the extent to which this intermediate can be built up. No diazo intermediate (1900–2300 cm^{-1}) was observed, nor were chlorooxirane[13] or methyl chloride formed.[13] Ketene and HCl do not arise from secondary photolysis of acetyl chloride. Independent experiments showed acetyl chloride to be photochemically stable at these wavelengths ($\lambda > 340$ nm), although higher energy irradiation ($\lambda > 270$ nm) cleanly produces ketene and HCl in an argon matrix.[14]

The identity of **1** as the initial photoproduct was confirmed by bimolecular reaction. Irradiation of a 3-methylpentane glass of **2** at 10 K produced the same IR absorptions as observed in argon. Warming the matrix to 80 K caused the disappearance of the photoproduct IR bands with concurrent generation of a mixture of azine **3** and dimer **4**.[13] These products are sensibly derived from reaction of carbene **1** with starting diazirine or with another carbene molecule, respectively.

The IR spectrum of **1** provides significant insight into its electronic structure. Of particular concern is the relative importance of π-bonding as indicated in **5**. A logical comparison may be made between the IR spectrum of **1** and that of chloromethyl methyl ether, for which a complete vibrational analysis has been reported.[15] Chloromethyl methyl ether shows a C–O–C antisymmetric stretch at 1120 cm^{-1} and has only CH_2 deformations in the region from 1200 to 1400 cm^{-1}.[15] In contrast, a major band in the IR spectrum of **1** appears at 1300 cm^{-1}. Deuterium labeling shows that this absorption is not due to a methyl deformation. Irradiation of the trideuterio analogue of **2** in an argon matrix gives the trideuterio carbene, with IR absorptions at 2178, 1370 (weak), 1330, 1324, 1073, 1053, 926, 807, 755, 680, and 386 cm^{-1}. In particular, methyl absorptions in the 1450-cm^{-1} region are absent, but the ca. 1300-cm^{-1} band remains. We thus assign this absorption to the C–O–C antisymmetric stretch of the carbene.[16] The higher frequency compared to the corresponding stretch in $ClCH_2OCH_3$ indicates considerable C–O double-bond character. This conclusion is supported by ab initio calculations by Schaefer and co-workers, who predict the C–O stretch in hydroxymethylene to be 1381 cm^{-1}.[17,18]

In contrast to thermolysis results,[8,9] matrix isolated **1** rearranges photochemically to acetyl chloride. This transformation likely involves either a concerted 1,2-methyl migration or C–O bond cleavage to give a radical pair followed by recombination. More enigmatic is the conversion of **1** to ketene and HCl. Although it is a logical intermediate, acetyl chloride gives ketene at shorter wavelengths only. An appealing mechanism thus involves the adiabatic conversion[19] of electronically excited carbene to electronically excited acetyl chloride. The excited acetyl chloride subsequently cleaves to HCl and ketene. Simple-minded calculations indicate that the adiabatic conversion is energetically feasible. Rondan, Houk, and Moss[4] have calculated (4-31G) the energy for the isodesmic reaction, $:CH_2 + CH_3Cl + CH_3OCH_3 \rightarrow ClCOCH_3 + 2CH_4$, to be −60.3 kcal/mol. Using the known heats of formation for the other species[20] in this equation gives an estimated $\Delta H_f°$ of 2.9 kcal/mol for **1**. Our photochemical results indicate that the longest wavelength where rearrangement of the carbene takes place is 360 nm. Combining this energy, the heats of formation of acetyl chloride[20] and **1** and the UV spectrum of acetyl chloride[21] indicates that rearrangement of excited singlet **1** to excited singlet acetyl chloride is exothermic by ca. 30 kcal/mol. Although the topology of the relevant energy surface is not known, the reaction clearly fulfills at least the energetic requirements for an adiabatic photoreaction.[19] Since acetyl chloride does not fluoresce, this mechanism must remain tentative at this time. Further studies on the chemistry and structure of **1** are in progress.[22]

Acknowledgment. We thank the Camille and Henry Dreyfus Foundation for support of this work. The infrared spectrometer and lamp were purchased in part with funds from the National Science Foundation (CHE-81173180).

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A General Procedure for Assigning the ^{31}P Spectra of Nucleic Acids

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One of the more promising approaches to the investigation of the conformations and molecular dynamics of polynucleotides is the use of phosphorus-31 nuclear magnetic resonance (^{31}P NMR).[1-5] The full potential of ^{31}P NMR has not been utilized since there has been no unambiguous method for assigning the signals. Thus, the information present in the chemical shifts has not been fully exploited, nor have the molecular dynamics of distinct sites been well explored.

Unlike many other elements, phosphorus does not have a suitable isotope for the purpose of assigning NMR resonances. Thus, we were led to develop the use of ^{17}O directly bonded to the phosphorus of polynucleotides as a means of specifically and unobtrusively labeling particular sites. The introduction of ^{17}O induces an appreciable increase in the line width of the ^{31}P resonances of polynucleotides and hence presents an unambiguous assignment procedure that is also applicable to nucleic acid–protein complexes.

For the phosphorus nuclei of polynucleotides there are two main contributions to transverse relaxation: dipolar and chemical shift anisotropy.[1-5] The two pathways contribute about equally to the transverse relaxation of ^{31}P when the experiment is performed at 4.7 T.[1-5] The dipolar relaxation is due to the 5′, 5″, and 3′ protons of the polynucleotide, which are between 0.26 and 0.28 nm away from the phosphorus nucleus. When the phosphorus is directly bonded to ^{17}O two additional relaxation pathways come into play. Scalar relaxation of the second kind arises from rapid modulation of the ^{31}P–^{17}O scalar coupling due to the quadrupolar relaxation of the ^{17}O and is given by[6]

$$\frac{1}{T_{2SK}} = \frac{1}{3}S_Q(S_Q + 1)J^2\left(T_{1Q} + \frac{T_{2Q}}{1 + (\omega_P - \omega_Q)^2 T_{2Q}^2}\right) \quad (1)$$

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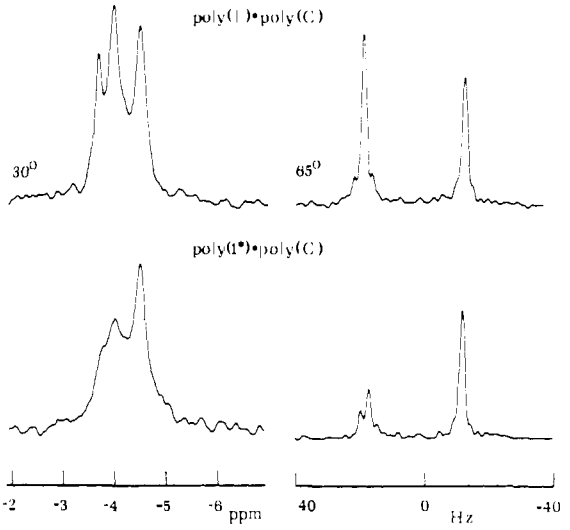

Figure 1. 81-MHz ^{31}P NMR spectra of labeled, poly(I*)–poly(C), and unlabeled samples at 30 and 65 °C. The chemical shifts are relative to trimethyl phosphate. The buffer was 0.1 M in NaCl and contained 2 mM ethylenediaminetetraacetic acid, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 10 mM cacodylic acid and was 90% ^2H_2O and the pD was 7.0. The samples consisted of a poly(I) to poly(C) ratio of 1.15:1.0 determined both by integration of the high-temperature NMR spectrum of the unlabeled sample and spectrophotometrically. The labeled and unlabeled samples were prepared identically except for the ^{17}O labeling, and the intensities of the two sets of spectra are directly comparable.

where T_{2sk} is the transverse relaxation due to scalar relaxation of the second kind; S_Q is the spin of the quadrupolar, ^{17}O nucleus; J is the heteronuclear coupling; T_{1Q} and T_{2Q} are the T_1 and T_2 of ^{17}O; ω_P and ω_Q are the Larmor frequencies of ^{31}P and ^{17}O.

For duplex polynucleotides scalar relaxation of the second kind will typically not be important. This is because T_{1Q} will be short due to the nanosecond time scale motions of the backbone[1-5] whereas T_{2Q} will be short due to the relatively slow overall motion of the duplex.[1-5] The minimum contribution of scalar relaxation of the second kind to the ^{31}P line width is only 2–4 Hz. For single-stranded polynucleotides and oligonucleotides, scalar relaxation of the second kind will be the dominant mechanism since T_{2Q} will not be short.

The important relaxation mechanism for duplex polynucleotides introduced by the presence of ^{17}O is dipolar. The dipolar contribution to transverse relaxation is given by[1-6]

$$\frac{1}{T_{2D}} = \frac{1}{30}\left(\frac{\hbar^2\gamma_P^2\gamma_I^2}{r_I^6}\right)S_I(S_I + 1)X_I \qquad (2)$$

$$X_I = 5J(0) + 3J(\omega_P) + 6J(\omega_I) + 6J(\omega_P + \omega_I)$$

where γ_P and γ_I are the gyromagnetic ratios of ^{31}P and nucleus I; S_I is the spin of nucleus I; P_I is the distance between ^{31}P and nucleus I; $J(\omega)$ is the spectral density of frequency ω.[1-5]

Since ^{17}O has a spin of $^5/_2$ and is 0.15 nm away from the phosphorus when directly bonded at a nonbridging site, the dipolar transverse relaxation contribution from ^{17}O will be 3–5 times greater than that of the three protons. Therefore, the introduction of ^{17}O will increase the line widths of the ^{31}P signals of 2.5- to 3.5-fold due to ^{17}O dipolar relaxation when the applied field is 4.7 T. The magnitude of the line width increase depends on the distances of the protons from the phosphorus and the details of the relative contributions of the chemical shift anisotropy and proton dipolar relaxation mechanisms.[12]

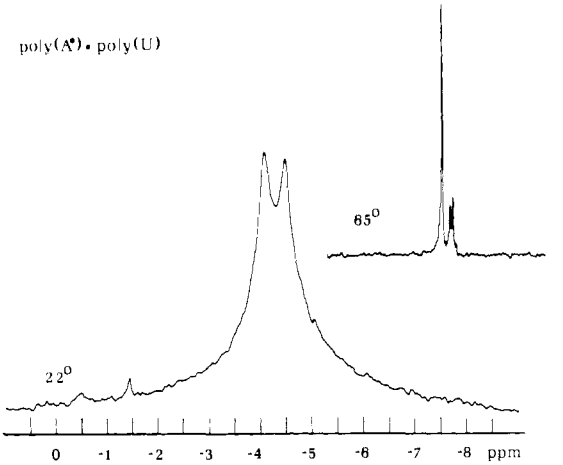

Figure 2. 81-MHz ^{31}P NMR spectra of poly(A)–poly(U) which has been prepared with ^{17}O in the nonbridging positions of poly(A) in the same buffer as used for poly(I)–poly(C). The labeled poly(A) is designated poly(A*). All of the NMR spectra were obtained using a Varian XL-200. The chemical shift scale applies only to the 22 °C spectrum with the 65 °C spectrum shown at the same scale but with arbitrary chemical shift offset.

A demonstration of this method is illustrated by the spectra for poly(I)–poly(C) with and without ^{17}O in the nonbridging positions of poly(I) shown in Figure 1. The high-temperature spectra show the ^{18}O isotope shifts of the poly(I) signal and analysis of the intensities of the isotope-shifted peaks in conjunction with the known ^{18}O:^{17}O ratio of 7:10 indicates that 60% of the phosphorus nuclei are directly bonded to one or more ^{17}O.[8] The low-temperature spectrum of the unlabeled sample contains three signals with the lowest field one arising from free poly(I), there being a 15% excess of poly(I) over poly(C). The low-temperature spectrum of the labeled sample, which was prepared from concentrations identical with the unlabeled one, shows that the two signals to low field are from poly(I) and the high-field signal is from poly(C). Thus, the ^{17}O labeling allows ready assignment of the ^{31}P signals of duplex poly(I)–poly(C), and the assignments are in agreement with those previously suggested.[7]

This method has also been applied to poly(A)–poly(U) with labeling of 75% of the phosphorus of poly(A) with at least one directly bonded ^{17}O.[8] The spectrum in Figure 2 of duplex poly(A)–poly(U) is equivalent to a spectrum of an unlabeled sample.[13]

(8) The labeled polynucleotides were prepared from adenosine diphosphate (ADP), which was prepared by S. Medhi and J. A. Gerlt. The original sample of labeled ADP had an oxygen isotope distribution of 15:50:35 for ^{16}O:^{17}O:^{18}O in both nonbridging positions. This ADP was polymerized with polynucleotide phosphorylase to prepare ^{17}O-labeled poly(A). The isotopic enrichment of the poly(A) was found to be identical with that of the labeled ADP via examination of the high-temperature ^{31}P spectrum of the poly(A), which was of sufficient resolution to quantify the relative intensities of the ^{18}O-shifted resonances. The labeled poly(I) was prepared from ^{17}O-labeled inosine diphosphate (IDP), which in turn was prepared from the labeled ADP by deamination. During the 12 months or so between the preparation of the poly(A) and poly(I) the ADP lost some of its isotopic enrichment while sotred at −4 °C as a lypholized sample. There was no loss of isotopic enrichment going from the ADP to poly(I). The mechanism of the loss of isotopic enrichment is currently being investigated as this may lead to a simpler and more economical labeling procedure.

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(13) It is known that poly(A) and poly(U) form a duplex as well as an A–2U triplex.[7] The chemical shifts of the duplex are −4.1 and −4.45 ppm whereas those for the triplex are −3.39, −3.84, and −4.36 ppm referenced to trimethyl phosphate.[7] The spectrum shown in Figure 2 shows no evidence of signals from the triplex both in terms of the chemical shifts observed and the spacing between the peaks. The chemical shifts for the lableled duplex are the same as those for an unlabeled sample. The spectrum of the labeled sample differs from that of unlabeled sample only in having greater apparent line widths.

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This implies that the residues of both homopolymers may have alternating conformations. Thus, each homopolymer might be syndiotactic or have some other alternating form. Alternating forms of polynucleotides have been observed previously but in all cases there has also been an alternating sequence.[9] The NMR results do not discriminate between the wide variety of possible alternating forms nor are they readily interpretable in terms of conformational or other property, such as solvation,[10] which might induce chemical shift differences. However, now that ^{31}P signals can be reliably assigned the information from additional assignments may well lead to an understanding of the origin of the chemical shift dispersion of nucleic acids. The following communication describes the applications of this method to oligonucleotides.[11]

Acknowledgment. We thank John A. Gerlt for supplying the ^{17}O-labeled ADP and Shujaath Medhi for his aid in converting the labeled ADP into IDP. This research was supported, in part, by the National Institutes of Health (GM-29184), the Camille and Henry Dreyfus Foundation, and the Research Corporation.

Registry No. ^{17}O, 13968-48-4; [^{17}O]poly(I)·[^{17}O]poly(C), 88295-88-9; [^{17}O]poly(A)·[^{17}O]poly(U), 88295-92-5.

A General Procedure for Assigning the ^{31}P Spectra of Nucleic Acids

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Although the ^{31}P NMR resonances associated with the phosphodiester groups in the sugar–phosphate backbones of oligonucleotides frequently show sufficient dispersion in chemical shift to permit detection of individual resonances, little quantitative use of the chemical shift data in characterizing conformation and dynamics has been possible due to the difficulty in assigning the resonances to particular nuclei in the backbone. A recently reported solution to this problem is to identify the 3′- and 5′-^1H nuclei coupled to each ^{31}P nucleus either by decoupling experiments[2] or by two-dimensional heteronuclear chemical shift correlation spectroscopy,[3] but both of these techniques require resolved and assigned ^1H NMR spectra. In this communication we describe a general method for the assignment of the resonances in the ^{31}P NMR spectra of oligonucleotides. Our approach is based on labeling the nonesterified phosphoryl oxygens of the backbone with ^{17}O in a sequence-specific manner; the efficient scalar relaxation of the second kind provided to the ^{31}P nucleus by the directly bonded ^{17}O nucleus causes extensive line broadening and a decrease in the apparent intensity of the associated ^{31}P NMR resonance.[4,5] To illustrate this method, we have prepared two samples of the self-complementary tetradeoxynucleoside triphosphate d(CpGpCpG) labeled in each d(CpG) unit with a single atom of ^{17}O and used their spectra to assign the ^{31}P NMR resonances of the oligonucleotide both in the absence and in the presence of the intercalative drug actinomycin D.

A solution phosphotriester method was used for the synthesis of unlabeled d(CpGpCpG) and two samples labeled in each d(CpG) unit with a single atom of ^{17}O.[6,7] This chemistry involves the coupling of a protected d(CpGp) having a 3′-(chlorophenyl phosphate) group with a protected d(CpG) having a free 5′-hydroxyl group; 1-(*p*-tolylsulfonyl)-4-nitroimidazole was used as the coupling reagent. The labeled d(CpGp) and d(CpG) were prepared from [^{17}O]$POCl_3$ by the hydroxybenzotriazole method described by van Boom and co-workers;[8] the isotopic composition of the labeled phosphoryl oxygen was 35.5% ^{16}O, 38.3% ^{17}O, and 26.2% ^{18}O.[9] Following purification of the completely protected tetramers on silica gel and removal of the protecting groups,[10] the samples of d(CpGpCpG) were isolated by ion-exchange chromatography on DEAE cellulose using a triethylammonium bicarbonate gradient as the eluent.[11] The ^1H NMR spectra at 500 MHz were in excellent agreement with literature data[12-14] and demonstrated a very high degree of chemical purity.

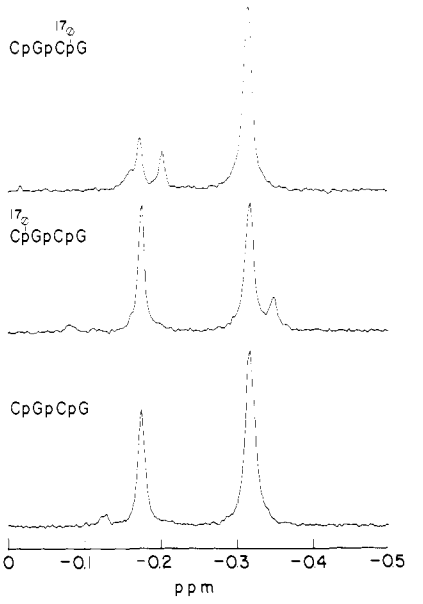

Figure 1. Proton-decoupled ^{31}P NMR spectra at 202.5 MHz and 30 °C of samples of 10 mM d(CpGpCpG) dissolved in D_2O containing 0.1 M sodium cacodylate, pH 7.0, and 2 mM EGTA. The spectra are of the unlabeled sample (bottom), the sample labeled with ^{17}O in the 3′-terminal d(CpG) unit (top), and the sample labeled with ^{17}O in the 5′-terminal d(CpG) unit (middle). Chemical shifts are measured relative to external 85% H_3PO_4.

The ^{31}P NMR spectra of the unlabeled and labeled oligonucleotides were obtained at 202.5 MHz and 30 °C, and these are reproduced in Figure 1. The spectrum of the unlabeled material (bottom spectrum) reveals two resonances in an intensity ratio of 1:2. The spectrum of the material labeled in the 3′-terminal d(CpG) unit (top spectrum) shows that the intensity of the downfield signal is diminished by the degree of ^{17}O enrichment. The remaining signal for this phosphodiester group is composed of two resonances of approximately equal intensity that are separated by 0.030 ppm; these resonances are associated with unlabeled and ^{18}O-labeled oligonucleotides which are present by virtue of the isotopic composition of the $POCl_3$ used to prepare the dimeric units. The spectrum of the material labeled in the

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