Observation of Resonances from Some Minor Bases in the Natural-Abundance Carbon-13 Nuclear Magnetic Resonance Spectrum of Unfractionated Yeast Transfer Ribonucleic Acid. Evidence for Fast Internal Motion of the Dihydrouracil Rings†

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ABSTRACT: The development of a probe for sample tubes of 20-mm o.d. has increased the sensitivity of natural-abundance ^{13}C Fourier transform nmr to the point that resonances of some minor base carbons of tRNA in concentrated solutions can be studied. In order to facilitate the identification of minor base resonances of tRNA, we first recorded and assigned the ^{13}C spectra of some minor base mononucleosides. We then obtained the proton-decoupled natural-abundance ^{13}C Fourier transform nmr spectrum (at 15.18 MHz) of aqueous (150 mg/ml) unfractionated tRNA from baker's yeast in the presence of Mg^{2+} (about 8 ions per tRNA molecule) at 41°. On the basis of ^{13}C chemical shifts of mononucleosides and mononucleotides, some ring carbons of dihydrouridine, pseudouridine, inosine, and ribosylthymine residues, as well as all methyl carbons, should resonate in regions of the spectrum free of interference from ribose and major base resonances. The ^{13}C spectrum of tRNA yields resonances of C-4, -5, and -6 of dihydrouridine residues, with a signal-to-noise ratio of about 8 after 14 hr of signal accumulation time. There is also evidence of methyl carbon resonances, and of a peak arising from C-5 of pseudouridine and/or ribosylthymine residues. In addition, we show that dihydrouracil rings of folded tRNA undergo fast internal motion (with a correlation time $\lesssim 2 \times 10^{-10}$ sec), a result that suggests a lack of direct participation of dihydrouridine residues in the determination of the secondary and tertiary structures of tRNA.

In a previous paper (Komoroski and Allerhand, 1972) we demonstrated the feasibility of examining some properties of tRNA in solution by means of natural-abundance carbon-13 Fourier transform nuclear magnetic resonance (nmr). The only clearly observed signals were those resulting from the overlap of the resonances of the numerous adenosine (A), guanosine (G), cytosine (C), and uridine (U) units. After 20 hr of spectral accumulation, two barely discernible signals were observed upfield from the main resonances. These were tentatively attributed to minor bases, but not assigned. In this paper, we reexamine the ^{13}C nmr spectrum of folded tRNA in aqueous solution, with the use of improved nmr instrumentation recently developed in our laboratory.

Most ^{13}C nmr instruments utilize sample tubes with an outside diameter of 10, 12, or 13 mm. We have recently constructed a probe which uses spinning sample tubes with an outside diameter of 20 mm (18-mm i.d.). This relatively inexpensive development (Allerhand *et al.*, 1972), together with the incorporation of a special crystal filter (Allerhand *et al.*, 1973a), has given our nmr apparatus an increase in sensitivity (in concentration-limited studies) of about a factor of 4 with respect to commercial Fourier transform nmr equipment (Allerhand *et al.*, 1973a), in spite of the fact that we work at 15.18 MHz while most commercial instruments operate at ^{13}C resonance frequencies in the range 23–25 MHz. Fortunately, the magnetic field inhomogeneity over our large sample volume (about 12 ml) is still sufficiently small (about 0.3 Hz inhomogeneity broadening) for high-resolution studies. Observation of minor base ^{13}C resonances of tRNA now becomes practical.

We report here the observation and assignment of various resolved resonances of minor bases in the proton-decoupled natural-abundance ^{13}C spectrum of unfractionated baker's yeast tRNA in its folded conformation. In addition, we show that the dihydrouracil rings of the dihydrouridine residues of tRNA undergo fast internal rotation. We also present the chemical shifts and assignments of the ^{13}C resonances of some mononucleosides which occur as minor base residues of tRNA

Experimental Section

Materials. Unfractionated tRNA from baker's yeast was obtained from Plenum Scientific Research Inc., Hackensack, N. J. Commercial material (3 g) was dialyzed twice (6 hr each time) at 4° against an aqueous solution 0.1 M in NaCl and 0.005 M in EDTA. Then the material was dialyzed three times (6 hr each time) against deionized water and lyophilized. The sample for nmr studies contained 1.8 g of tRNA in 12 ml of aqueous solution. The only added counterion was Mg^{2+} (45 mM $MgCl_2$). The pH was adjusted to 7.1 with 5 N NaOH. Mononucleosides were purchased from Sigma Chemical Co., St. Louis, Mo., and used without further purification.

Spectra. Natural-abundance proton-decoupled ^{13}C nmr spectra were recorded at 15.18 MHz on a "home-built" Fourier transform nmr apparatus which has been described (Allerhand *et al.*, 1970, 1971, 1972, 1973a). The probe uses spinning sample tubes with a 20-mm o.d. (Allerhand *et al.*, 1972). A crystal filter provides an additional improvement in the signal-to-noise ratio (Allerhand *et al.*, 1973a). A Nicolet-1083 computer was used for recording the tRNA spectrum, with 8192 memory addresses for the time-domain accumulation and a spectral width of 4000 Hz. In order to increase the signal-to-

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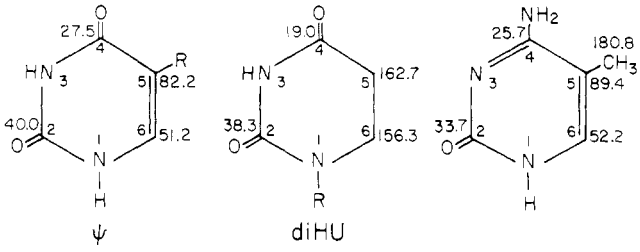

FIGURE 1: ^{13}C chemical shifts, in ppm upfield from CS_2, of aqueous pseudouridine (ψ), dihydrouridine (diHU), and 5-methylcytosine; R = ribosyl group. Molarities, pH, and temperatures were: 0.02, 7.1, 52° (ψ); 0.035, 7.0, 50° (dihydrouridine); 0.20, 6.9, 52° (5-methylcytosine). Other details are given in the Experimental Section.

noise ratio, the time domain data were multiplied by an exponential function with a negative time constant (which added 3.1 Hz to the line widths). Spectra of mononucleosides were recorded in a Nicolet-1074 digital accumulator (4096 addresses) and processed with the use of a PDP-8/E computer (Digital Equipment Corporation). Chemical shifts of the mononucleosides and of tRNA were measured digitally and have an estimated accuracy of ±0.2 ppm. Dioxane was used as an internal reference (at 126.2 ppm upfield from CS_2) for the mononucleoside chemical-shift measurements. In the case of tRNA, no reference material was added. Instead, the tRNA peak at 27.0 ppm (Komoroski and Allerhand, 1972) was used as the reference.

Results and Discussion

Model Compounds. Only those minor base resonances which do not overlap with resonances of the major bases can yield identifiable peaks in the ^{13}C spectrum of tRNA. In order to establish which are the most promising minor bases in this respect, it is useful to examine the ^{13}C chemical shifts of model compounds such as mononucleosides and mononucleotides. The ^{13}C chemical shifts of some mononucleosides in dimethyl sulfoxide have been reported by Jones *et al.* (1970a,b). The ^{13}C spectra of mononucleotides of major bases in aqueous solution have also been published (Dorman and Roberts, 1970; Mantsch and Smith, 1972). Dorman and Roberts (1970) have also reported the ^{13}C chemical shifts of inosine 5′-monophosphate and thymidine 5′-monophosphate. Even though the

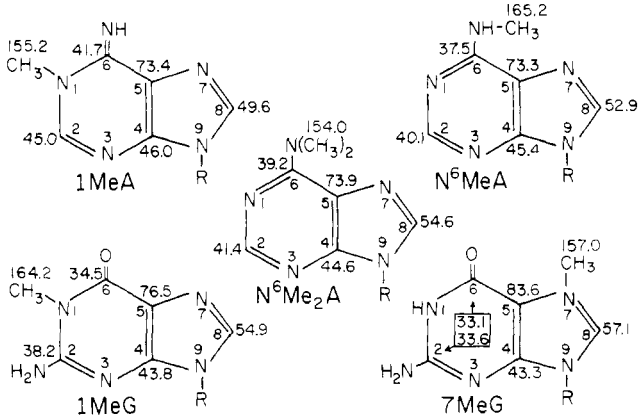

FIGURE 2: ^{13}C chemical shifts, in ppm upfield from CS_2, of aqueous 1-methyladenosine (1MeA), N^6-methyladenosine (N^6MeA), N^6,N^6-dimethyladenosine (N^6Me_2A), 1-methylguanosine (1MeG), and 7-methylguanosine (7MeG); R = ribosyl group. Molarities, pH, and temperatures were: 0.077, 7.1, 42° (1MeA); 0.048, 6.9, 52° (N^6-MeA); 0.15, 6.8, 39° (N^6Me_2A); 0.073, 7.0, 51° (1MeG); 0.073, 7.0, 53° (7MeG). Other details are given in the Experimental Section.

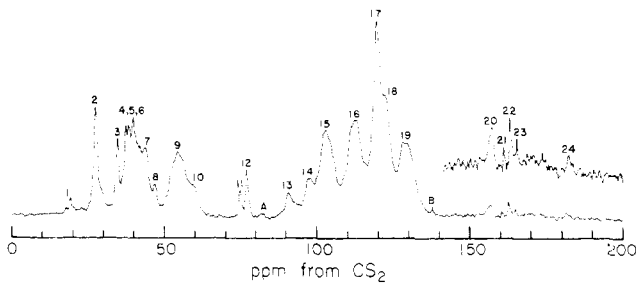

FIGURE 3: Proton-decoupled natural-abundance ^{13}C nmr spectrum of unfractionated baker's yeast tRNA in water (150 mg/ml) with about 8 Mg^{2+} ions per tRNA molecule, at pH 7.1, and 41°. The spectrum was recorded at 15.18 MHz, by means of the Fourier transform method, with 49,152 accumulations and a recycle time of 1.035 sec (14.1 hr total time). A digital broadening of 3.1 Hz was used to improve the signal-to-noise ratio. Other details are given in the Experimental Section.

latter compound is a deoxyribonucleotide, it is a good model for the ^{13}C chemical shifts of the base carbons of ribosylthymine groups.

We have recorded and assigned the ^{13}C spectra of some additional model compounds (Figures 1 and 2). Most of the assignments resulted directly from those reported for the mononucleotides of the major bases (Dorman and Roberts, 1970). In the case of dihydrouridine, the resonances at 156.3 and 162.7 ppm were assigned to C-6 and -5 on the basis of known chemical shifts of saturated carbons (Stothers, 1972). The specific assignment of the resonance at 156.3 ppm to C-6 (Figure 1) is based on the fact that this carbon is bonded to nitrogen and should therefore resonate downfield from C-5. C-2 and C-4 of 1-methyladenosine had very similar chemical shifts (Figure 2) and could not be specifically assigned just by comparing with the corresponding chemical shifts of adenosine 5′-monophosphate (AMP). However, C-2 has a directly attached hydrogen, while C-4 does not. Therefore, C-2 should have a much shorter spin–lattice relaxation time (T_1) than C-4 (Allerhand and Doddrell, 1971), an expectation that has been verified experimentally in the case of aqueous AMP (Allerhand *et al.*, 1971). We distinguished the resonances of C-2 and -4 of 1-methyladenosine on the basis of large differences in T_1 values. The same procedure was used to distinguish the closely spaced resonances of C-2 and -6 of N^6,N^6-dimethyladenosine (Figure 2). The closely spaced resonances of nonprotonated C-2 and -6 of 7-methylguanosine (Figure 2) could not be assigned on a one-to-one basis.

Carbon-13 Spectrum of tRNA. In Figure 3 we show the proton-decoupled natural-abundance ^{13}C Fourier transform nmr spectrum of aqueous unfractionated baker's yeast tRNA obtained after 14 hr of signal accumulation. The chemical shifts and assignments of the resonances of Figure 3 are given in Table I. The assignments of the resonances of the ribose carbons and major base carbons have been given previously (Komoroski and Allerhand, 1972). Identification of minor base resonances follows from the chemical shifts of Figures 1 and 2, and from the chemical shifts of IMP and TMP reported by Dorman and Roberts (1970).

Listed in Table II are the ranges of ^{13}C chemical shifts that are free from any resonances of ribose and of the major bases. Only minor base carbons with chemical shifts in these ranges are expected to yield clearly resolved resonances. Table II lists such minor base carbons taken from Figures 1 and 2 and from the work of Dorman and Roberts (1970). These are promising candidates for use as ^{13}C probes of individual carbon sites of tRNA. It should be noted, however, that our list is

TABLE I: Spectral Assignments of the ^{13}C Resonances of Unfractionated Baker's Yeast tRNA.

Peak[a]	Chem Shift[b]	Assignment[c]	Peak[a]	Chem Shift[b]	Assignment[c]
1	18.9	H_2U-4	13	90.5	U-5
2	27.0	C-4, U-4	14	97.4	C-5
3	34.1	G-6	15	~102	1′
4	36.8	C-2	16	~113	4′
5	37.7	A-6	17	~120	2′
6[d]	39.2	G-2	18	~122[e]	3′
e	41.3	U-2	19	~129	5′
7	42.9	G-4	B	137.6	OCH_3
8	46.4	A-4	20	156.7	H_2U-6, NCH_3
9	53.8	U-6, C-6	21	160.3	*f*
10	58.7	A-8, G-8	22	162.5	H_2U-5
11	74.5	A-5	23	164.8	NCH_3
12	76.6	G-5	24	181.6	CCH_3
A	~82	ψ-5, T-5[g]			

[a] Peak numbers are those of Figure 3. [b] In ppm upfield from CS_2, accurate to ±0.2 ppm. [c] Letter designates base in nucleotide unit. Number is standard carbon designation, as in Figures 1 and 2. No assignments are given for minor base carbon resonances that are not resolved from major base resonances. H_2U is dihydrouridine. [d] C-2 of the adenosine residues, which is a protonated carbon, should yield a broad resonance in the region of peak 7. [e] Shoulder. [f] Probably *N*-methyl carbon (see text). [g] C-5 of ribosylthymine. This assignment is based on the chemical shift of the corresponding carbon in TMP (Dorman and Roberts, 1970).

TABLE II: Ranges of ^{13}C Chemical Shifts Devoid of Ribose and Major Base Resonances.

Range[a]	Minor Base Carbons[b]
<24	H_2U-4
29–32	
62–72	I-5[c]
78–87	ψ-5, 7MeG-5, T-5[d]
>135	H_2U-5, H_2U-6, methyl[e]

[a] Chemical shifts in ppm upfield from CS_2. [b] Carbons expected to resonate in the given region, on the basis of known chemical shifts of model compounds. Model compounds and their designations are those of Figures 1 and 2, unless otherwise stated. Number after hyphen is standard carbon designation. H_2U is dihydrouridine. [c] C-5 of inosine, based on its chemical shift in inosine 5′-monophosphate, at 69.4 ppm (Dorman and Roberts, 1970). [d] C-5 of ribosylthymine, based on the chemical shift of C-5 in thymidine 5′-monophosphate, at 81.1 ppm (Dorman and Roberts, 1970). [e] All methyl carbons.

probably not complete because the ^{13}C chemical shifts of a number of minor bases present in tRNA (Zachau, 1969; Dayhoff, 1972, 1973) have not yet been measured.

The carbons of dihydrouridine residues of tRNA yield clearly identifiable resonances, namely peaks 1, 20, and 22 of Figure 3, which can be assigned to C-4, -6, and -5, respectively (Table I). It should be noted, however, that peak 20 is broader and has a greater integrated intensity than peak 22. It follows from Figure 2 that some methyl carbons attached to nitrogen should contribute to peak 20.

Methyl carbons bonded to unsaturated carbons (Figure 1) give rise to the weak resonance at 181.6 ppm (peak 24 of Figure 3). On the basis of the chemical shifts of Figure 2, we assign the resonance at 164.8 ppm (peak 23 of Figure 3) to *N*-methyl carbons. There is no chemical shift in Figure 2 corresponding to the tRNA resonance at 160.3 ppm (peak 21). This peak may arise from some *N*-methyl carbons. However, we cannot exclude the possibility of chemical-shift nonequivalence of C-5 of the various dihydrouridines. Therefore, peak 21 may be a shifted dihydrouridine resonance.

The very weak but reproducible resonance at about 82 ppm (peak A) can be assigned to C-5 of pseudouridine residues and/or C-5 of ribosylthymine residues. The corresponding carbons of pseudouridine and thymidine 5′-monophosphate resonate at 82.1 (Figure 1) and 81.1 ppm (Dorman and Roberts, 1970), respectively. The remaining identifiable minor base resonance in Figure 3 is peak B at 137.6 ppm. Its chemical shift is consistent with that of a methoxy carbon (Stothers, 1972; Breitmaier *et al.*, 1971). There are various minor bases of tRNA with methoxy groups at C-2′ (Zachau, 1969; Dayhoff, 1972, 1973). They are all expected to yield OCH_3 resonances with practically identical chemical shifts.

No additional minor base resonances can be identified in Figure 3. C-5 of inosine residues is expected to yield a well-resolved resonance on the basis of its chemical shift (69.4 ppm) in inosine 5′-monophosphate (Dorman and Roberts, 1970). However, no resonance can be detected in the vicinity of 70 ppm (Figure 3). The content of inosine and of some other minor bases in unfractionated baker's yeast tRNA is not sufficient to yield an adequate signal-to-noise ratio under the conditions of Figure 3. It is not surprising that dihydrouridine, the most prevalent minor base in unfractionated baker's yeast tRNA (Dayhoff, 1972), yields stronger ^{13}C resonances than any other minor base.

The line widths of the resonances in Figure 3 range from less than 10 Hz to more than 50 Hz. The instrumental broadening is about 3 Hz. The other important contributions to the observed line widths are the natural line width ($W = 1/\pi T_2$, where T_2 is the spin–spin relaxation time) and chemical-shift nonequivalence. The narrow major base resonances have been assigned to nonprotonated carbons (Table I). Ribose carbons and major base protonated carbons yield broad resonances.

The value of $1/T_2$, and therefore the natural line width, of a *protonated* carbon in a large molecule is overwhelmingly dominated by ^{13}C–^{1}H dipolar relaxation with the directly bonded hydrogen or hydrogens (Allerhand *et al.*, 1971). Because of the dependence of dipolar relaxation on the inverse sixth power of the distance between the nuclei (Abragam, 1961) and because of the short C–H bond length (about 1.09 Å), ^{13}C–^{1}H dipolar relaxation is much more effective for protonated carbons than for nonprotonated ones. As a result, nonprotonated carbons have longer T_2 values and therefore narrower resonances than protonated carbons which have comparable rotational correlation times (Allerhand *et al.*, 1971). The rotational correlation time for overall rotation (τ_R) of aqueous tRNA under conditions of Figure 3 is about 3×10^{-8} sec (Komoroski and Allerhand, 1972). In the absence of internal motions, the line width of a methine carbon resonance (at 14.2 kG) should then be (Doddrell *et al.*, 1972) about 45 Hz, and that of a methylene carbon resonance should be about 90

Hz. On the other hand, even in the absence of internal motions, nonprotonated carbons of tRNA should have much narrower resonances.

A protonated carbon of tRNA *can* yield a narrow resonance if its effective rotational correlation time is considerably shorter than τ_R, as a result of contributions from fast internal motion (Doddrell *et al.*, 1972). C-5 of dihydrouridine has two directly attached hydrogens. Therefore, the line width of the resonance of this carbon in the spectrum of tRNA in its folded conformation would be about 90 Hz in the absence of internal rotation of the dihydrouracil ring. The observed line width (peak 22 in Figure 3) is 13 Hz, a result that is only consistent with *fast internal rotation* of the dihydrouracil rings of folded tRNA at 41° (see below).

Without the 3-Hz instrumental contribution, the line width of the resonance of C-5 of the dihydrouridine residues would be about 10 Hz. In the absence of information about chemical-shift nonequivalence of C-5 of the several dihydrouridine residues in the various species of unfractionated tRNA, we cannot determine the natural line width. However, we know that 10 Hz is the upper limit to the natural line width. With this information we compute below a lower limit to the rate of internal rotation of the dihydrouridine bases.

For simplicity, we assume that both C–H bond lengths (r) of the CH_2 group are equal, that the angle (θ) between the axis of internal rotation and the C–H vector is the same for both C–H bonds (see below, however), that the stochastic diffusion model for internal rotation (Woessner, 1962) is valid, that rotation about the (C-1′)–(N-1) bond is the only internal motion of the dihydrouracil group, and that the overall rotation of the tRNA molecules is isotropic. Then the spin–spin relaxation time of C-5 of dihydrouridine residues of tRNA is given by eq 37 of Doddrell *et al.* (1972).

It is easy to show that our experimental upper limit to the line width of C-5 of dihydrouridine residues (10 Hz), and our value of the correlation time for overall rotation (3×10^{-8} sec) (Komoroski and Allerhand, 1972), are only consistent with values of θ between 110 and 138° (or between 42 and 70°), in agreement with an expected value, on the basis of bond geometries, of about 120°. It is also easily shown with the use of the theoretical results of Doddrell *et al.* (1972) that the correlation time for internal rotation (τ_G) of the dihydrouracil rings is $\lesssim 2 \times 10^{-10}$ sec. The derivation of this result does not require knowing the value of θ, or a precise knowledge of the correlation time for overall rotation (τ_R). It is enough to assume that τ_R is in the range 10–50 nsec. This assumption leaves a very ample margin for error in our experimental τ_R of 30 nsec (Komoroski and Allerhand, 1972).

We conclude that the dihydrouracil rings of folded tRNA in aqueous solution are undergoing internal motion at a rate that is more than 100 times faster than that of overall molecular rotation. This result suggests a lack of direct participation of the dihydrouracil rings in the determination of the conformation of folded tRNA. It should be noted that we have only determined an upper limit to τ_G (or lower limit to the rate of rotation) because our ^{13}C spectrum only yields an upper limit to the *natural line width* ($W = 1/\pi T_2$) of C-5 of dihydrouridine residues. We would be able to determine W if we knew the contribution to the observed line width from chemical-shift nonequivalence of the numerous dihydrouridine resonances of unfractionated tRNA. A preferable alternative would be to record the ^{13}C spectrum of a pure strain of tRNA, and thus perhaps observe resolved *single carbon resonances* of minor bases. Numerous resolved single carbon resonances have been recently observed in natural-abundance ^{13}C spectra of small proteins in their native conformation (Allerhand *et al.*, 1973a,b; Oldfield and Allerhand, 1973).

In the absence of internal rotation, the methyl carbon resonances of folded tRNA would have line widths of about 135 Hz (three times the line width of a methine carbon without internal rotation), and would be difficult to detect. The observed methyl carbon resonances are relatively narrow. Peak 24 (Figure 3) has a line width of about 25 Hz, and the other observed methyl carbon resonances are even narrower. This behavior indicates fast internal rotation of the C–H vectors of the methyl groups, but it cannot be taken as evidence for internal rotation of the methylated purine and pyrimidine rings as a whole, because the methyl groups themselves can rotate relative to the rings.

References

Abragam, A. (1961), The Principles of Nuclear Magnetism, London, Oxford University Press.

Allerhand, A., Childers, R. F., Goodman, R., Oldfield, E., and Ysern, X. (1972), *Amer. Lab. 4* (11), 19.

Allerhand, A., Childers, R. F., and Oldfield, E. (1973a), *J. Magn. Resonance 11*, 272.

Allerhand, A., Childers, R. F., and Oldfield, E. (1973b), *Biochemistry 12*, 1335.

Allerhand, A., Cochran, D. W., and Doddrell, D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 1093.

Allerhand, A., and Doddrell, D. (1971), *J. Amer. Chem. Soc. 93*, 2777.

Allerhand, A., Doddrell, D., and Komoroski, R. (1971), *J. Chem. Phys. 55*, 189.

Breitmaier, E., Voelter, W., Jung, G., and Tanzer, C. (1971), *Chem. Ber. 104*, 1147.

Dayhoff, M. O. (1972), Atlas of Protein Sequence and Structure, Vol. 5, Washington, D. C., National Biomedical Research Foundation.

Dayhoff, M. O. (1973), Atlas of Protein Sequence and Structure, Vol. 5, Washington, D. C., National Biomedical Research Foundation, Suppl. I.

Doddrell, D., Glushko, V., and Allerhand, A. (1972), *J. Chem. Phys. 56*, 3683.

Dorman, D. E., and Roberts, J. D. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 19.

Jones, A. J., Grant, D. M., Winkley, M. W., and Robins, R. K. (1970a), *J. Amer. Chem. Soc. 92*, 4079.

Jones, A. J., Winkley, M. W., Grant, D. M., and Robins, R. K. (1970b), *Proc. Nat. Acad. Sci. U. S. 65*, 27.

Komoroski, R. A., and Allerhand, A. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1804.

Mantsch, H. H., and Smith, I. C. P. (1972), *Biochem. Biophys. Res. Commun. 46*, 808.

Oldfield, E., and Allerhand, A. (1973), *Proc. Nat. Acad. Sci. U. S. 70*, 3531.

Stothers, J. B. (1972), Carbon-13 NMR Spectroscopy, New York, N. Y., Academic Press.

Woessner, D. E. (1962), *J. Chem. Phys. 36*, 1.

Zachau, H. G. (1969), *Angew. Chem., Int. Ed. Engl. 8*, 711.