′-O-diacetylguanylyl-(3′–5′)-2′-O-acetyluridine 3′-phosphate, was synthesized using the 2′,3′-cyclic phosphate as protection for the terminal phosphomonoester and the 2′-hydroxyl group. The synthetic steps involved (1) the condensation of uridine 2′(3′)-phosphate with 5′-O-monomethoxytrityl-N,2′-diacetylguanosine 3′-phosphate using dicyclohexylcarbodiimide, (2) acidic removal of the monomethoxytrityl group, and (3) condensation with 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate. The terminal uridine cyclic phosphate was hydrolyzed with pancreatic RNase and the 2′-hydroxyl group was acetylated. The overall yield of the protected trinucleotide from the protected mononucleotide was 10%. Methods for the synthesis of protected guanosine 3′-phosphate using an extraction procedure are described.

Suitably protected ribooligonucleotides are required as key intermediates for chemical synthesis of ribonucleotides. The preceding paper reports the synthesis of a protected trinucleotide with 3′-phosphate using aromatic phosphoramidates as a protecting group for phosphomonoesters.[1] In this paper, another approach, one using a 2′,3′-cyclic phosphate as protection for the hydroxyl and phosphomonoester groups, is used to synthesize the protected trinucleotide 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidylyl-(3′–5′)-N,2′-O-diacetylguanylyl-(3′–5′)-2′-O-acetyluridine 3′-phosphate (MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-OAc-*p*).[2] The trinucleotide, CpGpUp, is the third triplet from the 3′ end of yeast alanine $tRNA_1$.[3] For

(1) E. Ohtsuka, K. Murao, M. Ubasawa, and M. Ikehara, *J. Amer. Chem. Soc.*, **92**, 3441 (1970).

(2) The system of abbreviation is the same as described in the preceding paper.[1]

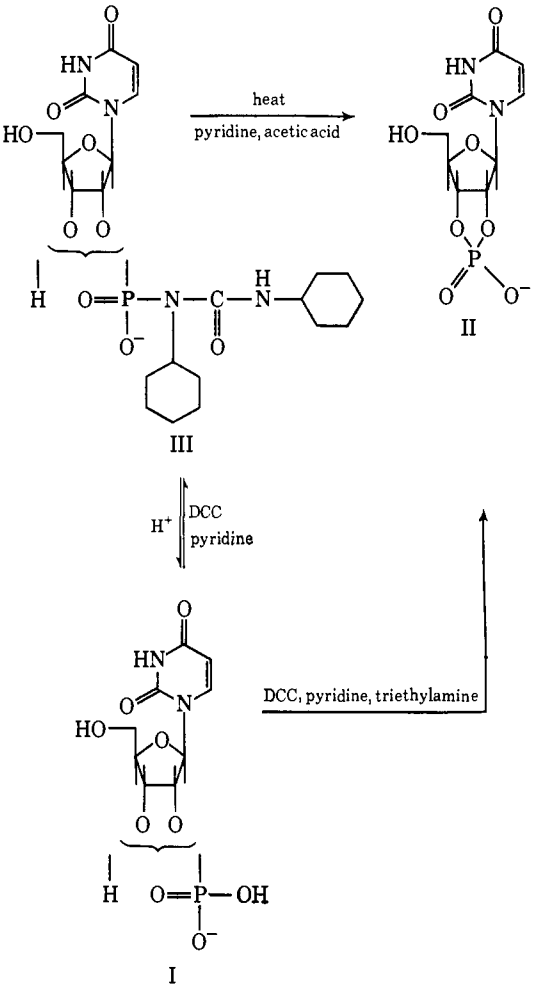
the chemical synthesis of the polynucleotide corresponding to the 3′ end of the RNA using blockwise condensation, this blocked trinucleotide would be needed in substantial amounts since it must be used in excess in subsequent condensations.

Söll and Khorana reported the synthesis of ribodinucleotides with a pyrimidine nucleotide at the 3′ end.[4] The 2′,3′-cyclic phosphate of uridine was used for protection of the 2′-hydroxyl and 3′-phosphate. This cyclic phosphate was hydrolyzed by pancreatic RNase after the condensation. Similar use of cyclic phosphates on blocked nucleosides is more problematic: the rate of hydrolysis of N-anisoyl and N-benzoylcytidine 2′,3′-cyclic phosphate with RNase M[5] was too slow for preparative purposes.[6] Similar results were obtained with N-benzoyladenosine 2′,3′-cyclic phosphate and N-acetylguanosine 2′,3′-cyclic phosphate using RNase M and RNase T_1, respectively.[6] Therefore, the base of the terminal nucleotide must be deblocked without damage to internal 2′-O-acyl groups if protected oligonucleotides with 3′-phosphate are to be synthesized *via* cyclic phosphate formation. Fortunately, the present trinucleotide block contains uridine at the 3′ terminus, making RNase hydrolysis applicable without further manipulation.

The protection of the trinucleotide CpGpUp was designed in such a way that the 5′-hydroxyl group could be deprotected selectively after the condensation of the trinucleotide with the growing chain. The monomethoxytrityl group was chosen as protection for the 5′-hydroxyl group of the block because of its selectivity and stability.[7] Heterocyclic amino groups and the 2′-hydroxyl group were protected by acylation. The 2′-hydroxyl group was acylated with acid anhydrides in the presence of acyl anions to prevent the formation of the cyclic phosphate.[8]

It was reported that five-membered cyclic phosphates react with dicyclohexylcarbodiimide (DCC) to yield phosphorylureas, which can be degraded by treatment with acid.[9] The phosphorylurea of uridine 2′(3′)-phosphate and the oligonucleotide would be formed during the condensation using DCC. In the presence of 5′-O-monomethoxytrityl and acyl groups, the phosphorylurea of the trinucleotide has to be decomposed selectively. We have found that a mixture of pyridine and acetic acid (1:1) degraded N-(uridylyl)-N,N′-dicyclohexylurea (III) mostly to the 2′,3′-cyclic phosphate (II) and to a small amount of the 2′(3′)-phosphate (I). The decomposition and formation of the phosphorylurea are shown in Chart I. Uridine 2′(3′)-phosphate (I) could be cyclized without forming the phosphorylurea in the presence of alkyl amines.[9] Rhaese, *et al.*, used tetraethylammonium salt during the condensation with DCC for the synthesis of di-

Chart I. Formation and Decomposition of the Phosphorylurea



nucleotides.[10] However, alkylamines reduce the rate and extent of the DCC reaction.[11]

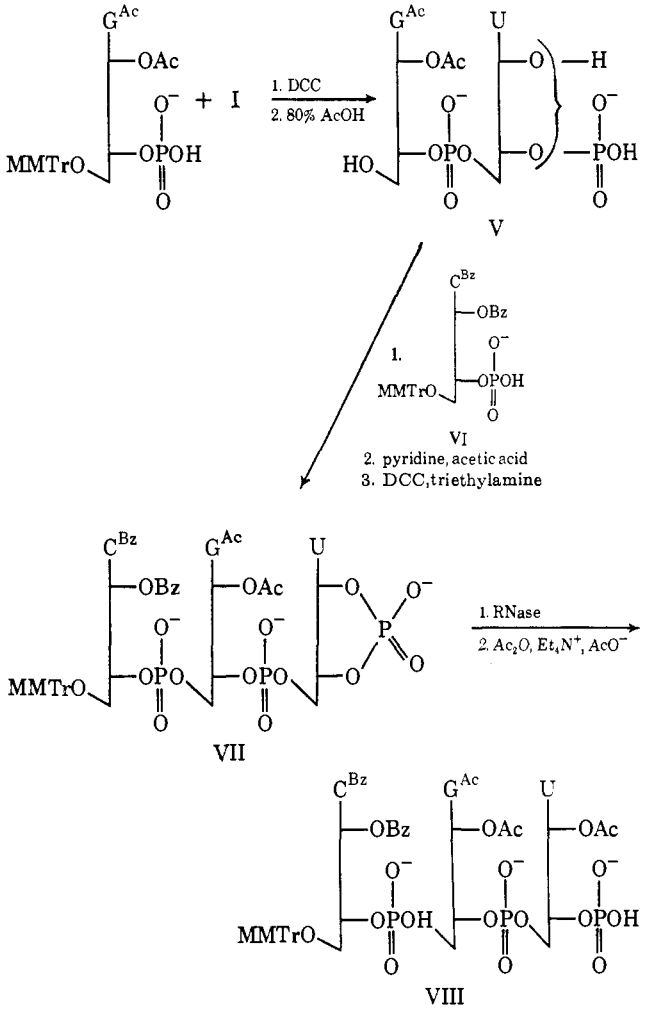
When pyridinium uridine 2′(3′)-phosphate was treated with DCC in pyridine for 20 hr, 70% of the starting material was converted to the phosphorylurea III and the rest remained as the cyclic phosphate II. The phosphorylurea III was not degraded with isoamyl nitrite because of the lack of hydrogen on phosphoryl nitrogen. However, the acidic buffer, a 1:1 mixture of acetic acid and pyridine, used as a medium for the isoamyl nitrite reaction with phosphoramidate, was found to degrade the phosphorylurea mainly to the cyclic phosphate (Figure 1). Detritylation of 5′-O-monomethoxytritylcytidine 3′-phosphate was not detected during degradation of the corresponding phosphorylurea.

Using this procedure, the trinucleotide, MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-OAc-*p*, was synthesized as shown in Chart II. For this approach the preparation of the protected mononucleotide was studied.

The Protected Mononucleotide, 5′-O-Monomethoxytrityl-N,2′-O-diacetylguanosine 3′-Phosphate. The synthesis of 5′-O-monomethoxytrityl-N,2′-O-diacetylguanosine 3′-phosphate has previously been reported.[12] Column chromatography on DEAE–cellulose was used to isolate 5′-O-monomethoxytritylguanosine 3′-phos-

(3) R. W. Holley, J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merril, J. R. Penswick, and A. Zamir, *Science*, **147**, 1462 (1965).
(4) D. Söll and H. G. Khorana, *J. Amer. Chem. Soc.*, **87**, 350 (1965).
(5) (a) M. Irie, *J. Biochem.* (Tokyo), **62**, 509 (1967); (b) M. Imazawa, M. Irie, and T. Ukita, *ibid.*, **64**, 595 (1968).
(6) Unpublished work by E. Ohtsuka and M. Ikehara. RNase M was a generous gift of Dr. M. Irie of the University of Kyoto.
(7) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Amer. Chem. Soc.*, **84**, 430 (1962).
(8) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963).
(9) (a) C. A. Dekker and H. G. Khorana, *ibid.*, **76**, 3522 (1954); (b) G. M. Tener and H. G. Khorana, *ibid.*, **77**, 5348 (1955); (c) H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *ibid.*, **79**, 430 (1957).
(10) H. J. Rhaese, W. Siehr, and F. Cramer, *Ann.*, **703**, 215 (1967).
(11) P. T. Gilham and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6212 (1958).
(12) R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *ibid.*, **88**, 819 (1966).

Chart II. Synthesis of the Fully Protected Trinucleotide



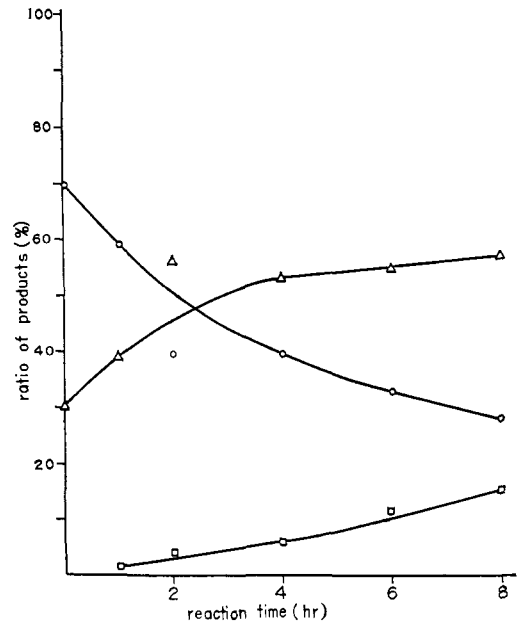


Figure 1. Degradation of N-(uridylyl)-N,N′-dicyclohexylurea in a mixture of pyridine and acetic acid (1:1) at 40°. For the reaction condition, see the Experimental Section. Circles represent N-(uridylyl)-N,N′-dicyclohexylurea (III), triangles, uridine 2′,3′-cyclic phosphate, and squares, uridine 2′(3′)-phosphate.

phate. In this paper, we report two different approaches for synthesizing this compound. Guanosine or guanosine 3′-phosphate was used as the starting material and the intermediates were purified without column chromatography.

As shown in Chart III, the amino group of guanosine was protected with dimethylformamide dimethylacetal[13] to yield the dimethylaminomethylene derivative[14] (X) which was then used for monomethoxytritylation. 5′-O-Monomethoxytrityl-N-dimethylaminomethyleneguanosine (XI, 55% yield from guanosine) was phosphorylated with morpholinophosphorodichloridate[15] and cyclized with dilute ammonia.[16] The dimethylaminomethylene group was removed using methanolic ammonia. Since the cyclic phosphate was partially hydrolyzed during the methanolic ammonia treatment, the mixture was treated with DCC in the presence of triethylamine. The yield of the cyclic phosphate XII from XI was 94%. XII was then hydrolyzed with RNase T_1 to yield the phosphate XIII, which was acetylated with acetic anhydride in tetraethylammonium acetate.[12] The overall yield was 95% from XII. This method may be generally useful when a nucleoside is available providing the 2′,3′-cyclic phosphate of the nucleoside is susceptible to a nuclease.

The other approach, starting from guanosine 3′-phosphate,[17] is shown in Chart IV. The amino group of guanosine 3′-phosphate (XV) was protected to give the N-dimethylaminomethyleneguanosine 3′-phosphate[18] (XVI) and the product was allowed to react with monomethoxytrityl chloride without purification. After removal of the dimethylaminomethylene group with ammonia, the ditritylated compound (XVII) was removed by extraction with a mixture of chloroform and isoamyl alcohol. The 5′-O-monomethoxytrityl-guanosine 3′-phosphate (XIII) was then obtained by extraction with a mixture of *n*-butyl alcohol and ethyl acetate. The yield of XIII from XV was 80%. XIII was acetylated as above.

Synthesis of the Partially Protected Dinucleotide, G^{Ac}-OAc-*p*-Up-2′(3′) (V). The protected mononucleotide (XIV) was condensed with uridine 2′(3′)-phosphate using DCC as shown in Chart II. Uridine 2′(3′)-phosphate was converted to the cyclic phosphate immediately and the formation of N-(uridyl)-N,N′-dicyclohexylurea (III) occurred gradually during the condensation. After condensation, the monomethoxytrityl group, cyclic phosphates, and phosphorylureas were hydrolyzed with 80% acetic acid for 2 hr. The dinucleotide V was isolated by anion-exchange chromagraphy on TEAE-cellulose. The elution pattern and the conditions are shown in Figure 2. The yield of the dinucleotide (peak IV) was 52%. The purity of the product was checked by paper chromatography and paper electrophoresis. R_f values of the protected and

(13) (a) H. Bredereck, F. Effenberger, and G. Simchen, *Angew. Chem.*, 73, 493 (1961); (b) Z. Arnold and M. Kornilov, *Collect. Czech. Chem. Commun.*, **29**, 645 (1964).

(14) J. Zemlicka and A. Holly, *ibid.*, **32**, 3159 (1967).

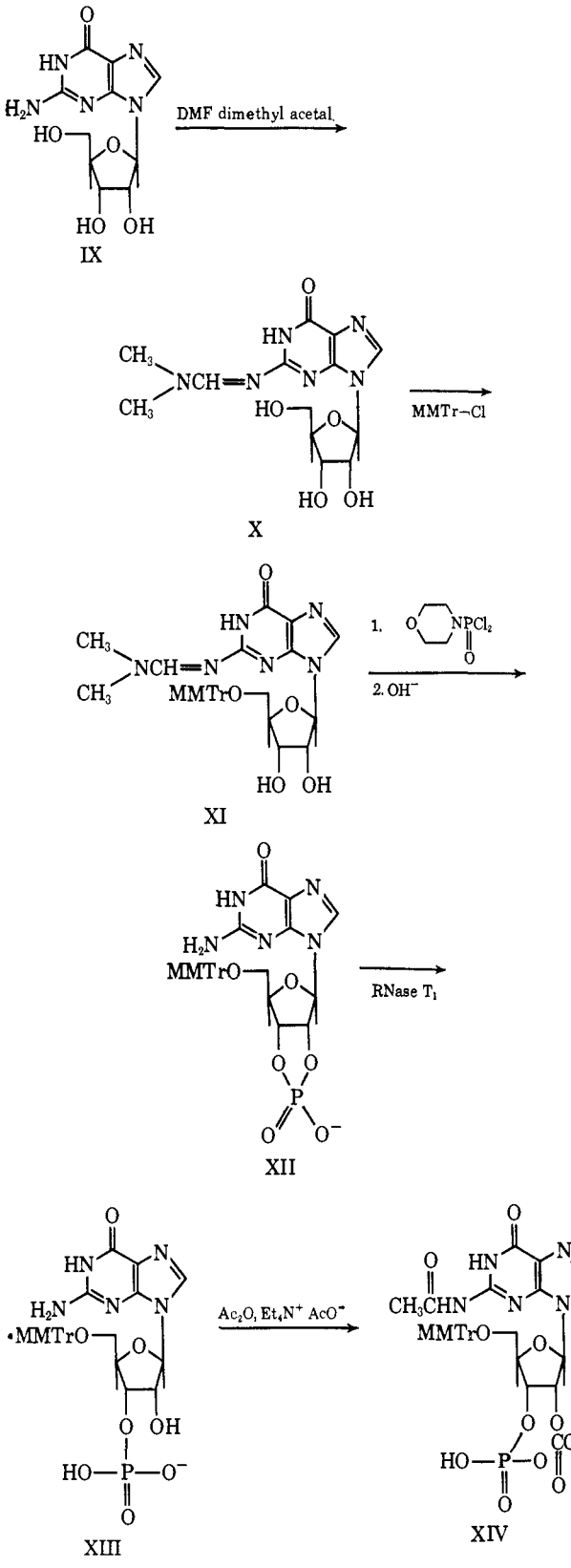
(15) M. Ikehara and E. Ohtsuka, *Chem. Pharm. Bull.* (Tokyo), **11**, 435 (1963).

(16) (a) M. Ikehara and I. Tazawa, *J. Org. Chem.*, **31**, 819 (1966); (b) M. Ikehara and I. Tazawa in "Synthetic Procedure in Nucleic Acid Chemistry," W. W. Sorbach and R. S. Tipson, Ed., Interscience Publishers, New York, N. Y., 1968, p 445.

(17) R. Lohrmann and H. G. Khorana, *J. Amer. Chem. Soc.*, **86**, 4188 (1964).

(18) A. Holy, S. Chladek, and J. Zemlicka, *Collect. Czech. Chem. Commun.*, **34**, 253 (1969).

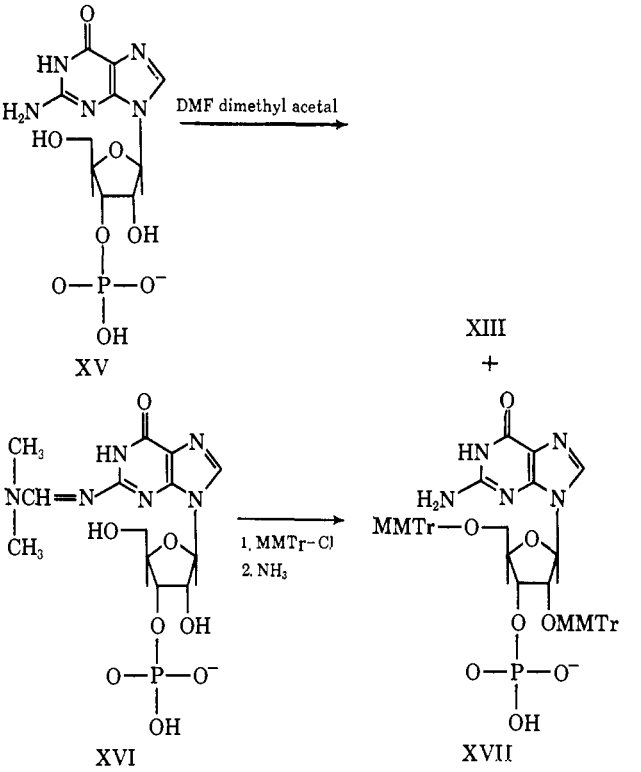
Chart III. Synthesis of the Protected Mononucleotide from the Nucleoside



deprotected dinucleotide are given in Table I. The unprotected dinucleotide, GpUp-2′(3′), was completely hydrolyzed by RNase M to give Gp and Up-2′(3′).

Synthesis of the Fully Protected Trinucleotide, MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-OAc-*p* (VIII). The dinucleotide V and 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate (VI) were treated with DCC in

Chart IV. Protection of the Mononucleotide



pyridine. Compound VI was synthesized by a method similar to the one used for preparation of the dimethoxytrityl derivative.[1,12] The compound with terminal phosphorylurea was converted to the corresponding cyclic phosphate by treatment with pyridine–acetic acid at 40° for 5 hr. When hydrolysis of the cyclic phosphate was detected, the mixture was treated with DCC in pyridine in the presence of trialkylamines to ensure the formation of cyclic phosphates. The trinucleotide with 2′,3′-cyclic phosphate VII was isolated by column chromatography using TEAE–cellulose. The elution pattern and conditions are shown in Figure 3. Peak IV contained the product VII. The yield was 17% from the dinucleotide neglecting hypochromicity. The low yield may be due to the formation of higher compounds (peaks V and VI). One of the major compounds from peaks V and VI was estimated as the polymerized product of the dinucleotide V. RNase M hydrolysis of the unprotected higher compound gave Gp, Up, and an undigested dinucleotide, probably uridylyl-(2′–5′)-guanosine 3′-phosphate. The cyclic phosphate (VII) was hydrolyzed with pancreatic RNase in aqueous DMF and the 2′-hydroxyl group of the product was acetylated with acetic anhydride in tetraethylammonium acetate to yield VIII. R_f values of the protected and unprotected trinucleotide are given in Table I. The completely unprotected trinucleotide, CpGpUp, was hydrolyzed by RNase M to give the expected mononucleotide in the correct ratio.

General Comments

The protected trinucleotide, MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-OAc, was obtained using a combination of chemical and enzymatic procedures. Although the method was applied to the synthesis of a ribooligonucleotide bearing a terminal uridine 3′-phosphate, it is

Table I. Paper Chromatography and Electrophoresis

Compounds	Paper chromatography, R_f Solvent A	Solvent C	Solvent D	Paper electrophoresis, relative mobility to Cp pH 7.5	pH 3.5
Up	0.08	0.29	0.49	1.0	1.0
Gp	0.05	0.17	0.29	0.91	0.90
Cp	0.08	0.27	0.50	0.91	0.80
GpUp 2′(3′)	0.03		0.24		
CpGpUp	0.07 (R_{Cp})		0.17		
MMTr-Gp	0.31	0.46		0.70	
MMTr-G-cyclic *p*	0.70	0.80		0.38	
MMTr-Cp		0.68		0.73	
MMTr-CpGpUp	0.06	0.21	0.22		
MMTr-CpGpU-cyclic *p*		0.27			
G^{Ac}-OAc-*p*-Up2′(3′)		0.22			
C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-OAc-*p*		0.35			
MMTr-G^{Ac}-OAc-*p*		0.78		0.63	
MMTr-C^{Bz}-OBz-*p*		0.88			
MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-Up				0.83	
MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-cyclic *p*				0.64	
MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-OAc-*p*		0.83		0.73	

possible to extend the enzymatic hydrolysis of the terminal cyclic phosphate to the synthesis of protected oligonucleotides with different bases at the 3′ end. However, protecting groups which can be selectively removed from the 3′-terminal nucleotide must be devised. Otherwise the enzymatic cleavage would be very slow. Letsinger's new procedure of selective N-debenzoylation using hydrazin[19] would be suitable for the synthesis of oligonucleotides having cytidine or adenosine at the 3′ end. Even if N-protecting groups of the oligonucleotide are removed, the 2′-O-acyl groups prevent the enzymatic hydrolysis of internucleotide linkages. This method does not require the protection of phosphomonoesters. On the other hand, a relatively large number of steps is involved after forming the internucleotide bond. Comparison with another approach[1] toward a general synthesis of protected ribooligonucleotides and studies with different protecting groups are in progress.

(19) R. L. Letsinger, P. S. Miller, and G. W. Grams, *Tetrahedron Lett.*, 2621 (1968).

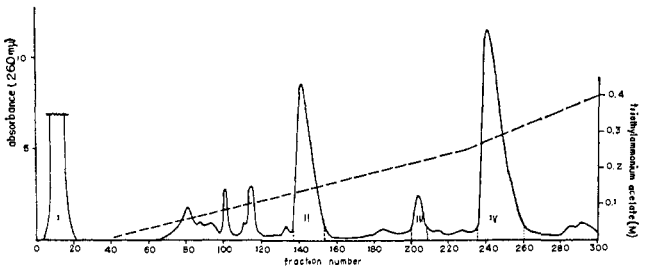


Figure 2. Chromatography of the products obtained in the synthesis of the partially protected dinucleotide, G^{Ac}-OAc-*p*-Up2′(3′) (V), on a column (2.3 × 34 cm) of TEAE-cellulose (acetate) preequilibrated with 0.005 *M* triethylammonium acetate in 30% ethyl alcohol. The column was washed with the equilibration buffer. Elution was carried out using a linear salt gradient of triethylammonium acetate, pH 6.5 in 30% ethyl alcohol (2 l. of 0.005 *M* salt in the mixing vessel and the equal volume of 0.25 *M* salt in the reservoir, followed by the elution using 2 l. of 0.25 *M* salt and 2 l. of 0.4 *M* salt). Fractions of 19 ml were collected every 15 min. Peak II was identified as Up and peak III was the dinucleotide with terminal 2′,3′-cyclic phosphate. Peak IV contained the product, G^{Ac}-OAc-*p*-Up2′(3′).

Experimental Section

General Methods. Paper chromatography was performed on Toyoroshi No. 51A paper using the descending technique unless otherwise mentioned: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent B (ascending), *n*-butyl alcohol–water (86:14, v/v); solvent C; ethyl alcohol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v); solvent D, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v). Paper electrophoresis was performed at 900 V/40 cm using 0.05 *M* triethylammonium bicarbonate (pH 7.5) or 0.05 *M* ammonium formate (pH 3.5).

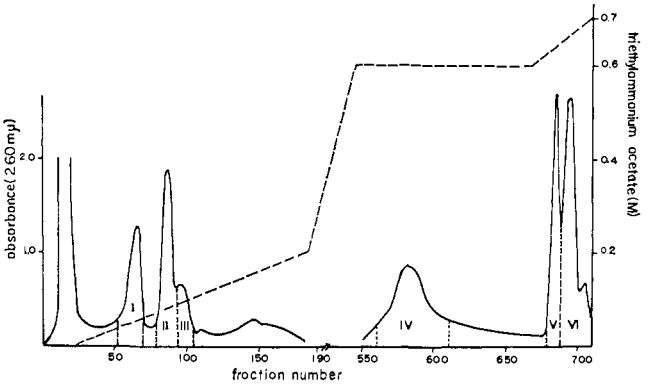


Figure 3. Chromatography of the products obtained in the synthesis of the trinucleotide, MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-U-cyclic *p* (VII), on a column (3.0 × 32 cm) of TEAE-cellulose (acetate) preequilibrated with 0.005 *M* triethylammonium acetate, pH 6.5 in 95% ethyl alcohol. Elution was performed using a linear salt gradient of triethylammonium acetate from 0.005 *M* to 0.6 *M* in 95% ethyl alcohol. The total volume was 12 l. After fraction 547, 0.6 *M* salt in 90% ethyl alcohol was used. Fractions 669–708 were eluted with a salt gradient using 0.6 *M* salt (1 l.) and 0.7 *M* salt (1 l.). Fractions of 20 ml were collected every 15 min. Peaks I and II contained MMTr-C^{Bz}-OBz-*p* and the pyrophosphate of this mononucleotide, respectively. Peak III was characterized as C^{Bz}-OBz-*p*. Peak IV contained the product VII. Peak V and VI are not identified (see text for an estimated structure of the higher compound).

The molar extinction values for the nucleotides are as follows: Up, 10,000 (260 nm at pH 2); Cp, 13,000 (280 nm at pH 2); Gp, 11,300 (260 nm at pH 2); MMTr-C^{Bz}-OBz-*p*, 29,000 (260 nm); G^{Ac}-OAc-*p*, 16,700 (260 nm); G^{Ac}-OAc-*p*-Up, 21,300 (260 nm); MMTr-C^{Bz}-OBz-*p*-G^{Ac}-OAc-*p*-Up, 50,300 (260 nm).

Enzymatic hydrolyses and other general procedures are the same as described in the preceding paper.[1]

Decomposition of N-(Uridylyl)-N,N′-dicyclohexylurea. Pyridinium uridine 2′(3′)-phosphate (0.2 mmol) was treated with DCC (1 mmol) in pyridine (4 ml) for 21 hr at room temperature. Water (10 ml) was added and the aqueous pyridine solution was extracted with ether after the filtration. The solution was rendered anhydrous by repeated evaporation with added pyridine. The mixture contained N-(uridilyl)-N,N′-dicyclohexylurea (70%) and uridine 2′,3′-cyclic phosphate (30%) as determined by paper chromatography. The mixture (50 OD_{260} units) was dissolved in a 1:1 mixture of pyridine and acetic acid (0.2 ml), and the solution was kept at 40° in a stoppered tube. Aliquots (20 μl) were taken in intervals as shown in Figure 1. Products were analyzed by paper chromatography (solvent A or D).

N-Dimethylaminomethylene-5′-O-monomethoxytritylguanosine (XI). N-Dimethylaminomethylene guanosine was synthesized from guanosine (2.83 g, 10 mmol) with DMF dimethyl acetal (5 ml) in DMF (50 ml) under stirring at 70° for 30 min and at room temperature for 5 hr. Thin layer chromatography on silica gel (chloroform:ethyl alcohol, 1:1) showed no starting material at this stage. The volatile materials were evaporated *in vacuo* and an aliquot was crystallized from DMF for identification [mp 246–248°, lit.[14] 247–248°; R_f 0.12 (solvent B), 0.22 (solvent A, ascending); $\lambda_{max}^{H_2O}$ 302 nm] and the rest was allowed to react with monomethoxytrityl chloride (3.4 g, 11 mmol) in DMF (25 ml) and pyridine (25 ml) for 2 days with shaking at room temperature. Monomethoxytrityl chloride (1 mmol) was further added as the starting material was detected by thin layer chromatography (chloroform:ethyl alcohol, 5:1, v/v). After 16 hr the reaction mixture was added to ice–water (200 ml) and extracted with chloroform (40 ml) for four times. The product was crystallized from chloroform. The yield was 55%: mp 204°; λ_{max}^{EtOH} 235 and 305 nm; R_f 0.78 (solvent A and B).

Anal. Calcd for $C_{33}H_{34}N_6O_6$: C, 64.90; H, 5.61; N, 13.76. Found: C, 65.14; H, 5.49; N, 13.96.

Ammonium 5′-O-Monomethoxytritylguanosine 2′,3′-Cyclic Phosphate (XII). N-Dimethylaminomethylene 5′-O-monomethoxytritylguanosine (XI) (1.83 g, 3 mmol) was dried *in vacuo* at 100° for 5 hr and suspended in dioxane (20 ml) and DMF (10 ml). The suspension was added to a mixture of morpholinophosphorodichloridate (1.22 g, 6 mmol) and 2,6-lutidine (1.36 ml) in dioxane (1 ml). The mixture was stirred at room temperature for 6 days. The reaction extent was checked by paper electrophoresis after treatment of an aliquot with 80% acetic acid. The reaction mixture was poured into a mixture of 0.05 *N* ammonium hydroxide and DMF (100 ml) and stirred overnight. Morpholine was extracted with ether and the solution was filtered to remove a precipitate. The nucleotide was extracted with *n*-butyl alcohol (500 ml) and the solution was evaporated with added pyridine. The residue was treated with methanolic ammonia for 21 hr and evaporated to a syrup. The syrup was allowed to react with DCC (6.18 g) in pyridine (75 ml) and triethylamine (3.75 ml) at room temperature for 24 hr. Water (200 ml) was added to the mixture and extracted with ether (40 ml) five times. The solution was filtered to remove dicyclohexylureas and evaporated with pyridine. The residue was dissolved in anhydrous pyridine and added to ether with stirring. The precipitate was collected by centrifugation and washed with ether three times. The yield was 31.8×10^3 OD_{260} units (2.8 mmol), 94%; $\lambda_{max}^{H_2O}$ 255 nm; R_f values are given in Table I.

Pyridinium 5′-O-Monomethoxytritylguanosine 3′-Phosphate (XIII). The triethylammonium salt of XII (31.8×10^3 OD_{26} units, 2.8 mmol) was dissolved in 1 *N* ammonium hydroxide (12 ml), DMF (30 ml), and water (60 ml). The solution was adjusted to pH 7.5 immediately by introducing carbon dioxide gas. RNase T_1 (13.2 mg/ml, 6 ml) was added and pH of the solution was kept at pH 7.5 using a pH stat. After 20 hr at 37° the enzyme solution was added (5 ml) and incubation was continued for 24 hr at 37°. The completely hydrolyzed compound was checked by paper electrophoresis and the enzyme was removed by shaking with a mixture of chloroform and isoamyl alcohol (2:1, v/v). The aqueous layer was made a 10% pyridine solution and passed through a column (1.9×20 cm) of ion-exchange resin pyridinium Dowex 50X2. The column was washed with 10% pyridine. The effluent and washings were evaporated with added pyridine. The yield was 30×10^3 OD_{260} units, 95%. The R_f's are shown in Table I.

Pyridinium 5′-O-Monomethoxytrityl-N,2′-O-diacetylguanosine 3′-Phosphate (XIV). Pyridinium salt of XIII (25,1000 OD_{260} units, 2.2 mmol) and tetraethylammonium acetate (22 mmol) were rendered anhydrous by evaporation with pyridine for four times. The residue was evaporated with toluene six times and dried over phosphorus pentoxide for 30 min at room temperature. Acetic anhydride (4.2 ml, 44 mmol) was added to the mixture and kept at room temperature for 13 days in the dark. Cooled 50% aqueous pyridine (40 ml) was added in an ice bath and the solution was passed through a column (2.5×25 cm) of pyridinium Dowex 50X2. The column was washed with 50% pyridine. The effluent and the washings were kept for 2 hr at room temperature and evaporated with added pyridine. The anhydrous pyridine solution was added to ether by vigorous stirring and the precipitate was collected by centrifugation. The yield was almost quantitative.

4-Morpholine N,N′-Dicyclohexylcarboxamidinium 5′-O-Monomethoxytritylguanosine 3′-Phosphate (XIII) from Guanosine 3′-Phosphate. 4-Morpholine N,N′-dicyclohexylcarboxamidinium N-dimethylaminomethylene guanosine 3′-phosphate was synthesized from guanosine 3′-phosphate (212 mg, 0.5 mmol) of the same salt using DMF dimethyl acetal (0.25 ml) in anhydrous DMF (3 ml). After 24 hr, 80% of the nucleotide was converted to the N-protected form: $\lambda_{max}^{H_2O}$ 293 nm, R_f 0.23 (solvent A). The solution was evaporated with added pyridine and the residue was dissolved in DMF (20 ml). Monomethoxytrityl chloride (950 mg, 3 mmol) was added under stirring and a mixture of DMF (200 ml) and pyridine (0.32 ml) was added at 30-min intervals four times. After 5 hr, 40% aqueous pyridine (50 ml) was added and the solution was concentrated with added pyridine to a small volume. Methanolic ammonia was added and the mixture was kept at room temperature for 13 hr. Ammonia was evaporated and the residue was dissolved in 1% ammonium hydroxide (15 ml) and DMF (3 ml). The solution was extracted with ether (20 ml, 3 portions) and the aqueous phase was extracted with a mixture of chloroform and isoamyl alcohol (4:1, v/v) (10 ml, 2 portions) to remove the bistrityl compound. The aqueous phase was then extracted with a 1:1 mixture of *n*-butyl alcohol and ethyl acetate (20 ml, 2 portions). The organic layer was evaporated with pyridine. The yield was 84%, 4800 OD_{260} units, 0.42 mmol.

Triethylammonium N,2′-O-Diacetylguanylyl-(3′–5′)-uridine 2′-(3′)-Phosphate (V). Pyridinuim 5′-O-monomethoxytrityl-N,2′-O-diacetylguanosine 3′-phosphate (7900 OD_{260} units, 0.47 mmol) and pyridinium uridine 2′(3′)-phosphate (0.9 mmol) were rendered anhydrous by coevaporation with pyridine. The pyridine solution was added to a mixture of ether and *n*-pentane (3:2, v/v) and the precipitate was collected by centrifugation and dissolved in 50% pyridine. The solution was passed through a column (1.2×15 cm) of pyridinium Dowex 50X2 and the column was washed with 50% pyridine. The solution and pyridinium Dowex 50X2 (1.5 g) were evaporated with pyridine. The anhydrous residue was dissolved in pyridine (7 ml) and treated with DCC (1.65 g) for 5 days after pyridine was evaporated to *ca.* 2 ml. Aqueous pyridine (50%, 7 ml) was added to the reaction mixture and the mixture was extracted with *n*-pentane (12 ml) three times. The solution was filtered to remove the urea and kept at room temperature for 16 hr. One-half of the solution was evaporated with water repeatedly to remove pyridine and the residue was treated with 80% acetic acid (10 ml) for 2 hr at room temperature. The volatile materials were evaporated and the residue was coevaporated with pyridine. The anhydrous pyridine solution (5 ml) was added to a mixture of ether and *n*-pentane (3:2, v/v) (200 ml). The precipitate was collected by centrifugation and dissolved in a mixture of DMF (40 ml) and 30% ethyl alcohol (100 ml). The solution was applied to a column of TEAE-cellulose (acetate). The elution pattern and conditions are shown in Figure 2. Peak IV contained the dinucleotide, G^{Ac}-OAc-*p*-Up2′(3′) (2650 OD_{260} units), 52% yield. The buffer was removed by evaporation with added pyridine and the last trace of the buffer was removed by precipitating the product from pyridine solution with a mixture of ether and *n*-pentane. R_f values of the dinucleotide with and without protecting groups are given in Table I. The unprotected dinucleotide, GpUp, was hydrolyzed by RNase M to yield Gp (0.50 OD_{260} unit) and Up (0.47 OD_{260} unit).

Triethylammonium 5′-O-Monomethoxytrityl-N-2′-O-dibenzoylcytidylyl-(3′–5′)-N,2′-O-diacetylguanylyl-(3′–5′)-uridine 2′,3′-Cyclic Phosphate (VII). The dinucleotide V (2600 OD_{260} units, 0.12 mmol) and 5′-O-monomethoxytrityl-N,2′-O-dibenzoylcytidine 3′-phosphate (1500 OD_{304} units) were dissolved in 50% aqueous pyridine and passed through a column (1.2×8 cm) of pyridinium Dowex 50X2. The effluent and washings were evaporated with added pyridine and the anhydrous pyridine solution was added to a mixture of ether and *n*-pentane. The precipitate was collected by centrifugation and washed with ether. The dried precipitate was dissolved in pyridine and evaporated with Dowex 50X2 (210 mg). The evaporation was repeated three times and the residue was treated with DCC (450 mg) in pyridine (3 ml). The solution was concentrated to 2 ml and kept at 20° for 7 days. Aqueous pyri-

dine (50%, 20 ml) was added and the mixture was extracted with *n*-pentane. The solution was filtered to remove the urea, rendered anhydrous after 18 hr, and evaporated with pyridine. The residue was dissolved in a mixture of dry pyridine (2.5 ml) and acetic acid (2.5 ml). The solution was heated at 40° for 5 hr and precipitated with ether and *n*-pentane (3:2). The precipitate was collected by centrifugation and washed with ether. It was treated with DCC (310 mg) in pyridine (10 ml) and triethylamine (0.7 ml, 0.088 mmol) for 2 days at room temperature. Water (10 ml) was added and the solution was extracted with *n*-pentane (10 ml, 3 portions). The solution was filtered and diluted with 95% ethyl alcohol (20 ml) and applied to a column of TEAE-cellulose (acetate). The elution pattern and conditions are shown in Figure 3. The trinucleotide VII was eluted in peak IV. Fractions of 561–610 were combined and evaporated with pyridine. The residual syrup was dissolved in water (200 ml) and extracted with *n*-butyl alcohol (100 ml and 50 ml, 3 portions). *n*-Butyl alcohol was evaporated and the product was precipitated from its pyridine solution with ether and *n*-pentane (3:2). The yield was 1060 OD_{260} units, 17%.

Pyridinium 5′-O-Monomethoxytrityl-N,2′-O-dibenzoylcytidylyl-(3′–5′)-N,2′-O-diacetylguanylyl-(3′–5′)-uridine 3′-Phosphate. The cyclic phosphate VII was treated with pancreatic RNase (2.5 mg) in a mixture of DMF (1.5 ml), 1 *M* ammonium acetate (1 ml), and water (7.5 ml) at 37° for 9 hr. Paper chromatography and electrophoresis at this stage showed a small amount of the starting material and the incubation was further continued with addition of the enzyme (1 mg) for 8 hr. The reaction mixture was passed through a column (1.2 × 10 cm) of pyridinium Dowex 50X2. The column was washed with a mixture of pyridine (5 ml), ethyl alcohol (50 ml), DMF (20 ml), and water (25 ml). The effluent and washings were used for the next step.

Pyridinium 5′-O-Monomethoxytrityl-N,2′-O-dibenzoylcytidylyl-(3′–5′)-N-2′,O-diacetylguanylyl-(3′–5′)-2′-O-acetyluridine 3′-Phosphate (VIII). The trinucleotide from the above experiment was added to tetraethylammonium acetate (6 mmol) and evaporated with added pyridine five times. The residue was coevaporated with toluene four times to remove pyridine and treated with acetic anhydride (0.57 ml, 6 mmol) for 3 days at room temperature. The reaction mixture was cooled in an ice bath and cooled 50% aqueous pyridine (200 ml) was added. The solution was passed through a column (1.2 × 25 cm) of pyridinium Dowex 50X2 and the column was washed with 50% pyridine. The effluent and washings were evaporated with pyridine and the anhydrous pyridine solution was added to a mixture of ether and *n*-pentane (3:2, v/v) with vigorous stirring. The precipitate was collected by centrifugation and washed with the same mixture of ether and *n*-pentane. The purity of the trinucleotide was checked by paper chromatography. R_f values of the trinucleotide derivatives are shown in Table I. Acetic acid (80%) treatment and successive methanolic ammonia treatment gave the unprotected trinucleotide, CpGpUp, which was completely digested by RNase M to yield Gp (1.1 OD_{260} units), Up (0.96 OD_{260} unit), and Cp (1.28 OD_{280} units) in 0.05 *N* HCl after separation by paper chromatography (solvent C) and paper electrophoresis (pH 3.5).

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π-Cation Radicals and Dications of Metalloporphyrins[1]

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Abstract: Zinc tetraphenylporphyrin (ZnTPP) and magnesium octaethylporphyrin (MgOEP) undergo reversible one- and two-electron oxidations. Removal of the first electron by controlled potential electrolysis or by oxidation with xenon difluoride or bromine results in cation radicals whose esr spectra clearly indicate electron abstraction from the porphyrin ring. The observed hyperfine structure is assigned, with the help of deuterium labeling, to interaction of the unpaired electron with the *meso* protons of $MgOEP^+$ and with the protons on the *phenyl* groups of $ZnTPP^+$. Electronic absorption spectra of the radicals, dications, and of a dimer of $MgOEP^+$ which exists at low temperatures are presented. The results of an SCF–MO study are compared with the experimental esr and optical data. These calculations suggest that a radical may occupy either of two close-lying ground states. We conclude that the ground-state symmetries of $ZnTPP^+$ and $MgOEP^+$ are, respectively, $^2A_{2u}$ and $^2A_{1u}$ of the D_{4h} point group.

Crucial steps in photosynthetic and metabolic processes involve metalloporphyrin redox properties. Prior studies of heme function have stressed the redox characteristics of the central metal;[3,4] in contradistinction, primary photosynthetic events involving chlorophyll appear to depend upon ring redox properties.[5,6]

A striking feature of the porphyrin ring is its ability to undergo facile reduction and oxidation. Reductions of chlorophyll[7] and metalloporphyrins,[8] dissolved in aprotic solvents, have been accomplished by electrochemical and chemical techniques, and the results

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