Synthesis and Properties of Nucleoside 5′-Phosphoazidates Derived from Guanosine and Adenosine Nucleotides: Effect on Elongation Factors G and T_u Dependent Reactions†

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ABSTRACT: A new type of nucleoside poly(5′-phosphate) analogue, nucleoside 5′-phosphoazidate, with an azido group on the terminal phosphate of GTP, ATP, GDP, GMP, and AMP, has been synthesized by nucleophilic displacement of the corresponding activated nucleosides by azide ion in yields of 25–45%. Guanosine 5′-phosphoazidate is readily photolyzed by ultraviolet light; the corresponding adenosine derivative photolyzes more slowly. Guanosine 5′-*O*-(2-azidodiphosphate) and guanosine 5′-*O*-(3-azidotriphosphate) are competitive inhibitors of the formation of the ribosome·EF-G·GDP·fusidic acid complex and of the ribosome·EF-G GTPase. The dissociation constants of the former reaction are calculated to be 27 and 7 μM, respectively, or 270 and 73 times that of GDP. In the latter reaction, which is conducted in the absence of fusidic acid, the K_i values are 330 and 28 μM, respectively, or 12 and 1 times that of GDP. Guanosine 5′-*O*-(2-azidodiphosphate) and guanosine 5′-*O*-(3-azidotriphosphate) also compete with GTP in the formation of the binary complex. EF-T_u·GTP, with respective K_d values of 750 and 75 relative to GTP.

During ribosomal protein synthesis incorporation of each internal amino acid is associated with the hydrolysis of two molecules of GTP (Cabrer et al., 1976). Although the exact role of GTP hydrolysis in polypeptide chain elongation is not yet fully understood, it is well known that the ribosome-dependent GTPase reaction is mediated by the two elongation factors, EF-T_u and EF-G.[1] Since both factors appear to bind to the same area on the ribosome (Cabrer et al., 1972; Richman and Bodley, 1972; Miller, 1972; Richter, 1972), it is reasonable to assume that there is a single enzymatic site for GTP hydrolysis (the GTPase center) located on the ribosome whose activity is controlled by either factor. Alternatively, it has been suggested that the site(s) for GTP hydrolysis may be located on the elongation factors themselves (Wolf et al., 1974; Marsh et al., 1975) and that this center may be activated and controlled by the interaction of EF-T_u and EF-G with the ribosome.

Maassen and Möller (1974) attempted to identify the ribosomal proteins involved in the binding of GTP to the EF-G·ribosome·fusidic acid complex by affinity labeling with guanosine 5′-*O*-[2-(*p*-azidophenyl)diphosphate] as a photolabile analogue of GDP. Their results implicated proteins L5, L11, L18, and L30 in GDP binding. Other experiments, however, with a similar probe attached either to the 2′,3′ position of the ribose moiety of GTP or to the γ-phosphate group, indicated labeling of EF-G only (Girshovich et al., 1974, 1976). The apparent discrepancy in these results shows how the length and position of the probe, as well as the affinity of the labeling reagent for the recognition site, may play an extremely important role in the covalent linking observed.

We felt that further progress in locating the GTP binding site on either EF-T_u, EF-G, or the ribosome could be achieved by using a new type of label such that the length of the probe would be very short and the probe would be located on that part of the GTP molecule directly interacting with the enzyme active center.

We have chosen as a probe the photolabile azido group, placed at the terminal phosphate of GTP and GDP. In this paper, we describe our initial studies of the synthesis and properties of these new compounds, the nucleoside 5′-phosphoazidates. We also report on the behavior of these analogues of GTP and GDP in enzyme systems containing EF-T_u and EF-G. An outline of this work has been reported in abstract form (Chládek et al., 1977).

Experimental Procedure

General Methods

Chromatography. Paper chromatography was performed by the descending technique on Whatman No. 1 paper using the following solvent system: S_1, isobutyric acid–1 M NH_4OH–0.1 M EDTA (100:60:1.6); S_2, ethanol–1 M ammonium acetate (pH 6.5) (7:2); S_3, ethanol–0.5 M ammonium acetate (pH 3.8) (5:2). Thin-layer chromatography (TLC) was done on PEI cellulose (PEI/UV/254 cellulose MN 300, Brinkmann Instruments, Westbury, N.Y.) in systems S_4, 1 M NaCl, and S_5, 0.75 M KH_2PO_4, adjusted to pH 3.8 with phosphoric acid; and on silica gel coated aluminum foil (silica gel 60 F-254, Brinkmann Instruments) in systems S_6, CH_3CN–0.1 M NH_4Cl (7:3).

Paper electrophoresis was conducted on a Savant flat plate apparatus using 0.02 M Na_2HPO_4 (pH 7.0) and 0.1 M CH_3COONH_4 (pH 3.8) as buffers.

UV-absorbing compounds were detected using a Mineralight lamp. UV spectra were obtained with a Cary 14 recording

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[1] Abbreviations used are: EF-G, elongation factor G; EF-T_u, elongation factor T_u; GMP-N_3, guanosine 5′-phosphoazidate; GTP-N_3, guanosine 5′-*O*-(3-azidotriphosphate), similar abbreviations for other nucleotide derivatives; GMP-NH_2, guanosine 5′-phosphoamidate; Ap_nA, α,ω-diadenosine poly(5′-phosphates); GDPCP, guanylyl 5′-methylenediphosphonate; $Et_3NH_2CO_3$, triethylammonium bicarbonate; EDTA, ethylenediaminetetraacetic acid (tetrasodium salt); DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

UV spectrophotometer. Yields of nucleotides were determined spectrophotometrically based on the extinction coefficients for AMP, ATP, GMP, GDP, and GTP. Nitrogen and phosphorus analyses were performed by Micro-Tech Laboratories, Skokie, Ill. Infrared spectra in KBr pellets were made on a Perkin-Elmer 21 infrared recording spectrophotometer. ^{31}P NMR spectra were obtained with a Bruker 90-MHz spectrometer in D_2O (pH 6) with 85% H_3PO_4 as the external standard. The spectral data are reported in Table II. The pK values (basic) (Sober 1973) were obtained by potentiometric titrations at 24 °C on a Metrohm Combititrator 3D. The ionic strength for each determination was adjusted to 0.01 M by adding $(Me)_4NBr$. Under these conditions, the pK of AMp was 3.96 (c 6.96×10^{-6} M), and GMP was 2.80 (c 7.20×10^{-4} M).

All evaporations were performed in vacuo with bath temperature below 35 °C.

Enzymatic Degradation. Alkaline Phosphatase. Nucleotide (IIIa-f, ca. 1 μmol) was dissolved in 50 μL of aqueous bacterial alkaline phosphatase (Sigma, III-R, 2.5 unit) and the pH was adjusted to 7.8. The solution was incubated at 37 °C for 1 h, and then analyzed by paper electrophoresis at pH 3.8 or by TLC on PEI cellulose.

Snake Venom Phosphodiesterase. Nucleotide (IIIa-f, ca. 1 μmol) was dissolved in 0.1 M Tris-acetate (pH 8.5, 60 μL), 0.3 M $Mg(OAc)_2$ (10 μL), and phosphodiesterase (Russell's viper venom, Calbiochem, 10 units in 50 μL of water), and the solution was incubated for 2 h at 37 °C. The products of the degradation were electrophoresed at pH 3.8.

Photolysis Experiments. Compounds were photolyzed using a 450-W Hanovia lamp (Ace Glass). The sample was dissolved in water (concentration is given in the legend of Figure 1) and maintained at 4 °C in a water-jacketed quartz cuvette during photolysis. For the photolysis experiments at higher concentration (5 mM), 10-μL aliquots were taken at regular intervals during photolysis and applied to silica gel TLC plates (developed in S_6); the spots corresponding to unphotolyzed material were scraped off, eluted, and measured spectrophotometrically. For the low concentration (0.05 mM) photolysis of GMP-N_3, 1.2-mL samples were photolyzed for varying times, 1.0-mL aliquots were taken, freeze-dried, and redissolved in 0.05 mL of water; an aliquot (0.02 mL) of the resulting solution was run on TLC as above, and the spots corresponding to photolyzed and unphotolyzed material were scraped off separately, eluted, and measured spectrophotometrically. The ratio of photolyzed to unphotolyzed material gave the percent photolysis. Measurements were made with no additional filter other than the quartz cuvettes, with a Pyrex filter, and with a WG320 Schott glass filter. Preliminary experiments indicated that N_2 bubbling had a negligible effect on the rate of photolysis.

Materials

AMP, ATP, GMP, GDP, and GTP were purchased from Sigma Chemical Co. and were checked by TLC (PEI cellulose). Diphenyl phosphochloridate, tri-*n*-butylamine and tri-*n*-octylamine (the latter two dried over Linde 3-Å molecular sieves) were obtained from Aldrich (Milwaukee, Wis.). Lithium azide was a product of Eastman Kodak. Pyridine (reagent grade) was distilled from ninhydrin, distilled again from KOH, and stored over 3-Å molecular sieves in the dark. Dimethylformamide was vacuum distilled from P_2O_5 and stored over 3-Å molecular sieves.

Synthesis of Nucleoside 5′-Phosphoazidates IIIa-f. Starting nucleotide I (1 mmol, sodium salt) was converted to the pyridinium salt by passage through a column of Dowex 50 (Py^+ cycle, ca. 15 mL) and elution with 50% pyridine. The eluate was evaporated in vacuo and made anhydrous by coevaporation with dry pyridine. The residue was dissolved in dry methanol (10 mL), tri-*n*-octylamine was added (1.26 mL), and the mixture briefly heated with a hot-air blower. The clear solution was evaporated in vacuo to dryness, and dried by coevaporation with pyridine and DMF. After the residue was dissolved in dry dioxane (5 mL), diphenyl phosphochloridate (0.3 mL) and tri-*n*-butylamine (0.45 mL) were added, and the solution was kept at room temperature for 2 h. The solution was evaporated in vacuo, and the residue was extracted twice with diethyl ether–petroleum ether (1:1) at 4 °C for 30 min. The solvents were decanted and discarded. The residue was dissolved in dioxane (ca. 15 mL), evaporated in vacuo, coevaporated with anhydrous pyridine (two times), dissolved in anhydrous pyridine (ca. 3 mL), and a solution of lithium azide (0.15 g, 3 mmol, predried by coevaporation with anhydrous pyridine) in pyridine (5 mL) was added. Upon mixing, a red color developed, followed by precipitation of a red material. The mixture was stirred for 2 h, and then evaporated to dryness. The residue was extracted with water, applied to a DEAE-cellulose column (3.5 × 54 cm), and eluted with a 3-L linear gradient of $Et_3NH_2CO_3$ in water. For the monophosphate derivatives the final buffer concentration was 0.30 M, for the diphosphate derivatives, 0.40 M, and for the triphosphate derivatives the gradient was 0 to 0.50 M. For triphosphate derivative IIIf, where the analysis of side products was attempted, a second gradient from 0.5 to 1.0 M $Et_3NH_2CO_3$ was used. The flow rate was 2 mL/min. Although several peaks were detected, particularly with the triphosphate derivatives IIIc and IIIf, the desired products were always the most prominent. Their position in the elution profile is given in Table I. The products were recovered by evaporation in vacuo and coevaporation several times with methanol. Because minute contamination with unsubstituted nucleotides could cause serious problems during the subsequent biochemical studies, the products were treated with alkaline phosphatase (Eckstein et al., 1975) to convert any 5′-nucleotides present to nucleosides. The whole product was dissolved in 0.2 M Tris-acetate (pH 7.5, 0.2 mL) plus 0.5 mL of a chicken intestine alkaline phosphatase solution (0.05 M Tris-HCl, pH 7.8, 0.036 M $CaCl_2$, 0.0024 M $MgCl_2$, 2 mg of enzyme protein/mL). The solution was adjusted with dilute ammonia to pH 7.8 and incubated for 2 h at 37 °C. After a fivefold dilution with water, the solution was applied to a column of DEAE-Sephadex A-25 (3 × 85 cm), and eluted with a 2-L linear gradient (0–1 M $Et_3NH_2CO_3$ for diphosphate derivative IIIb, or 0–2 M $Et_3NH_2CO_3$ for triphosphate derivatives IIIc and IIIf). Alkaline phosphatase treatment was omitted for the mononucleotide derivatives Ia and Id. Usually a single large peak was eluted, which was pooled, evaporated in vacuo, and the residue coevaporated several times with methanol. A stock solution of the product was made in methanol and found to be homogeneous both by TLC on PEI cellulose and by electrophoresis at pH 3.8. The yield was determined spectrophotometrically after appropriate dilution in 0.01 N HCl. The compounds were then isolated as the sodium salt according to Moffatt (1964). The triethylammonium salt (ca. 0.2 mmol) of the reaction product was treated in methanol (ca. 3 mL) with a 1 M solution of sodium iodide (6 equiv) in acetone, and the sodium salt of the product was precipitated by addition of anhydrous acetone (30 mL) and isolated by centrifugation and washing with 30-mL portions of dry acetone. The products were obtained as white nonhygroscopic powders (with the exception of IIe, which was very hygroscopic). The yields of this conversion were practically quantitative. The overall yields of compounds IIIa-f, their spectral properties, and the analysis for phosphorus and nitrogen are reported in Table I. Chromatographic

TABLE I: Nucleoside 5′-Phosphoazidates: Yields and Properties.

Compound	% Yield	Elution[a] molarity	pK_a basic (concn × 10^4 M)	UV spectra (0.01 N HCl) λ_{max} (ϵ)	λ_{min} (ϵ)	250/260 nm	280/260 nm	Formula (mol wt)	Analysis %P calcd (found)	%N calcd (found)
GMP-N_3 IIIa	43	0.20	2.95 (6.44)	256 (12.2)	227 (1.11)	0.98	0.66	$C_{10}H_{12}N_8O_7PNa + 3H_2O$ (464.3)	6.67 (6.50)	24.13 (24.15)
GDP-N_3 IIIb	30	0.34		256 (13.0)	228 (2.9)	0.95	0.67	$C_{10}H_{12}N_8O_{10}P_2Na_2 + 3H_2O$ (566.3)	10.94 (10.66)	19.79 (19.78)
GTP-N_3 IIIc	28	0.42	3.26 (7.06)	256 (12.4)	228 (2.83)	1.02	0.65	$C_{10}H_{12}N_8O_{13}P_3Na_3 + 7H_2O$ (740.3)	12.55 (12.93)	15.13 (15.25)
AMP-N_3 IIId	34	0.17	4.00 (4.13)	258 (13.7)[b]	230 (1.79)[b]	0.78	0.23	$C_{10}H_{12}N_8O_6PNa + 1H_2O$ (412.3)	7.51 (7.70)	27.18 (26.90)
ATP-N_3 IIIf	26	0.40	4.55 (4.98)	257 (14.6)	230 (2.99)	0.83	0.21	$C_{10}H_{12}N_8O_{12}P_3Na_3 + 4H_2O$ (673.3)	13.86 (13.72)	16.72 (16.84)

[a] Elution molarity of $Et_3NH_2CO_3$ buffer during DEAE-cellulose chromatography (center of peak). [b] These values are approximate because of the hygroscopic nature of the preparation of this compound.

TABLE II: ^{31}P NMR Spectra of Nucleoside 5′-Phosphoazidates and Standard Nucleoside 5′-Phosphates.[a]

Compound	δ (ppm) P_α	P_β	P_γ	J(Hz) $P_\alpha P_\beta$	$P_\gamma P_\beta$	$P_\beta P_\alpha$ and $P_\beta P_\gamma$
GMP	0.18 s					
GMP-N_3 IIIa	0.34 s					
AMP	0.21 s					
AMP-N_3 IIId	1.33 s					
GTP	11.29 d	22.92 t	9.78 d	19.5	19.5	19.5; 19.5
GTP-N_3 IIIc	11.59 d	22.91 t	13.25 d	19.5	20.7	20.7; 22.0
ATP	11.32 d	23.12 t	10.81 d	19.5	18.3	19.5; 19.5
ATP-N_3 IIIf	11.56 d	23.26 t	13.28 d	19.1	20.6	20.6; 22.2

[a] Abbreviations used are: s, singlet; d, doublet; t, triplet.

properties are reported in Table IV. The compounds were found to be quantitatively hydrolyzed by snake venom diesterase to AMP or GMP and to be resistant to alkaline phosphatase. GTP or GDP contamination was absent as assessed by electrophoresis [ca. 18 optical density units of GTP-N_3 (IIIc) applied as a 1-cm band to Whatman 3MM paper, 0.02 M phosphate buffer pH 7, 60 V/cm for 1.5 h). By purposely contaminating the GTP-N_3 (IIIc) with small amounts of GTP, it was determined that 0.2–0.3% could have been detected. Therefore, the GTP-N_3 is >99.7% pure.

Analysis of Side Products of the Reaction of Activated ATP with Lithium Azide. All peaks separated on the DEAE column, as described above, were pooled as indicated in Table III, evaporated in vacuo, and investigated by chromatography in systems S1, S5 and electrophoresis at pH 3.8. All peaks with the exception of peaks B, D, and E were found to be mixtures. They were treated with alkaline phosphatase and chromatographed on DEAE-Sephadex A-25 (gradient of 0–2 M aqueous $Et_3NH_2CO_3$). As indicated in Table III, some of the peaks separated into several major peaks. In addition, there were always minor peaks which were not identified for lack of material, and usually a peak of adenosine. All of the peaks in Table III were isolated as the sodium salts described above. The yields, molecular weights, IR properties, and stability to nucleolytic enzymes are reported in Table III.

Guanosine 5′-Phosphoamidate. (a) By the Method of Michelson (1964). The triethylammonium salt of guanosine 5′-phosphoramidate was prepared in 47% yield. It was determined to be chromatographically identical to the compound prepared by hydrogenation (see below) in systems S_2 and S_6.

(b) By Hydrogenation of IIIa. The sodium salt of compound IIIa (25.3 mg, ca. 60 μmol) was dissolved in methanol (4 mL), 10% Pd/carbon catalyst (60 mg) was added, and hydrogen was bubbled in with ice-bath cooling for 10 h. After this time, hydrogenation was practically quantitative as shown by TLC in system S_6. The catalyst was filtered off through Celite and washed with methanol, and the combined filtrates were evaporated to dryness in vacuo. The product was homogeneous in systems S_2, S_6, and by electrophoresis in phosphate buffer (pH 7.0). The barium salt was prepared from an aqueous solution of the product by precipitation with barium acetate and ethanol (1:4), centrifugation, and washing with ethanol and acetone. After drying in vacuo, a molecular weight of 596 was determined spectrophotometrically (0.01 N HCl).

Biochemical Assays. Binding of GTP analogues IIIb and IIIc to EF-T_u of *E. coli* was measured by competition with [^{3}H]GTP, since the analogues were not radioactive. Two incubation tubes were prepared. Tube A contained, per 75 μL, 67 mM Tris-HCl, pH 7.4, 67 mM NH_4Cl, 13.3 mM $MgCl_2$, 6.7 mM dithiothreitol, 13.3 mM phosphoenolpyruvate, 0.21 mg/mL pyruvate kinase in 3 M $(NH_4)_2SO_4$, and [^{3}H]GDP (900 cpm/pmol). Tube B contained 50 mM Tris-HCl, pH 7.4, 50 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM phosphoenolpyruvate, 0.13 mg/mL pyruvate kinase, and 35 μg/mL EF-T_u·GDP (specific activity, 10.4 nmol of GDP bound/mg, 44% of theory). After incubation of tube A for 20 min at 37 °C to convert the GDP to GTP, 75-μL aliquots were combined with an appropriate amount of the GTP analogue IIIb or IIIc, adjusted to a 100-μL volume with water, and incubated for a further 15 min at 37 °C. Tube B was also incubated for 15 min at 37 °C. To start the binding reaction, 50 μL of tube B was added to each tube of 100 μL containing prein-

TABLE III: Products of the Reaction of Activated ATP with LiN_3.

Peak[a]	Elution molarity[a]	Yield[a] %	Peak[b]	Yield[b] %	IR spectra azide band	Mol wt of Na salt[c]	Enzymatic hydrolysis: Snake venom diesterase	Enzymatic hydrolysis: Alkaline phosphatase	Structure
A	0.12								Nonnucleotide
B[d]	0.19	11.1			Yes		Yes	No	AMP-N_3 IIId
C[e]	0.34	4.7	1	1.3	No	1044	Yes	No	Ap_2A[f]
			2	1.3	Yes	631	Yes	No	ADP-N_3 IIIe[f]
D	0.40	26.4		21.5	Yes	678	Yes	No	ATP-N_3 IIIf
E[d]	0.43	3.3			Yes	607 or 1414[g]	Partly	No	?
F[e]	0.47	4.6	1	0.7	No	1440	Partly	Partly	Contaminated Ap_4A
			2	2.3	Yes	783	Yes	Trace	Ap_4N_3 or Ap_5N_3 IIIg or h
G	0.53	6.1		4.7	No	1300	Partly	No	Ap_5A[f,h]
H[e]	0.65	3.4	1	0.5	No		?	Yes	?
			2	0.8	No	1400	Yes	Partly	Ap_6A[f]

[a] DEAE-cellulose column. [b] DEAE-Sephadex column. [c] Based on ϵ value of the parent nucleotide. [d] Treatment with alkaline phosphatase and DEAE-Sephadex chromatography omitted. [e] Resolved in two peaks on DEAE-Sephadex column. [f] Probable structure. [g] Based on ϵ 15.0 or 30.0, respectively. [h] Ratio of P/N corresponds to this structure.

TABLE IV: Chromatography and Electrophoresis of Products and Authentic Specimens.

Compound	R_f in the following systems: S2	S4[a]	S5[b]	S6	Electrophoresis: pH 3.8[b]	pH 7.0[a]
(A) Guanosine derivatives						
GTP			1.00		1.0	1.0
GDP			3.1		0.85	
GMP	0.02	1.0	4.2	0.18	0.55	1.00
GMP-N_3 IIIa	0.31	6.09	5.7	0.52	0.65	0.73
GDP-N_3 IIIb			5.3		0.94	
GTP-N_3 IIIc			4.0		1.12	1.3
GMP-NH_2	0.08			0.21	0.53	0.67

Compound	R_f in the following systems: S1	S2	S3[c]	S5[c]	Electrophoresis: pH 3.8[c]	pH 7.0[d]
(B) Adenosine derivatives						
ATP	1.00		1.00	1.00	1.00	
ADP	1.45		4.85	2.11	0.89	1.06
AMP	1.95	0.16	14.3	2.58	0.50	1.00
AMP-N_3 IIId	2.16	0.63		2.79	0.55	0.64
ADP-N_3 IIIe (peak C2)	1.59		9.54		0.99	0.81
ATP-N_3 IIIf	1.06		3.95	2.73	1.15	
AMP-NH_2		0.30				

[a] Relative to GMP. [b] Relative to GTP. [c] Relative to ATP. [d] Relative to AMP.

cubated [^{3}H]GTP and GTP analogue IIIb or IIIc. After 10 min at 37 °C, 100-μL aliquots were passed through Millipore filters, washed, and counted (Ofengand, 1974).

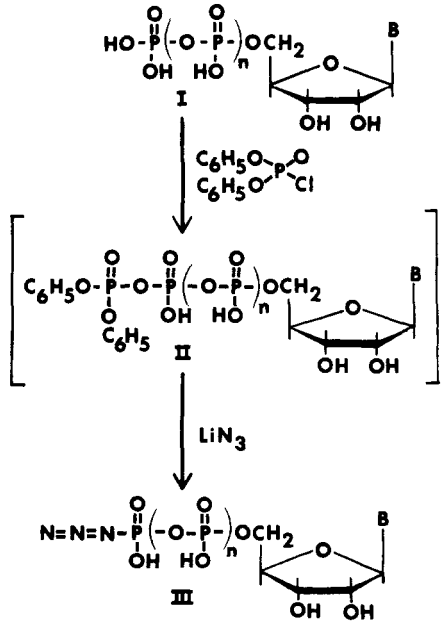
Binding of the analogues to EF-G was also measured by competition. Each 50-μL reaction mixture contained 50 mM Tris, pH 7.8, 20 mM NH_4Cl, 1 mM dithiothreitol, 2.2 A_{260} units of two times high-salt-washed ribosomes (Bodley et al., 1974), 1 mM fusidic acid (Leo Pharmaceuticals), 21 pmol of EF-G, [^{3}H]GDP (750–820 cpm/pmol), and analogues IIIb or IIIc. In order to ensure proper competition conditions, GDP and analogue were mixed first, and the reaction was started by addition of the ribosome/EF-G/fusidic acid mixture. After 20 min at 30 °C, 40-μL aliquots were washed and filtered through Millipore filters under 10 mL of wash buffer (20 mM Tris, pH 7.8, 20 mM NH_4Cl, 10 mM $MgCl_2$) as described by Eckstein et al. (1975) and counted. In some experiments, 10^{-5} M fusidic acid was added to the wash buffer (Bodley et al., 1974), without changing the results obtained.

Ribosome·EF-G GTPase activity was measured essentially as described by Eckstein et al. (1975) using a 100-μL reaction mixture containing 20 mM Tris-HCl, pH 7.8, 14 mM $MgCl_2$, 80 mM NH_4Cl, 1 mM dithiothreitol, 0.1 μM ribosomes, 0.3 μM EF-G and GTP and analogues as indicated in Figure 5.

Results

Synthesis and Proof of Structure of Nucleoside 5′-Phosphoazidates IIIa–f. Michelson (1964) reported the synthesis of various nucleoside anhydrides as well as nucleotide amidates by anion displacement on p^1-nucleoside 5′-p^2-diphenylpyrophosphates. It was found that a variety of anions of acids weaker than diphenylphosphoric acid could attack triesterified pyrophosphates of the general type II (Scheme I). Eckstein et

Scheme I



I–III	B	n
a	guanine	0
b	guanine	1
c	guanine	2
d	adenine	0
e	adenine	1
f	adenine	2
g	adenine	3
h	adenine	4

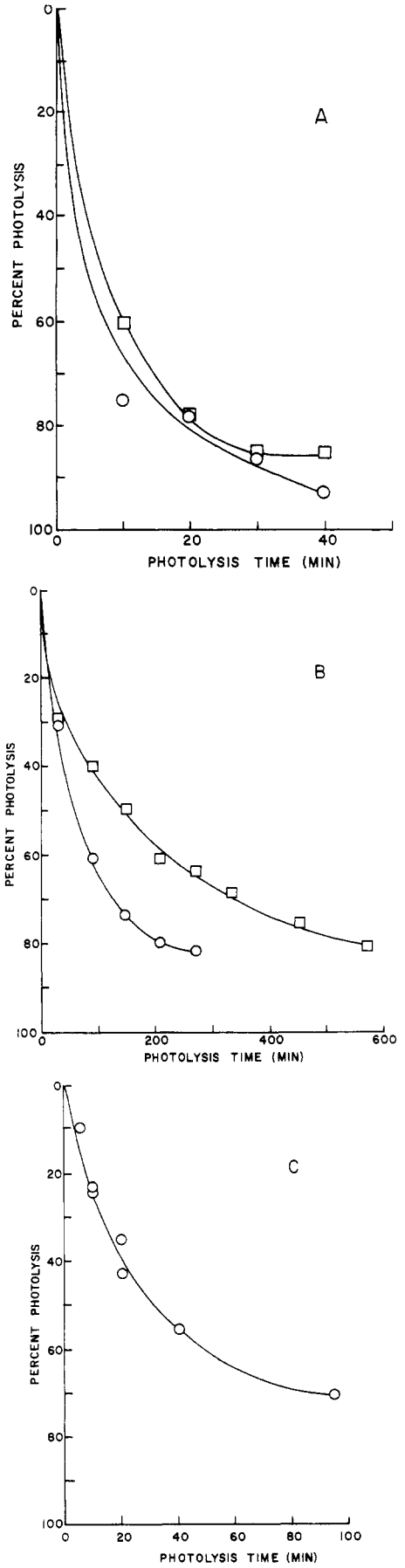


FIGURE 1: Rate of photolysis of GMP-N_3 (Ia) and AMP-N_3 (IIId): (A) quartz filter; (B) Pyrex filter. The reaction conditions are given under Experimental Procedures: (○) GMP-N_3 (Ia, 5 mM); (□) AMP-N_3 (IIId, 5 mM). (C) Pyrex filter: (○) GMP-N_3 (Ia, 0.05 mM). Negligible photolysis occurred using Schott filter.

al. (1975) prepared a series of γ-esters of GTP by the anion-exchange reaction and proved that esterification had occurred on the terminal phosphate. We have used the method of Michelson (1964) for the synthesis of the novel nucleotide analogues, nucleoside 5′-phosphoazidates (IIIa–f). The triesterified nucleoside 5′-pyrophosphates (II) react smoothly with lithium azide in pyridine solution,[2] furnishing good yields of the desired compounds.

The compounds IIIa–f were characterized by elemental analysis, UV, and ^{31}P NMR spectra, and by chromatography and electrophoresis in several systems. The IR spectra of nucleoside 5′-phosphoazidates show a strong azido group absorbance at ca. 2150 cm^{-1}. Resistance of compounds IIIa–f to bacterial alkaline phosphatase shows the absence of a monoester phosphate group, indicating substitution exclusively on the terminal phosphorus atom of GDP, GTP, or ATP. The ^{31}P NMR spectra of GTP-N_3 (IIIc) and ATP-N_3 (IIIf) show a significant chemical shift only on the γ-phosphorus atom (about 3.5 ppm) which is in agreement with the proposed structures. Eckstein et al. (1975) also observed a similar chemical shift in the spectra of the γ-methyl ester of GTP. In agreement with this structure, nucleoside 5′-phosphoazidates IIIa–f were quantitatively hydrolyzed by snake venom phosphodiesterase to nucleoside 5′-phosphates.

Chemical Stability. The nucleoside 5′-phosphoazidates IIIa–f are stable at near-neutral pH and thus can be used for biochemical investigations without danger of hydrolysis to the parent compound. They are, however, unstable in strongly alkaline media. Thus, GMP-N_3 (IIIa) and AMP-N_3 (IIId) are partially hydrolyzed to their parent nucleotide by 0.5 N NaOH in 5 h at room temperature, and converted almost quantitatively to the corresponding nucleoside 5′-phosphoamidates (IVa and IVc) by treatment with concentrated aqueous ammonia (5 h, room temperature). In this respect, compounds III seem to behave similarly to active esters, and under appropriate conditions may serve as phosphorylating reagents. On the other hand, phosphoazidates IIIa and IIId are stable in 80% acetic acid for at least 5 h at room temperature.

Photolysis. Breslow et al. (1974) reported the smooth generation of phosphonitrenes by UV irradiation of various phosphoazidates derived from dialkyl or diaryl phosphoric acids. AMP-N_3 (IIId) and GMP-N_3 (IIIa) are both readily photolyzed using either a Pyrex filter (wavelengths above 280 nm) or no filter (see General Methods). Interestingly, photolysis of the guanosine derivative IIIa is much faster than that of the adenosine derivative IIId (Figure 1). As is expected, photolysis proceeds more rapidly at lower concentrations, due to decreased internal shielding by the photolabel itself. Photolysis under these conditions is not expected to cause significant photo-damage to ribosomes. Although the photolysis of IIIa gives rise to several products, we have been able to identify only GMP-NH_2 (undoubtedly generated by the hydrogen abstraction reaction) as a major photolytic product. A second major product of photolysis which was observed may be guanosine 5′-phosphohydroxyamidate arising from insertion of the phosphonitrene into the OH bond of water (Breslow et al., (1974). Authentic GMP-NH_2 was prepared by the method of Michelson (1964) from the activated derivative of GMP (II) and ammonia.

Side Reactions. As indicated under Experimental Section, the desired nucleoside 5′-phosphoazidates IIIa–f were always the major product of displacement of activated phosphates II by lithium azide. Nevertheless, the reaction mixtures resulting

[2] Hydrazoic acid (pK_a 0.8 × 10^{-5}) is a much weaker acid than diphenylphosphoric acid.

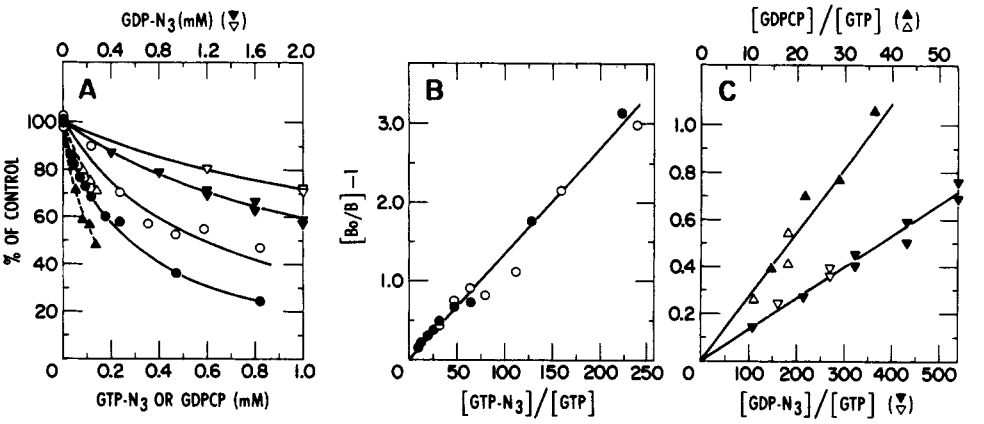


FIGURE 2: Competitive binding of GTP-N_3 (IIIc), GDP-N_3 (IIIb), and GDPCP to EF-T_u of *E. coli*. (A) Binding assays were carried out as described under Experimental Procedures using 3.69 (●, ▲, ▼) or 7.37 μM (○, △, ▽) [^{3}H]GTP: GTP-N_3 (IIIc) (●, ○); GDP-N_3 (IIIb) (▼, ▽); GDPCP (▲, △). One hundred percent binding corresponds to 11–13 pmol bound to the filter. (B) The data of part A for GTP-N_3 (IIIc) was replotted as described in the text. The total concentration of GTP or analogue was used as an approximation to the unbound concentration: (●) 3.69 μM GTP; (○) 7.37 μM GTP. (C) The data of part A for GDP-N_3 (IIIc) and GDPCP was replotted as described in part B: 3.69 (▼, ▲) or 7.37 μM (▽, △) GTP; GDP-N_3 (IIIb) (▼, ▽); GDPCP (▲, △).

from the displacement on ATP and GTP were quite complicated, giving rise to several peaks on the DEAE-cellulose column. Although the peak of desired product, ATP-N_3 (IIIf) or GTP-N_3 (IIIc), was always most prominent, the formation of side products deserves further comment. A partial analysis was performed for the reaction with ATP. Since most of the products were not chromatographically homogeneous, they were routinely treated with alkaline phosphatase to destroy any nucleoside poly(5′-phosphates) which may have been present, and purified by a subsequent DEAE-Sephadex chromatography. Identification of the side products was then performed according to the properties listed in Table III. As can be seen, altogether nine different nucleotides were obtained, in addition to the major product ATP-N_3 (IIIf).

Biochemical Activity. Since neither GTP-N_3 (IIIc) nor GDP-N_3 (IIIb) was available in radioactive form, their ability to interact with the elongation factors T_u and G of *E. coli* was measured by an indirect method. EF-T_u forms a binary complex with GTP or GDP which can be detected by quantitative trapping of the complex on a Millipore filter (Miller and Weissbach, 1974), since the association constants are very high (3×10^6 and 3×10^8 M^{-1} for GTP and GDP, respectively). The binding of nonradioactive analogues IIIb and IIIc was thus detected by their ability to reduce the amount of radioactive GTP complexed with EF-T_u, and the competitive nature of the effect was verified by its reversal upon increasing the GTP concentration (Figure 2A). For comparison, the effect of the analogue GDPCP is also shown. In such experiments, the concentration of GTP used must be sufficient to saturate its binding site. From the known dissociation constant of 3×10^{-7} M (Miller and Weissbach, 1974) and the concentrations of GTP and EF-T_u employed here, one can readily calculate that 93% or more of the added EF-T_u was complexed at all times.

Under these conditions, namely that the binding of both GTP and the analogue had reached equilibrium in the presence of a limiting number of binding sites, the data of Figure 2A can be quantitatively analyzed by the relation $(B_0/B) - 1 = K_r$[GTP analogue]/[GTP] (Ofengand and Henes, 1969), where B_0 and B are the amounts bound in the absence and presence of inhibitor, respectively, K_r is the ratio K_a[analogue]/K_a[GTP] and the nucleotide concentrations are those of the unbound species. Since the concentration of EF-T_u in these experiments was low compared to the nucleotides, the GTP analogue/GTP ratio has been approximated by the concentration ratio of added nucleotides.

Analysis of the experimental results by this procedure for both concentrations of GTP (Figure 2B,C) yields relative dissociation constants for GTP-N_3 (IIIc) and GDP-N_3 (IIIb) which are 75 and 750 times that for GTP, respectively. As a control, GDPCP was also tested. Its relative dissociation constant is 37. The published values are 4.5 (Arai et al., 1974) and 330 (Miller and Weissbach, 1974). Note that with both analogues true competition was demonstrated, since increasing the GTP concentration decreased the inhibition by the expected amount.

Contaminating trace amounts of GDP in the GTP-N_3 (IIIc) could have caused the inhibition observed, since the K_a for GDP is 100 times that for GTP. This problem was minimized in two ways. First, during purification the analogues were treated with alkaline phosphatase to degrade any GTP or GDP (see above) and, second, in the assay the analogues were treated with phosphoenolpyruvate and pyruvate kinase to convert any GDP to GTP before adding the EF-T_u. A 1.3% contamination of GTP-N_3 (IIIc) with GDP, subsequently converted to GTP, would be necessary to account for the results observed, and this amount would have been detected by the procedures used to monitor the purity of the preparation (see above).

Binding to EF-G was measured in an analogous way, using a modification of the procedure described by Eckstein et al. (1975). The major procedural difference between the two methods is the simultaneous addition of both GDP and analogues IIIc and IIIb to EF-G and the demonstration of a true competitive relationship (Figures 3 and 4). Figure 3 shows the competition with GTP-N_3 (IIIc) and Figure 4 that with GDP-N_3 (IIIb). The GTP-N_3 (IIIc) dissociation constant is 73 times that for GDP, and that for GDP-N_3 (IIIb) is 270 times greater. Since the apparent dissociation constant for GDP under these conditions is 0.07–0.10 μM (data not shown), the affinity of these analogues for the EF-G·ribosome·fusidic acid complex is still sufficiently high to make photoaffinity labeling studies feasible.

It is not clear whether or not the GTP-N_3 (IIIc) is completely resistant to hydrolysis by the EF-G·ribosome complex. However, even if there were some hydrolysis followed by exchange with the added [^{3}H]GDP despite the presence of fusidic acid, its magnitude must be very small relative to the effects observed here, since in a control experiment varying the in-

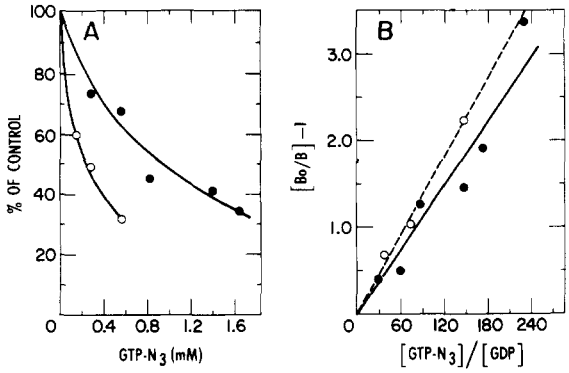


FIGURE 3: Competitive binding between GTP-N_3 (IIIc) and GDP for EF-G of *E. coli*. (A) Binding assays were carried out as described under Experimental Procedures using 3.82 μM (○) or 9.56 μM (●) [^{3}H]GDP. One hundred percent binding corresponds to 17 pmoles bound to the filter. (B). Reciprocal plot of the data in part A as described in the text. Symbols as in part A.

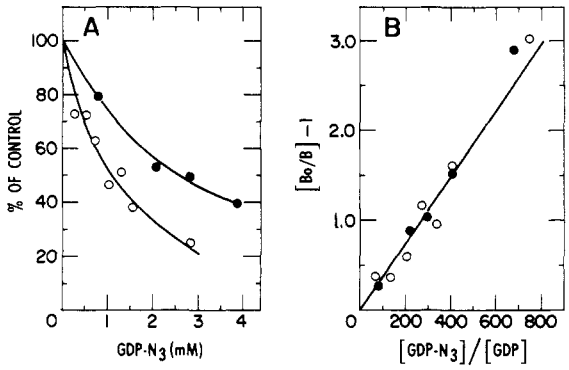


FIGURE 4: Competitive binding between GDP-N_3 (IIIb) and GDP for EF-G of *E. coli*. (A) Binding assays were carried out as described in the legend to Figure 3A. (B) Reciprocal plot of the data of part A as in Figure 3B.

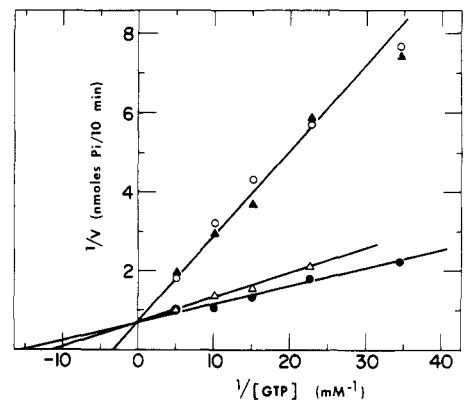


FIGURE 5: Competitive inhibition of the ribosome·EF-G GTPase by GTP-N_3 (IIIc) and GDP-N_3 (IIIb). Incubations were conducted as described under Experimental Procedures for 10 min at 30 °C with varying concentrations of GTP and 0.1 mM inhibitor, where present: (●) no inhibitor; (○) GDP; (▲) GTP-N_3 (IIIc); (△) GDP-N_3 (IIIb).

cubation time from 5 to 30 min had no effect on an approximately 50% inhibited reaction. If hydrolysis and exchange were significant, the amount of radioactivity bound in the complex should have continually decreased with time.

Interaction of the analogues with EF-G was also measured by inhibition of the ribosome·EF-G GTPase (Figure 5). The inhibition was clearly competitive, a K_i of 28 μM being obtained for GTP-N_3 (IIIc) and a K_i of 330 μM for GDP-N_3 (IIIb). For comparison, the K_i for GDP was 28 μM and the K_m for GTP was 67 μM. As judged by this assay, in the absence of fusidic acid, the GTP analogue is recognized as well or better than GTP. Similar differences between the GTPase and binding assays were also noted by Eckstein et al. (1975) for the analogues they studied.

Discussion

We were led to the search for suitable photolabile GTP analogues by the report of Breslow et al. (1974) which described intermolecular insertion reactions of various phosphorylnitrenes generated by UV irradiation of phosphoryl-azides. It was reported that phosphorylnitrenes are very resistant to rearrangement and thus lead to excellent yields of products from attack on neighboring molecules. It follows that phosphorylnitrenes should be particularly suitable for applications in affinity labeling. The relatively short length of the azido group (ca. 2.4 Å in methyl azide, Cotton and Wilkinson, 1972) should provide minimal distortion when substituted on the γ-phosphorus atom of nucleoside triphosphates such as GTP, since, as was shown by Eckstein et al. (1975), the inhibitory activity of γ-alkyl and aryl esters of GTP in the ribosome·EF-G GTPase reaction decreased with the size and length of the substituent. We have chosen GTP-N_3 (IIIc) and GDP-N_3 (IIIb) as the first compounds to be investigated as potential photoaffinity labeling reagents for the ribosomal GTPase site and for the recognition sites on both EF-G and T_u. In these compounds, the active nitrene is directly attached to the terminal phosphorus atom and occupies the place normally taken up by a hydroxyl group.

As we show in this report, nucleoside 5′-phosphoazidates III are readily synthesized by nucleophilic displacement of activated phosphates by the azide ion. The compounds can easily be isolated in high purity and are sufficiently stable for biochemical experiments.

Although the anion-exchange method of Michelson (1964) has been used quite often for the synthesis of ω-esters of nucleoside polyphosphates (Eckstein et al., 1975) or similar reactions (Haley and Yount, 1972; Feldhaus et al., 1975), there has been a general lack of interest in the various side products. Our results with the synthesis of ATP-N_3 (IIIg) (and also GTP-N_3, IIIc) show that many products are formed during the reaction, some of them in significant amounts. The formation of some of these side products may be caused by the inherent instability of nucleoside 5′-triphosphates in pyridine due to a dismutation reaction (Verheyden et al., 1965) or by activation on either the α- or β-phosphorus, followed by nucleophilic attack by azide anion and cleavage of an ortho or diphosphate residue. Another possibility is the formation of cyclic adenosine 5′-trimetaphosphate (Glonek et al., 1974) as an intermediate in the reaction of ATP with diphenyl phosphochloridate, followed by displacement by azide ion on either the α-, β-, or γ-phosphorus atom. The formation of nucleoside 5′-phosphoazidates derived from higher adenosine polyphosphates may arise from the availability of the corresponding higher adenosine poly(5′-phosphates), phosphate, and polyphosphate ions (generated by disproportionation of, e.g., α-azido-ATP) in the reaction mixture. Also, nucleoside 5′-phosphoazidates may behave as phosphorylating agents per se (vide supra), due to the pseudohalogen nature of the azido group.

The photolytic properties of nucleoside 5′-phosphoazidate have been studied using GMP-N_3 (IIIa) and AMP-N_3 (IIId) because the course of photolysis is more easily monitored chromatographically with the monophosphates than with the triphosphates. We found that GMP-N_3 (IIIa) is photolyzed

relatively smoothly at wavelengths above 280 nm. Quite surprisingly, the photolysis of AMP-N_3 (IIId) is much slower. The reason for this significant difference is not apparent at this time.

GTP-N_3 (IIIc) and GDP-N_3 (IIIb) were investigated as inhibitors of the formation of the EF-T_u·GTP binary complex, of the ribosome·EF-G·GDP·fusidic acid complex, and of the ribosome-dependent GTPase activity of EF-G in order to assess their ability to be recognized by these factors. As is clear from our results, both compounds IIIb and IIIc act as competitive inhibitors in all three assays. In the case of EF-T_u, the affinity of GTP-N_3 (IIIc) is similar to GDPCP which can also form a binary complex with EF-T_u (Arai et al., 1976; Miller and Weissbach, 1974), while the affinity of GDP-N_3 (IIIb) was ten times weaker. Since the affinity of GDP is 100 times greater even than GTP, it is clear that GDP-N_3 (IIIb) is a much poorer analogue of GDP than of GTP. In the case of EF-G, the affinity of GDP-N_3 (IIIb) was 12 times weaker than GTP-N_3 (IIIc) as a competitive inhibitor of the GTPase reaction. While both azides (IIIb and IIIc) were much less tightly bound than GDP in the binding assay, carried out in the presence of fusidic acid, GTP-N_3 (IIIc) was as good a competitor as GDP in the GTPase assay done in the absence of fusidic acid. Moreover, the K_i for GTP-N_3 (IIIc), 28 μM, was similar to the dissociation constant of 7 μM calculated for the binding reaction in the presence of fusidic acid. This result is in general agreement with the observations of Eckstein et al. (1975) that fusidic acid does not appear to stabilize the binding of unhydrolyzable GTP derivatives.

The extensive series of compounds studied by Eckstein et al. (1975) are mostly not comparable to our derivatives IIIc and IIIb. The one compound analogous to IIIb, namely, GDP-β-NH_2, had a K_i of 240 μM in the GTPase assay, while the K_i for GDP-N_3 (IIIb) was found here to be 330 μM. In general, these authors observed that the GTP analogues were better competitors than the GDP analogues in EF-G reactions and this trend is confirmed in our work. Eckstein et al. (1975) also reported that none of the γ-substituted analogues of GTP (with the exception of guanosine 5′-*O*-(3-thiotriphosphate)) were hydrolyzed by EF-G and ribosomes. Our work shows similarly that GTP-N_3 (IIIc) is not rapidly hydrolyzed to GDP, although it is not clear whether or not GTP-N_3 (IIIc) is completely resistant to hydrolysis. Thus, both the EF-G·ribosome complex and EF-T_u can tolerate the substitution by a relatively short azido group on the γ-phosphate of GTP without appreciable loss of affinity for the active site. GTP-N_3 in radioactive form should, therefore, be useful for investigation of the binding sites for GTP on EF-T_u, EF-G, and ribosomes.

Recently, Girshovich et al. (1976) have reported photoaffinity labeling studies of the GTP-binding site in the EF-G·ribosome complex using a γ-phosphate derivative of GTP whose active nitrene was located 8 Å from the terminal phosphorus atom. They observed only labeling of the EF-G molecule with this derivative, while Massen and Möller (1974) earlier had reported labeling only ribosomal proteins using a very similar GDP analogue whose nitrene was 7 Å from the terminal phosphorus. Since the active nitrene derived from GTP-N_3 is only ca. 1.5 Å from the terminal phosphorus, it should be possible to more specifically determine the nature of the GTPase catalytic center with this and similar compounds.

Finally, it should be pointed out that the relatively easy accessibility of hitherto unknown nucleoside 5′-phosphoazidates should make feasible structural studies with other enzymes which use nucleoside 5′-triphosphates as substrates or cofactors.

Acknowledgments

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