McPhalen, C. A., Svendsen, I., Jonassen, I., & James, M. N. G. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7242–7246.
Neurath, H. (1984) *Science (Washington, D.C.) 224*, 350–357.
North, A. C. T., Phillips, D. C., & Mathews, F. S. (1968) *Acta Crystallogr., Sect. A: Cryst. Phys., Diffr., Theor. Gen. Crystallogr. A24*, 351–359.
Ramakrishnan, C., & Ramanchandran, G. N. (1965) *Biophys. J. 5*, 909–933.
Read, R. J. (1986) *Acta Crystallogr., Sect. A: Found. Crystallogr. A42*, 140–149.
Read, R. J., & James, M. N. G. (1986) in *Proteinase Inhibitors* (Barrett, A. J., & Salvesen, J., Eds.) pp 301–336, Elsevier, Amsterdam.
Read, R. J., Fujinaga, M., Sielecki, A. R., & James, M. N. G. (1983) *Biochemistry 22*, 4420–4433.
Rossman, M. G. (1972) *The Molecular Replacement Method*, International Science Review 13, Gordon & Breach, New York.
Sielecki, A. R., James, M. N. G., & Broughton, C. G. (1982) in *Crystallographic Computing, Proceedings of the International Summer School* (Sayre, D., Ed.) pp 409–419, Carleton University, Ottawa, Oxford University Press, Oxford.
Svendsen, I., Jonassen, I., Hejgaard, J., & Boisen, S. (1980) *Carlsberg Res. Commun. 45*, 389–395.
Svendsen, I., Hejgaard, J., & Chavan, J. K. (1984) *Carlsberg Res. Commun. 49*, 493–502.
Thiessen, W. E., & Levy, H. A. (1973) *J. Appl. Crystallogr. 6*, 309.

Synthesis and Biological Properties of 5-Azido-2′-deoxyuridine 5′-Triphosphate, a Photoactive Nucleotide Suitable for Making Light-Sensitive DNA†

Robert K. Evans
Department of Microbiology/Biochemistry, The University of Wyoming, Laramie, Wyoming 82071
Boyd E. Haley*
Lucille Parker-Markey Cancer Center and Division of Medicinal Chemistry, University of Kentucky, Lexington, Kentucky 40536

Received June 24, 1986; Revised Manuscript Received September 18, 1986

ABSTRACT: A photoactive nucleotide analogue of dUTP, 5-azido-2′-deoxyuridine 5′-triphosphate (5-N_3dUTP), was synthesized from dUMP in five steps. The key reaction in the synthesis of 5-N_3dUTP is the nitration of dUMP in 98% yield in 5 min at 25 °C using an excess of nitrosonium tetrafluoroborate in anhydrous dimethylformamide. Reduction of the resulting 5-nitro compound with zinc and 20 mM HCl gave 5-aminodeoxyuridine monophosphate (5-NH_2dUMP). Diazotization of 5-NH_2dUMP with HNO_2 followed by the addition of NaN_3 to the acidic diazonium salt solution gave a photoactive nucleotide derivative in 80–90% yield. The monophosphate product was identified as 5-N_3dUMP by proton NMR, UV, IR, and chromatographic analysis as well as by the mode of synthesis and its photosensitivity. After formation of 5-N_3dUTP through a chemical coupling of pyrophosphate to 5-N_3dUMP, the triphosphate form of the nucleotide was found to support DNA synthesis by *Escherichia coli* DNA polymerase I at a rate indistinguishable from that supported by dTTP. When UMP was used as the starting compound, 5-N_3UTP was formed in an analogous fashion with similar yields and produced a photoactive nucleotide which is a substrate for *E. coli* RNA polymerase. To prepare [γ-^{32}P]-5-N_3dUTP for use as an active-site-directed photoaffinity labeling reagent, a simple method of preparing γ-^{32}P-labeled pyrimidine nucleotides was developed. [γ-^{32}P]-5-N_3dUTP is an effective photoaffinity labeling reagent for DNA polymerase I and was found to bind to the active site with a 2-fold higher affinity than dTTP. The photoactivity of 5-N_3dUMP is stable to extremes of pH, and [γ-^{32}P]-5-N_3dUTP was an effective photolabeling reagent even in the presence of 10 mM dithiothreitol. 5-Azidouracil-containing nucleotides have potential applications as active-site-directed photoaffinity labeling reagents and as tools for generating photoactive DNA and RNA to study nucleic acid binding proteins.

A variety of photoactive nucleotide analogues using aryl azides to generate nitrenes upon photolysis have been synthesized and used to study nucleotide binding proteins (Czarnecki et al., 1979; Bayley & Knowles, 1977; Guillory & Jeng, 1983). The 8-azidopurine nucleotides in particular have been used to study enzymes such as the (Na,K)-ATPase of the erythrocyte membrane (Haley & Hoffman, 1974), the Ca-ATPase of the sarcoplasmic reticulum (Briggs et al., 1980), and the beef heart mitochondrial F_1-ATPase (Wagenvoord et al., 1980). In addition, the 8-azidopurine nucleotide photoprobes have been used to label and identify amino acids in the active sites of the regulatory subunits of both type I and type II cAMP-dependent protein kinase (Bubis & Taylor, 1985; Kerlavage & Taylor, 1980) as well as in *Escherichia coli* RecA (Knight & McEntee, 1985). Because of the obvious importance of polymerases and of nucleic acid binding proteins in general, we were intrigued with the concept of developing photoactive nucleic acids to study protein–nucleic acid in-

†This work was supported by National Institutes of Health Grants GM35766 and CA 00563. This work was presented in partial fulfillment for the Ph.D. degree to R.K.E. from The University of Wyoming.

0006-2960/87/0426-0269$01.50/0

teractions. Since the 8-azidopurine nucleotides are not substrates for template-directed polymerases (Woody et al., 1984; Czarnecki, 1978), we elected to synthesize a photoactive derivative of a pyrimidine nucleotide which could serve as a substrate for DNA synthesis. We recently reported the preparation of 5-azidodeoxyuridine triphosphate (5-N_3dUTP)[1] and its use to synthesize a photoactive *lac* operator (Evans et al., 1986). Cross-linking of *lac* repressor to this photoactive *lac* operator was demonstrated and was found to be totally dependent on UV illumination. We report here the chemical synthesis, characterization, and some biological properties of 5-N_3dUTP.

In the study of protein–DNA interactions, 5-N_3dUTP could prove to be an effective tool to identify amino acids in the binding site of DNA binding proteins. It has been suggested (Pabo & Lewis, 1982; Steitz et al., 1983; Pabo & Sauer, 1984) and experimental evidence supports the idea that the sequence specificity of DNA binding proteins like the catabolite gene activator protein (CAP), *cro* repressor, *lac* repressor, and other DNA binding proteins resides in base–amino acid interactions in the major groove of right-handed B-type DNA (Takeda et al., 1983; Ebright et al., 1984; Metzler et al., 1985; Ebright, 1986). Since the 5-N_3 group on 5-N_3dUMP-containing DNA replaces the 5-methyl group on thymine, which is located in the major groove (Metzler et al., 1985; Ebright et al., 1984), the nitrene produced upon photolysis would be in the proper location to insert into amino acids that are normally very close to or interact with the 5-methyl group of the thymine base. The involvement of the 5-methyl group of thymine in forming specific hydrophobic interactions with DNA binding proteins has been shown for the thymine at position −5 in the *cro* operator (Metzler et al., 1985) as well as the thymines at positions 6, 7, and 13 in the *lac* operator (Caruthers et al., 1983; Goeddel et al., 1978). In addition, the technique of photochemical cross-linking to 5-bromouracil-substituted DNA has shown that 13 of 17 thymines in the *lac* operator region of a 55 bp DNA fragment make contact with bound *lac* repressor (Ogata & Gilbert, 1977).

Therefore, the use of 5-N_3dUMP-containing DNA should allow the study of protein–DNA interactions in a direct manner which probes for extremely close interactions between amino acids in a DNA binding protein and a specific photoactive base in the binding site. Through the study of such interactions, the question of how proteins specifically bind to the DNA may be directly addressed.

Materials and Methods

Nonradioactive nucleotides, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and nucleoside-5′-diphosphate kinase were purchased from Sigma. Nitrosonium tetrafluoroborate is a product of Aldrich.

Escherichia coli DNA polymerase I (pol I) and activated calf thymus DNA were obtained from Pharmacia. *Escherichia coli* RNA polymerase was a generous gift from Dr. A.-Young Woody (Colorado State University) and was purified according to the procedure of Reisbig et al. (1979). [γ-^{32}P]-8-N_3ATP was prepared by the procedure of Czarnecki et al. (1979) as modified by Potter and Haley (1982). Radioactive inorganic phosphate (^{32}P) was purchased from ICN.

Proton magnetic resonance spectra were recorded on a Joel JNM-FX 270 Fourier-transform NMR on the free-acid form of the nucleotide in D_2O. Chemical shifts were measured relative to 3-(trimethylsilyl)propionic acid as an internal standard.

Infrared spectra were recorded on an Analect 6260 Fourier-transform infrared spectrophotometer equipped with an MCT detector. Spectra were recorded on the free-acid form of the nucleotides in anhydrous dimethylformamide, using an attenuated total reflectance sample cell. The infrared absorbance of the dimethylformamide solvent was subtracted electronically to yield the final spectrum of the nucleotide. Photolyzed samples were irradiated with an UVS-11 Mineralight lamp (7000 μW/cm^2, at 254 nm) from a distance of 2 cm for 5 min. Ultraviolet spectra were recorded on a Beckman Model 25 spectrophotometer.

Thin-layer chromatography was performed by the ascending method using Eastman-Kodak cellulose plates (13255) with fluorescent indicator or with PEI–cellulose F plates (5579-7) from EM. Solvents for TLC were the following: solvent system A, isobutyric acid–NH_4OH–H_2O (66:1:33 v/v); system B, 0.3 M NH_4HCO_3; system C, 0.5 M NH_4HCO_3.

The molar extinction coefficient (ϵ) of 5-N_3dUMP was determined by measuring the total phosphate content of a sample of 5-N_3dUMP with a known UV absorbance. Phosphate release from 5-N_3dUMP was accomplished by Mg(NO_3)$_2$ oxidation over a Bunsen burner (Ames & Dubin, 1960). Phosphate determinations were performed after phosphate release from 5-N_3dUMP, by the procedure of Fiske and Subbarow (1925).

To test the synthesized nucleotide analogues for the presence of the deoxyribose residue, we employed the use of the Dische color reaction (Dische, 1944) as modified by Buchanan (1951).

Pyrophosphate coupling to 5-N_3dUMP to produce 5-N^3dUTP was done according to the procedure of Michelson (1964).

The synthesis of [γ-^{32}P]-5-N_3dUTP requires the use of the procedure of Glynn and Chappell (1964) to generate 0.05 μmol of [γ-^{32}P]GTP. When 5 units of an additional enzyme, nucleoside-5′-diphosphate kinase, and 1.0 μmol of 5-N_3dUTP were included in the reaction, [γ-^{32}P]-5-N_3dUTP was generated over a 30-min period. The [γ-^{32}P]-5-N_3dUTP produced was separated from [γ-^{32}P]GTP on a benzylated-DEAE (BD) cellulose column (30 cm × 1.5 cm) with a 400-mL NH_4HCO_3 gradient from 10 mM to 1.0 M. Under these conditions, [γ-^{32}P]-5-N_3dUTP elutes after 180 mL of the gradient (0.45 M NH_4HCO_3) followed by [γ-^{32}P]GTP at 204 mL (0.51 M NH_4HCO_3).

Photolabeling experiments with pol I were performed in 50-μL samples in a buffer consisting of 20 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$; 2.5 μg of pol I was added to each sample, followed by a 15-s incubation at 2 °C and a 1.5-min photolysis with a UVS-11 mineralight lamp (2000 μW/cm^2 at 254 nm) from a distance of 5.5 cm. After photolysis, each sample was solubilized and subjected to SDS–PAGE as previously described (Owens & Haley, 1976). To quantitate the incorporation of ^{32}P, the labeled protein band was excised from the gel and counted by liquid scintillation counting.

Synthesis of 5-NO_2dUMP. A 100–200-μmol sample of 5′-dUMP (sodium salt) was converted to the free-acid form by applying the nucleotide to a 10-mL column of Dowex 50W resin (H^+ form) and eluting with water. The free-acid form

[1] Abbreviations: 5-N_3dUTP, 5-azidodeoxyuridine triphosphate; 5-NO_2dUMP, 5-nitrodeoxyuridine monophosphate; 5-NH_2dUMP, 5-aminodeoxyuridine monophosphate; 8-N_3ATP, 8-azidoadenosine triphosphate; pol I, *E. coli* DNA polymerase I; TCA, trichloroacetic acid; BD-cellulose, benzylated (diethylaminoethyl)cellulose; DMF, dimethylformamide; PEI, poly(ethylenimine); Tris, tris(hydroxymethyl)aminomethane; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; bp, base pair(s); TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

of 5′-dUMP was then evaporated to dryness at 45 °C under reduced pressure. Several additions and evaporations of anhydrous methanol were performed at 45 °C to remove traces of water. The dried dUMP was redissolved in 5.0 mL of anhydrous dimethylformamide (DMF) followed by the addition of 300 mg (2500 μmol) of nitrosonium tetrafluoroborate with rapid stirring under a dry nitrogen atmosphere. After 5 min, the nitration reaction was terminated by the addition of 1 mL of H_2O. The reaction volume was then reduced to approximately 1 mL by repeated coevaporation with methanol under reduced pressure at 30 °C. Three milliliters of 1 N HCl was added and allowed to stir for 15 min. The acid was then neutralized by the addition of NH_4OH followed by evaporation of excess NH_4OH. The neutralized sample was applied to a Sephadex G-10 column (40 cm × 1.5 cm) equilibrated with distilled H_2O. The first UV-absorbing fraction off the G-10 column was applied to a BD-cellulose column (30 cm × 1.5 cm) and eluted with a 400-mL gradient of NH_4HCO_3 (10 mM–0.3 M). Fractions containing 5-NO_2dUMP were identified by using routine UV spectral analysis. On the basis of the λ_{max} and extinction coefficient of 5-NO_2dUMP (Huang & Torrence, 1977), the yield of 5-NO_2dUMP was routinely 95–98%. After NH_4HCO_3 was removed by repeated coevaporations with water under reduced pressure, the final product was resuspended in methanol and stored at −20 °C.

Synthesis of 5-NH_2dUMP. 5-NO_2dUMP in methanol (10–20 μmol) was evaporated to dryness and resuspended in 5 mL of 20 mM HCl. This solution was then poured over approximately 10 g of clean granulated zinc and stirred until the UV absorbance of a sample indicated a λ_{max} of 265 nm, indicative of 5-NH_2dUMP (Luhrmann et al., 1973). At this point, the nucleotide was separated from the remaining zinc metal and the zinc washed with 10 mL of 20 mM HCl. The entire sample, including the 10-mL wash, was then neutralized with NH_4OH, applied to a DEAE-cellulose column (30 cm × 1.5 cm), and eluted with an NH_4HCO_3 gradient (10 mM–0.3 M). The final product was desalted and stored in methanol at −20 °C as above. On the basis of the known λ_{max} and extinction coefficient of 5-aminouridine (Roberts & Visser, 1952), the yield of 5-NH_2dUMP was generally in the range of 60–70%.

Synthesis of 5-N_3dUMP. 5-N_3dUMP was produced by the addition of NaN_3 to an acidic solution containing the unisolated diazonium salt of the nucleotide. First, 5.0 mL of 1 N HCl containing 10 μmol of 5-NH_2dUMP was placed to stir in an ice–water bath for 15 min. Then the diazonium salt was generated by the addition of 11.0 μmol of $NaNO_2$ in 3.0 mL of H_2O. After stirring for 2 min at 0 °C, 1.5 mL of 4 M NaN_3 was added with rapid stirring. The reaction was allowed to stir for 5 min at 0 °C, then removed from the ice water, and allowed to warm to room temperature for 30 min. The reaction mixture was then neutralized to a pH of 7.0 with NH_4OH and desalted by gel filtration chromatography on a Sephadex G-10 column as described above. The first UV-absorbing fraction off the G-10 column was then applied to a BD-cellulose column (30 cm × 1.5 cm) and eluted with an NH_4HCO_3 gradient as above. The NH_4HCO_3 was removed as above followed by application of the sample to a 50-mL Dowex 50W column to remove residual NH_4^+. The free-acid form of the nucleotide was stored in methanol at −20 °C. The yield of 5-N_3dUMP was typically 80–90%, from 5-NH_2dUMP.

Results

There are two generalized methods for the synthesis of aryl azides (Guillory & Jeng, 1983). One is the nucleophilic displacement of a bromine, chlorine, or nitro group by azide ion. The other involves the addition of sodium azide to an acidic solution containing a diazotized primary aromatic amine. The first method is routinely used to synthesize the 8-azidoadenosine and 8-azidoguanosine compounds from their respective 8-bromonucleotides (Czarnecki et al., 1979). When this method was applied toward the synthesis of 5-azidouridine from 5-nitrouridine derivatives, 2-oxo-8-azapurines were formed which result from the intramolecular cyclization of the azido group after attack at C-6 (Blank & Fox, 1968). A similar result was obtained when 5-bromouridine derivatives were used (Bradshaw & Hutchinson, 1977). Because of these difficulties, we elected to synthesize 5-N_3dUMP using the diazotization method. It had previously been shown that 5-aminouridine could be synthesized and easily diazotized in high yield with nitrous acid (Roberts & Visser, 1952). Since the previous synthesis of 5-aminouridine required the displacement of the bromine on 5-bromouridine by liquid ammonia at 50 °C for 5 days, a simpler and more convenient method applicable to a nucleotide monophosphate starting material was needed. We felt that a pathway involving a nitration reaction followed by reduction to the amine would be a reasonable approach to a simpler synthesis of 5-NH_2dUMP.

The nitration of uridine nucleosides has traditionally been by procedures involving the use of nitric acid and sulfuric acid on nucleosides in which the ribose hydroxyls are protected (Wempen et al., 1960). More recently, Huang and Torrence (1977) adapted the strong nitrating agent nitronium tetrafluoroborate to the nitration of various unprotected pyrimidine bases with some success. The disadvantages of nitronium tetrafluoroborate under the conditions employed were that it caused extensive glycosyl bond rupture on uridine and deoxyuridine as well as low yields (28–42%) of 5-NO_2dUMP and the formation of a 3′-O-nitration product in 28–42% yield when nitrating dUMP.

It has been known for some time that nitration reactions can also proceed by a pathway in which nitrosation is followed by oxidation of the resulting nitroso group to a nitro group (March, 1977). With this approach in mind, we used nitrosonium tetrafluoroborate to synthesize 5-NO_2dUMP from dUMP in yields that routinely exceed 95%. On the basis of the known extinction coefficients for dUMP (1.0×10^4 M^{-1} cm^{-1}) and 5-NO_2dUMP (9.0×10^3 M^{-1} cm^{-1}), the conversion of dUMP to 5-NO_2dUMP was nearly quantitative. The nitration product produced by this reaction has the same λ_{max} as that produced by nitronium tetrafluoroborate in both acid (λ_{max} 302 nm, 1 N HCl) and base (λ_{max} 321 nm, pH 12.0). Only one UV-absorbing compound could be observed after separation on cellulose (R_f 0.48) or PEI–cellulose (R_f 0.52) TLC plates using solvent systems A or B, respectively (Table I). In all cases, the UV-absorbing compound observed on the developed TLC plate gave a positive Dische color reaction, indicating an intact glycosyl bond. Although it appears possible to produce a 3′-O-nitration product (R_f 0.63 on cellulose, solvent A) with nitrosonium tetrafluoroborate, this product was completely eliminated by the use of short reaction times (5 min) followed by treatment in 1 N HCl for 15 min.

When 5-NO_2dUMP was reduced with zinc and HCl, the reduced product had the same λ_{max} as 5-NH_2UMP produced previously (Luhrmann et al., 1973) in both acid (λ_{max} 265 nm, 1 N HCl) and base (λ_{max} 290 nm, pH 12.0). TLC analysis of the reduced product revealed only one UV-absorbing compound on both cellulose (R_f 0.44) and PEI–cellulose (R_f 0.67) using solvent systems A and B, respectively (Table I). The

Table I: Chromatographic Properties of Pyrimidine Nucleotide Analogues

nucleotide	R_f[a] values for PEI/solvent B	cellulose/solvent A	PEI/solvent C
dUMP	0.73	0.50	
5-NO_2dUMP	0.52	0.48	
5-NH_2dUMP	0.67	0.44	
5-N_3dUMP	0.55	0.42	
dUTP		0.22	0.65
5-N_3dUTP		0.29	0.58

[a] R_f values were determined by using two of three TLC systems. Solvent A = isobutyric acid–concentrated NH_4OH–H_2O (66:1:33 v/v); solvent B = 0.3 M NH_4HCO_3; solvent C = 0.5 M NH_4HCO_3. Compounds were observed by using UV illumination on the developed TLC plates, which include a fluorescent indicator.

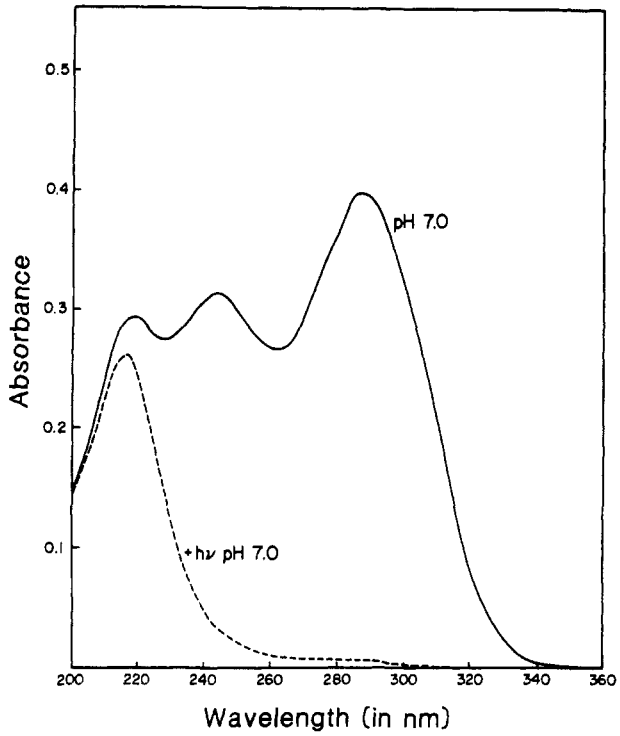

FIGURE 1: UV spectra of 5-N_3dUMP in 20 mM Tris, pH 7.0, before and after photolysis with a hand-held UVS-11 Mineralight lamp (7000 $\mu W/cm^2$ at 254 nm) from a distance of 3 cm for 1 min. ϵ for 5-N_3dUMP is 7.6×10^3 M^{-1} cm^{-1} at 288 nm.

yield of 5-NH_2dUMP from 5-NO_2dUMP was generally in the range of 60–70%.

To produce 5-N_3dUMP, the 5-NH_2dUMP product must first be diazotized with nitrous acid. Since diazo compounds are unstable above 0 °C, it was necessary to keep the reaction at or below this temperature and use the diazonium compound without isolation. When 5-NH_2dUMP was diazotized with nitrous acid and then placed in the presence of NaN_3, a photoactive nucleotide was generated. The photoactive product was first desalted on a Sephadex G-10 column and purified by BD-cellulose chromatography to yield a compound with the UV spectrum seen in Figure 1. From this figure, it is evident that the compound is very photoactive and produces an after-photolysis product that lacks the characteristic absorption spectrum of an aromatic compound. TLC analysis showed the presence of only one UV-absorbing compound on cellulose (R_f 0.42) and PEI–cellulose (R_f 0.55) using solvent systems A and B, respectively (Table I). A Dische color reaction on the developed TLC plate indicated the presence of deoxyribose at the same point where UV absorbance was observed.

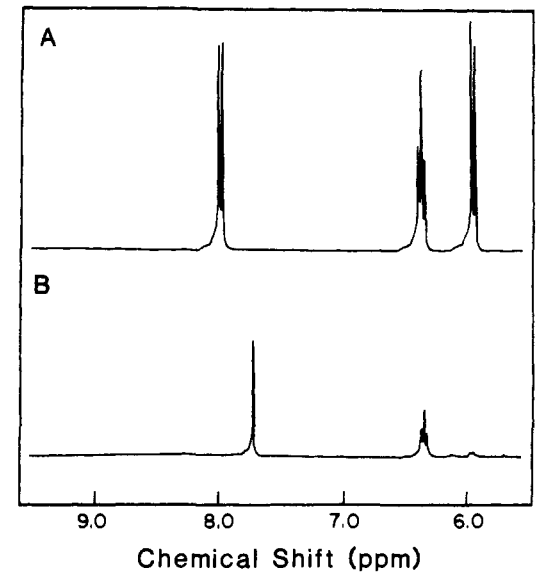

FIGURE 2: Fourier-transform proton NMR spectra of dUMP (A) and 5-N_3dUMP (B) in D_2O. Nucleotide concentrations were 26.5 mM for 5-N_3dUMP and 50 mM for dUMP.

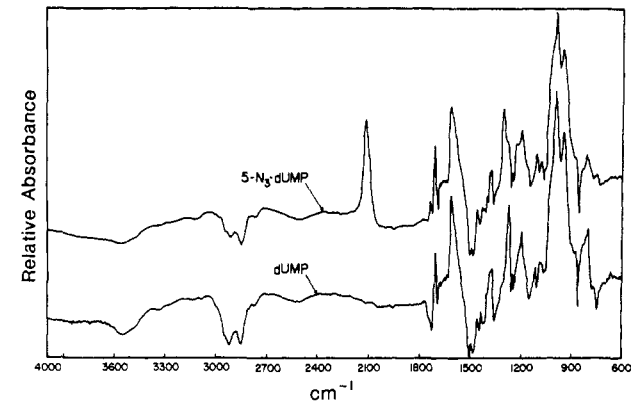

FIGURE 3: Fourier-transform infrared spectra of dUMP (lower) and 5-N_3dUMP (upper) in anhydrous DMF. Nucleotide concentrations were 26.5 mM for 5-N_3dUMP and 23.7 mM for dUMP.

Structural Characterization of 5-N_3dUMP. To unambiguously show that the photoactive product is substituted on the C-5 carbon, proton NMR spectra of dUMP and the photoactive product were recorded and are shown in Figure 2. Figure 2 shows how the NMR spectrum of dUMP changes after the addition of the photoactive group. Only the chemical shifts assigned to the H-6 (8.0 ppm), H-5 (6.0 ppm), and H–C_1 (6.39 ppm) are shown (Jardetzky & Jardetzky, 1960; Schweizer et al., 1968). It is apparent in Figure 2 that the addition of the photoactive group causes a collapse of the H-6 doublet to a singlet and the disappearance of the H-5 doublet. The resonance of H–C_1 appears unchanged. These data are entirely consistent with the photoactive substituent being located on the C-5 carbon.

To show that the substituent at C-5 contains double-bonded nitrogen atoms, we have used Fourier-transform infrared spectral analysis to look for the presence of the characteristic absorption band of the azide group near 2100 cm^{-1} (Treinin, 1971). Figure 3 shows the IR spectrum of dUMP and the photoactive product displaced from one another to illustrate the characteristic peak at 2117 cm^{-1}. This spectrum shows that the photoactive product contains at least two additional nitrogen atoms forming a double bond. This suggests the presence of either the azido group or the diazonium group produced via diazotization of 5-NH_2dUMP.

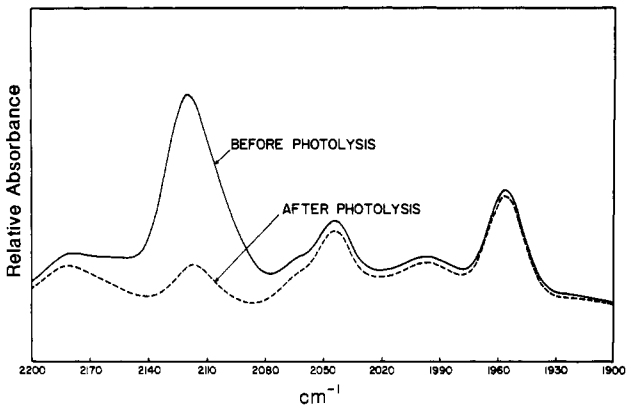

FIGURE 4: IR spectra of a portion of Figure 3 (for 5-N_3dUMP only) showing the effect of UV light on the azide absorption band at 2117 cm^{-1}. The sample was photolyzed for 5 min from 2 cm with the lamp described in Figure 1.

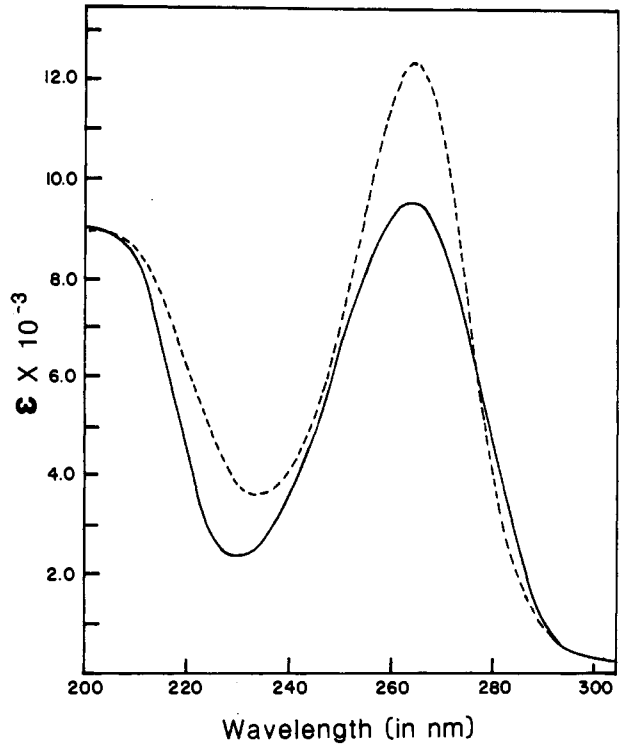

FIGURE 5: UV spectra of 5-NH_2dUMP (—) and 5-diazo-dUMP (---) showing the effect of nitrous acid on 5-NH_2dUMP. Spectra were recorded in 1 N HCl. ϵ for 5-diazo-dUMP is 1.24×10^4 M^{-1} cm^{-1}.

Figure 4 shows that the characteristic IR absorbance peak is greatly reduced by exposure of the compound to UV light. This suggests that the group producing the IR absorbance peak is photoactive. This is also consistent with the photoactive moiety being an azido or a diazonium group.

Figure 5 shows the change in the UV spectrum of 5-NH_2dUMP which occurs with diazotization of the amino group. It is apparent in this figure that the diazotized compound differs considerably from 5-NH_2dUMP in molar extinction coefficient. As reported previously, ϵ for 5-diazodeoxyuridine is 1.24×10^4 M^{-1} cm^{-1} (Paolini et al., 1963). As can be seen in Figure 5, the UV spectrum of 5-diazo-dUMP is quite different from that of the photoactive compound shown in Figure 1. Since NaN_3 addition to 5-diazo-dUMP is required to produce the compound analyzed in Figure 1, we feel this unambiguously identifies the photoactive base as 5-azidouracil. Since the triphosphate form is a substrate for pol I (Evans et al., 1986) and gives a positive Dische color reaction for deoxyribose, we feel confident that the photoactive compound

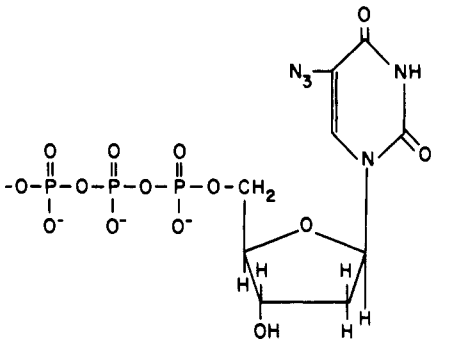

FIGURE 6: Structure of 5-N_3dUTP.

Table II: Effects of Solvent and Temperature on Stability of 5-N_3dUMP

solvent	temp (°C)	decrease in 5-N_3dUMP concn[a]/time
20 mM Tris, pH 7.5	22	2%/h
20 mM Tris, pH 7.5	37	10%/h
20 mM Tris, pH 7.5, + 10 mM MeSH	25	17.3%/h
H_2O (pH 7.0)	25	2%/h
H_2O (pH 7.0)	60	4%/min
H_2O (pH 7.0)	−20	5%/week
H_2O (0.1 N NH_4OH)	25	2%/h
H_2O (0.1 N HCl)	25	2%/h
CH_3OH	−20	stable for 1 year
CH_3OH	60	10%/min
DMF	5	stable
DMF	50	stable

[a] The decrease in 5-N_3 dUMP concentration was determined by the drop in absorbance at 288 nm as a percent of the initial absorbance. Since the decrease in concentration with time is not linear, only the initial decrease in absorbance was recorded. MeSH = β-mercaptoethanol.

is 5-N_3dUTP. The structure of 5-N_3dUTP is shown in Figure 6 in the normal "anti" configuration.

Stability of 5-N_3dUMP. The usefulness of 5-N_3dUMP as a research tool could be severely limited by an inherent instability toward factors such as visible light, heat, or chemical factors such as extremes of pH. Therefore, several tests were performed to determine the stability of 5-N_3dUMP under various conditions. Table II summarizes our findings with regard to solvent and temperature. It is evident from these data that 5-N_3dUMP is prone to self-destruction, presumably via intramolecular rearrangement initiated by nitrene production (Bayley & Knowles, 1977). The result of heating 5-N_3dUMP in aqueous solutions is similar to the result produced by photolysis as shown in Figure 1. The heat-destroyed product, like the after-photolysis product, lacks the characteristic UV absorbance of the heterocyclic aromatic ring. Fortunately, 5-N_3dUMP is stable to heat in nonaqueous solvents such as DMF (Table II) and is only moderately affected by room temperature in aqueous solutions. 5-N_3dUMP is completely stable in anhydrous methanol at −20 °C and has been stored for periods up to 1 year without significant loss of absorbance. The slight instability in aqueous solutions should pose no problems unless experiments are over long periods of time or done at elevated temperatures.

The photoactivity of 5-N_3dUMP is also stable to extremes of pH. Photolysis in 1 N NaOH or 1 N HCl after 1-h incubations showed no loss in photoactivity over control samples in distilled water (data not shown). Therefore, extremes of pH cause no loss in photoactivity within these limits and should not cause any problems in the design of experiments.

Biological Properties of 5-N_3dUTP. To test the biological properties of 5-N_3dUMP, it was first necessary to synthesize

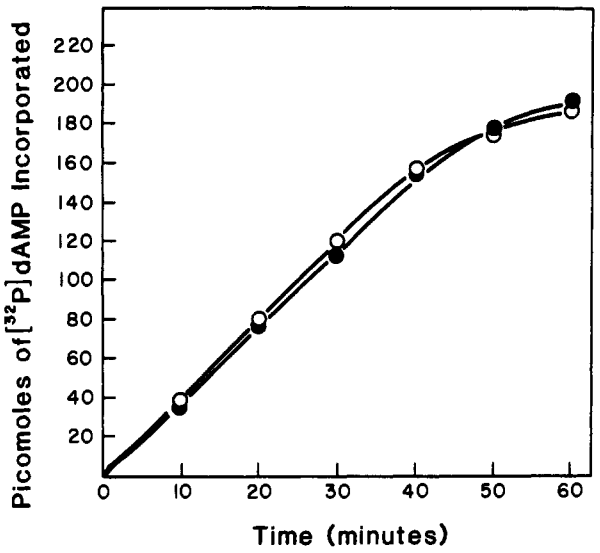

FIGURE 7: Nick translation assay showing the incorporation of [^{32}P]dAMP into activated calf thymus DNA by DNA polymerase I. The control reaction (○) contained dTTP at 10 μM, while the other reaction (●) contained 10 μM 5-N_3dUTP in place of dTTP. Each reaction also contained 40 μM dATP, dGTP, and dCTP as well as 2.9 μg of activated calf thymus DNA and 9 μCi of [α-^{32}P]dATP in a buffer consisting of 20 mM Tris–acetate, pH 7.4, 0.1 mM EDTA, and 10 mM $Mg(OAc)_2$. Each reaction was started by the addition of 1 unit of DNA polymerase I. Aliquots (2 μL) were removed from each 50-μL reaction at various times and spotted on a PEI–cellulose TLC plate. The dried TLC plate was developed in 0.5 M NH_4HCO_3 (ascending method) until the solvent front had moved 10 cm above the origin. Under these conditions, the activated calf thymus DNA remains within 1 cm of the origin while the [α-^{32}P]dATP migrates at R_f 0.65. The amount of [^{32}P]dAMP incorporated was determined by counting the TLC plate from the origin to R_f 0.2 by liquid scintillation counting.

the triphosphate form of the nucleotide. This was accomplished via a pyrophosphate coupling procedure (Michelson, 1964) to produce 5-N_3dUTP with recoveries in the range of 40%, an overall yield of 21% from dUMP.

Using 5-N_3dUTP produced in this manner, we have shown that it is a substrate for pol I and replaces only dTTP in the template-directed synthesis of photoactive DNA (Evans et al., 1986). To determine if the azido group reduces the rate of incorporation of 5-N_3dUTP as compared to dTTP, we have plotted the incorporation of [^{32}P]dAMP into calf thymus DNA over time, using either 5-N_3dUTP or dTTP as one substrate. The results are presented in Figure 7. The data clearly show that at 10 μM concentration, 5-N_3dUTP is just as effective a substrate as dTTP. Several other concentrations of 5-N_3dUTP and dTTP were used, both above and below 10 μM, with the same result (data not shown). Although all the positive control reactions incorporated approximately 110 pmol of [^{32}P]dAMP in 30 min, negative control reactions lacking dTTP incorporated less than 1 pmol of [^{32}P]dAMP in 30 min (data not shown).

To determine if the corresponding analogue 5-N_3UTP is a substrate for *E. coli* RNA polymerase, 5-N_3UTP was first made by using the same procedure as that used for 5-N_3dUTP. Table III shows the results of the RNA polymerase assay. Under the conditions employed, 5-N_3UTP is a substrate for RNA polymerase and was found to substitute for UTP but not for CTP. In addition, the after-photolysis product produced from 5-N_3UTP is obviously not a substrate for RNA polymerase or is an extremely poor one.

To synthesize [γ-^{32}P]-5-N_3dUTP for use as a photoaffinity labeling reagent, we developed an isotopic exchange procedure to exchange the γ-phosphate between [γ-^{32}P]GTP and 5-N_3dUTP. The principle of isotopic exchange is a common procedure used to label organic compounds (Purich & Allison, 1980) and is routinely used to produce [γ-^{32}P]ATP or [γ-^{32}P]GTP using an exchange between the γ-phosphate of ATP or GTP, and carrier-free ^{32}P, catalyzed by phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (Glynn & Chappell, 1964). Since pyrimidine nucleotides are not efficient substrates for these two enzymes, we included an additional enzyme, nucleoside-5′-diphosphate kinase, which has very broad substrate specificity at both the diphosphate and triphosphate nucleotide binding sites (Garces & Cleland, 1969). This allows the following reaction to be initiated when the additional enzyme is used.

$$5\text{-}N_3\text{dUTP} + \text{GDP} \rightleftharpoons 5\text{-}N_3\text{dUDP} + \text{GTP}$$

For this exchange process to operate, each of the four nucleotides must be present. Typically, 0.05 μmol of GTP and 1 μmol of 5-N_3dUTP are included in the reaction. GDP is provided in catalytic amounts from the reaction catalyzed by phosphoglycerate kinase, which also serves to prevent the accumulation of large amounts of GDP (Glynn & Chappel, 1964). When this exchange reaction was used, over 90% of the added ^{32}P was incorporated into GTP and 5-N_3dUTP. By using a ratio of 5-N_3dUTP to GTP of 20 or more, most (95%) of the incorporated ^{32}P will be in [γ-^{32}P]-5-N_3dUTP since [γ-^{32}P]GTP and [γ-^{32}P]-5-N_3dUTP will be of equal specific activity. GTP was chosen as one substrate over ATP because it is much easier to separate from 5-N_3dUTP using BD-cellulose chromatography. We have found this method to work for 5-N_3dUTP, 5-N_3UTP, UTP, and dUTP with equal success.

To determine the effect of the azido group of 5-N_3dUTP on the affinity of the nucleotide to DNA polymerase I, [γ-^{32}P]-5-N_3dUTP was used to photolabel the enzyme in the presence of increasing dTTP. Figure 8 shows the protection of [γ-^{32}P]-5-N_3dUTP photoincorporation obtained with increasing dTTP. This figure shows that photoinsertion occurs only in the presence of UV light and that dTTP protects less than would be expected if dTTP and 5-N_3dUTP bind to the

Table III: Incorporation of 5-N_3UMP into RNA by *E. coli* RNA Polymerase

reaction conditions[a]	cpm × 10^{-6} in TCA precipitate	cpm × 10^{-6} in incorporated [^{32}P]-AMP[b]	pmol of [^{32}P]AMP incorporated
complete	5.7	5.62	874
−UTP	1.08	1.0	156
−UTP, +5-N_3UTP	6.2	6.12	951
−CTP, +5-N_3UTP	1.0	0.92	144
−UTP, +prephotolyzed 5-N_3UTP	1.5	1.42	221
no enzyme control	0.08		

[a] Reaction conditions were the following: The complete 50-μL reaction contained 200 μM final concentration of all nucleotides except ATP, which was 227 μM. Each reaction also included 14.5 μg of activated calf thymus DNA, 33 μCi of [α-^{32}P]ATP (specific activity 25 mCi/μmol), and 3.6 μg of *E. coli* RNA polymerase in a buffer consisting of 5 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 100 mM KCl. Various deletions and additions to the complete reaction mix are indicated by − and +, respectively. All reactions were allowed to proceed for 30 min; then the RNA was precipitated by the addition of 1 mL of cold 7% trichloroacetic acid (TCA). The samples were then incubated on ice for 15 min and filtered through a Whatman GF/A glass fiber filter. Each filter was washed with 10 mL of cold 7% TCA, dried, and counted by liquid scintillation counting. [b] [^{32}P]AMP incorporation calculated by subtracting no enzyme control from total cpm in TCA precipitate. Each value recorded represents the average of three independent experiments.

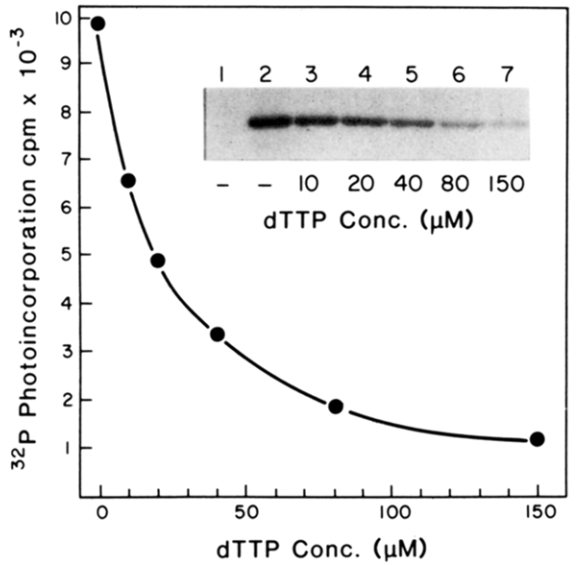

FIGURE 8: Photolabeling of DNA polymerase I by [γ-^{32}P]-5-N_3dUTP in the presence of an increasing concentration of dTTP. The photolabeled samples were subjected to SDS–PAGE on an 8% gel and autoradiography to show the labeled bands corresponding to pol I (see insert). The amount of ^{32}P incorporated in each band was determined by removing the radioactive band from the dried gel and counting it by using liquid scintillation counting. Lane 1 = no photolysis control.

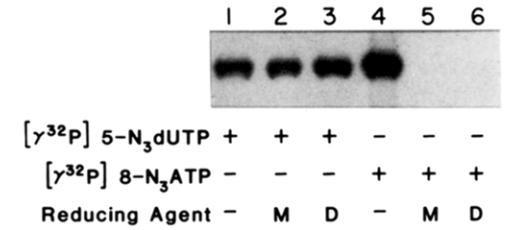

FIGURE 9: Photolabeling of DNA polymerase I with [γ-^{32}P]-5-N_3dUTP or [γ-^{32}P]-8-N_3ATP to show the effect of β-mercaptoethanol and dithiothreitol on photoincorporation. The presence or absence of each probe or reagent is indicated by a (+) or (–), respectively. Each nucleotide probe was present at a concentration of 20 μM. The specific activity of the [γ-^{32}P]-5-N_3dUTP was 5.5 mCi/μmol while the [γ-^{32}P]-8-N_3ATP was 6.5 mCi/μmol. β-Mercaptoethanol (M) and dithiothreitol (D) were present at a concentration of 10 mM. The photolabeling conditions are described under Materials and Methods.

active site of pol I with equal affinity. When the data from Figure 8 and the previously established K_d of 5-N_3dUTP binding to DNA polymerase I as 5 μM (Evans et al., 1986) are used, the K_d of dTTP binding to pol I is 9.5 μM (±1.0) (Ofengand & Henes, 1969). The dissociation constant for dTTP is thus 1.9 ± 0.2 times higher than that for 5-N_3dUTP under these conditions. This K_d is in the range observed by others for dTTP binding to pol I (Abraham & Modak, 1984; Englund et al., 1969). It is interesting to note that any steric hindrance the azido group might present to nucleotide binding is less important than the positive contributions to binding, possibly made through hydrogen bonding to the azido group.

When [γ-^{32}P]-5-N_3dUTP was used to photolabel DNA polymerase I in the presence of a reducing agent, an interesting result was obtained. Figure 9, lanes 2 and 3, shows that [γ-^{32}P]-5-N_3dUTP labels DNA polymerase I as well in the presence of 10 mM mercaptoethanol or 10 mM dithiothreitol as in the control lane (lane 1) without a reducing agent. In contrast, when [γ-^{32}P]-8-N_3ATP was used to label pol I, no labeling occurred in the presence of either reducing agent. Since [γ-^{32}P]-8-N_3ATP labels pol I very well without a reducing agent present (lane 4), this is undoubtedly caused by the reducing agent either reducing the azido group before photolysis or scavenging the nitrene produced from photolysis. Since the photolabeling conditions do not allow enough time for a significant portion of the azido analogue to be reduced to the amino compound (Potter & Haley, 1982; Cartwright et al., 1976), this reduction of photolabeling is probably due to the nitrene being scavenged by the reducing agent (Ruoho et al., 1973). Moreover, these reducing agents decrease photolabeling of many proteins in whole-cell homogenates and therefore do not seem to be affected by the orientation of the azido group on the protein-bound nucleotide (R. K. Evans and B. E. Haley, unpublished experiments). The scavenging effect of β-mercaptoethanol on [γ-^{32}P]-8-N_3ATP photolabeling has also been observed by King et al. (1982), when photolabeling rabbit skeletal muscle phosphorylase kinase. We suspect that for 5-N_3dUTP the intramolecular rearrangement believed to be responsible for loss of the conjugated ring system when photolyzed (Figure 1) also results in less susceptibility to nitrene scavenging by reducing agents. Generally, it is undesirable to have the photolabeling reagent undergo an intramolecular rearrangement upon photolysis because it reduces photoinsertion efficiency (Bayley & Knowles, 1977). However, in the case of 5-N_3dUTP, the intramolecular rearrangement may produce the unexpected benefit of the nitrene being less susceptible to scavenging from reducing agents used to keep proteins in the reduced state. Therefore, even though 5-N_3dUTP may have a lower photoinsertion efficiency than 8-N_3ATP, the benefit of being able to label proteins in the presence of high concentrations of reducing agents will increase its utility as a research tool.

Discussion

The synthesis of photoactive pyrimidine nucleotides provides access to a new class of nucleotide analogues which can serve as active-site-directed photoaffinity probes or as substrates for polymerizing enzymes to generate photoactive nucleic acids. Since these nucleotides are substrates for the template-directed synthesis of DNA and RNA, photoactive bases can be strategically positioned to resolve aspects of protein–nucleic acid interactions. In addition, the synthetic method provides a simple high-yielding route toward the synthesis of the nitro, amino, and diazo nucleotides. Considering the importance of the 5-substituted pyrimidine nucleotides in studying the inhibition of tumor cell growth (Balzarini et al., 1982) and viral replication (Allaudeen et al., 1981), we believe this will be an important contribution. Since diazonium groups are easily replaced by a variety of nucleophiles (March, 1977), easy access to 5-diazouridine nucleotides may provide a pathway for the synthesis of other useful 5-substituted uridine nucleotides. Since the conditions employed throughout the synthesis of 5-N_3dUMP are relatively mild, it may also be possible to synthesize homopolymers of 5-N_3dUMP. This approach could provide single-stranded photoactive DNA of perfectly defined length to study single-stranded DNA binding proteins. Some preliminary studies of our own have indicated that it is also possible to synthesize single-stranded poly(5-N_3dUMP) using terminal deoxynucleotidyl transferase with 5-N_3dUTP as the sole substrate (R. K. Evans and B. E. Haley, unpublished results).

Photochemical cross-linking is a technique that has been used with some success to study protein–nucleic acid interactions (Schimmel & Budzik, 1977). While this technique is potentially very powerful, its usefulness is limited by the low and comparatively similar photosensitivity of the nucleic acid bases. With the use of highly photoactive 5-azidouracil bases in the DNA, this technique may provide the increased efficiency of cross-linking necessary to form apple quantities of

a protein–DNA cross-linked complex for further study. Analysis of such complexes could then determine which amino acids were involved in forming the cross-link and the possibility of learning how particular amino acids interact with specific bases in the DNA.

ACKNOWLEDGMENTS

We thank Dr. Russell C. Middaugh, The University of Wyoming, for advice and help in performing the infrared spectra as well as Matthew Raymond for technical assistance.

Registry No. 5-N_3dUTP, 105449-09-0; 5-NO_2dUMP, 63689-79-2; 5-NH_2dUMP, 4603-58-1; UMP, 58-97-9; dUMP, 964-26-1; 5-N_3dUMP, 105426-34-4; 5-N_3UTP, 105518-68-1; [γ-^{32}P]GTP, 37156-72-2; [γ-^{32}P]-5-N_3dUTP, 105449-10-3; DNA polymerase, 9012-90-2; RNA polymerase, 9014-24-8; N_3^-, 14343-69-2; nitrosonium tetrafluoroborate, 14635-75-7; nucleoside-5′-diphosphate kinase, 9026-51-1.

REFERENCES

Abraham, K. I., & Modak, M. J. (1984) *Biochemistry 23*, 1176–1182.
Allaudeen, H. S., Kozarich, J. W., Bertino, J. R., & deClercq, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2698–2702.
Ames, B. N., & Dubin, D. T. (1960) *J. Biol. Chem. 235*, 769–775.
Balzarini, J., deClercq, E., Mertes, M. P., Shugar, D., & Torrence, P. F. (1982) *Biochem. Pharmacol.* 31, 3673–3682.
Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–114.
Blank, H. U., & Fox, J. J. (1968) *J. Am. Chem. Soc. 90*, 7175–7176.
Bradshaw, T. K., & Hutchinson, D. W. (1977) *Chem. Soc. Rev. 6*, 43–63.
Briggs, F. N., Al-Jumaily, W., & Haley, B. (1980) *Cell Calcium 1*, 205–215.
Bubis, J., & Taylor, S. S. (1985) *Biochemistry 24*, 2163–2170.
Buchanan, J. G. (1951) *Nature (London) 168*, 1091.
Cartwright, I. L., Hutchinson, D. W., & Armstrong, V. W. (1976) *Nucleic Acids Res. 3*, 2331–2339.
Caruthers, M. H., Beaucage, S. L., Efcavitch, J. W., Fisher, E. F., Goldman, R. A., deHaseth, P. L., Mandecki, W., Matteucci, M. D., Rosendahl, M. S., & Stabinsky, Y. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 411–418.
Czarnecki, J. J. (1978) Ph.D. Dissertation, The University of Wyoming, Laramie, WY.
Czarnecki, J., Geahlen, R., & Haley, B. (1979) *Methods Enzymol. 56*, 642–653.
Dische, Z. (1944) *Proc. Soc. Exp. Biol. Med. 55*, 217–218.
Ebright, R. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 303–307.
Ebright, R. H., Cossart, P., Gicquel-Sanzey, B., & Beckwith, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7274–7278.
Englund, P. T., Huberman, J. A., Jovin, T. M., & Kornberg, A. (1969) *J. Biol. Chem. 244*, 3038–3044.
Evans, R. K., Johnson, J. D., & Haley, B. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5382–5386.
Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.
Garces, E., & Cleland, W. W. (1969) *Biochemistry 8*, 633–640.
Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J. 90*, 147–149.
Goeddel, D. V., Yansura, D. G., & Caruthers, M. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3578–3582.
Guillory, R. J., & Jeng, S. J. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2826–2830.
Haley, B. E., & Hoffman, J. F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3367–3371.
Huang, G.-F., & Torrence, P. F. (1977) *J. Org. Chem. 42*, 3821–3824.
Jardetzky, C. D., & Jardetzky, O. (1960) *J. Am. Chem. Soc. 82*, 222–229.
Kerlavage, A. R., & Taylor, S. S. (1980) *J. Biol. Chem. 255*, 8483–8488.
King, M. M., Carlson, G. M., & Haley, B. E. (1982) *J. Biol. Chem. 257*, 14058–14065.
Knight, K. L., & McEntee, K. (1985) *J. Biol. Chem. 260*, 867–872.
Luhrmann, R., Schwarz, U., & Gassen, H. G. (1973) *FEBS Lett. 32*, 55–58.
March, J. (1977) in *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, pp 474, 610–612, McGraw-Hill, New York.
Metzler, W. J., Arndt, K., Tecza, E., Wasilewski, J., & Lu, P. (1985) *Biochemistry 24*, 1418–1424.
Michelson, A. M. (1964) *Biochim. Biophys. Acta 91*, 1–13.
Ofengand, J., & Henes, C. (1969) *J. Biol. Chem. 244*, 6241–6253.
Ogata, R., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4973–4976.
Owens, J. R., & Haley, B. E. (1976) *J. Supramol. Struct. 5*, 91–102.
Pabo, C. O., & Lewis, M. (1982) *Nature (London) 298*, 443–447.
Pabo, C. O., & Sauer, R. T. (1984) *Annu. Rev. Biochem. 53*, 293–321.
Paolini, J. P., Robins, R. K., & Cheng, C. C. (1963) *Biochim. Biophys. Acta 72*, 114–116.
Potter, R. L., & Haley, B. E. (1982) *Methods Enzymol. 91*, 613–633.
Pullman, B., & Pullman, A. (1969) *Prog. Nucleic Acid Res. Mol. Biol. 9*, 327–402.
Purich, D. L., & Allison, R. D. (1980) *Methods Enzymol. 64*, 3–46.
Reisbig, R. R., Woody, A.-Y. M., & Woody, R. W. (1979) *J. Biol. Chem. 254*, 11208–11217.
Roberts, M., & Visser, D. W. (1952) *J. Am. Chem. Soc. 74*, 668–669.
Ruoho, A. E., Kiefer, H., Roeder, P. E., & Singer, S. J. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2567–2571.
Schimmel, P. R., & Budzik, G. P. (1977) *Methods Enzymol. 46*, 168–180.
Schweizer, M. P., Broom, A. D., Ts'o, P. O. P., & Hollis, D. P. (1968) *J. Am. Chem. Soc. 90*, 1042–1055.
Steitz, T., Weber, I. T., & Matthew, J. B. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 419–426.
Takeda, Y., Ohlendorf, O. H., Anderson, W. F., & Matthews, B. W. (1983) *Science (Washington, D.C.) 221*, 1020–1026.
Treinin, A. (1971) in *The Chemistry of the Azido Group* (Patai, S., Ed.) p 22, Interscience, New York.
Wagenvoord, R. J., Kemp, A., & Slater, E. C. (1980) *Biochim. Biophys. Acta 593*, 204–211.
Wempen, I., Doerr, I. L., Kaplan, L., & Fox, J. J. (1960) *J. Am. Chem. Soc. 82*, 1624–1629.
Woody, A.-Y. M., Vader, C. R., Woody, R. W., & Haley, B. E. (1984) *Biochemistry 23*, 2843–2848.