Spin labeled nucleic acids.

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ABSTRACT

Homopolyribonucleotides and E. coli DNA were spin labeled with an iodoacetamide-nitroxide compound. The extent of labeling is highly dependent upon the nature of the base and the secondary structure of the nucleic acid. This spin label-polymer linkage is unstable at high temperatures and in phosphate buffers. In order to determine the effect of changes in the environment of nucleic acids on the esr signals of their attached spin labels, the polynucleotides were subjected to temperature and viscosity perturbations. An increase in temperature (T) affects a linear decrease in the anisotropy factor of the esr signal. The log τ (τ = correlation time) versus (1/T) profile is linear with a positive slope when the spin label is attached to single stranded polynucleotides but exhibits discontinuities at certain critical temperatures when attached to the duplexes poly (A₂-U) and poly (I·C₈). These critical temperatures are lower than the optical Tₘ. Logarithmic increase in viscosity was found to produce a linear increase in τ in aqueous sucrose solutions.

INTRODUCTION

While much is known about the properties and conformation of nucleic acid or other biopolymers in simple solutions, the conformational properties of nucleic acids in complex mixtures or inside the living cells so far have not been investigated quantitatively. Many of the physico-chemical techniques such as CD, IR, NMR and UV, which have been extensively used to study nucleic acid conformation, are not adaptable to studies of these biopolymers in complex biological media since signals arising from the environment often interfere with the signals of interest. Such measurements also represent averages over the whole molecular conformation of the biopolymer and usually provide limited information on the specificity or function of a particular site of the biopolymer.

The recent development of spin label technique in esr
spectroscopy has provided a new probe of biopolymers in complex systems. Since the concentration of free radicals in biological systems is small, a radical attached to a biopolymer will be easily distinguished from background signals. Nitroxide radical compounds (\(\cdot N-O\)) have proved to be ideally suited as spin labels for two reasons: (1) Their esr line-shapes are highly sensitive to the relaxation time of the unpaired electron which is, in part, determined by the molecular mobility of the spin label and the molecule to which it is attached. This property allows for their use as probes of regional mobility. (2) The molecular orbital configuration of the nitroxide group is anisotropic; therefore, its esr line-shape is also dependent upon the orientation of the nitroxide group with respect to the external magnetic field (for further discussion see ref. 1). This intrinsic property of nitroxides provides directional information of the regions of molecules to which the label is attached. Previous studies using nitroxide labels to study the properties of nucleic acids have generally indicated that these spin labels accurately monitor localized conformation events.\(^2\)\(^-\)\(^8\) These characteristics together with the inherent esr sensitivity of the spin label make this technique a potentially attractive one for studying the conformation of nucleic acids in biological or other complex environments.

However, prior to any detailed esr investigation of nucleic acids in a complex biochemical system or in a living cell, it is imperative to understand the basic behavior of spin labeled molecules in simpler systems. Thus, this investigation of the properties and behavior of spin labeled nucleic acids in model systems was initiated. Specifically, this report concerns the chemical reactivity of 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyl oxyl,\(^8\) IATMPO (Figure 1), with various polynucleotides, the stability of this linkage, the effects of temperature and viscosity on the esr spectra.

**Fig. 1** 3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyl oxyl, IATMPO
MATERIALS AND METHODS

Spin Label

The spin label reagent, 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (IATMPO) was obtained from Synvar Associates. E. coli K-12 DNA was purchased from General Biochemicals. The ribosyl homopolymers (poly A, poly I, poly C and poly U) were purchased from Miles Laboratories. Poly G was obtained from Sigma Chemical Corp. Pancreatic ribonuclease was purchased from Worthington. The phosphate buffer (HMP) consisted of 0.0025M disodium hydrogen phosphate and 0.005M sodium dihydrogen phosphate, adjusted, where necessary, with 1N sodium hydroxide to the indicated pH.

Electron Spin Resonance

ESR spectra were measured on a JEOL JES-MEX spectrometer which was interfaced with a Varian C1024 signal averager and a Data General Nova computer. The temperature was controlled by a JEOL JES-VT-3 temperature controller and JES-UCT-2A variable temperature adaptor. Samples were allowed to equilibrate for 5 minutes before measurement.

A study of the effect of microwave power on the line shape of the spectrum of poly (A₈·U) suggested that at high power levels (60 mW or greater) the microwave radiation heats up the sample. In the presence of nitrogen gas flowing through the cavity continuously, the line shape of the esr signal remained the same up to 200 mW (the power was not calibrated). All the spectra reported here were measured at 10 mW.

Quantitation of the spin label concentration was determined by use of a computer program designed to double integrate the peaks from both a MnO standard and the sample.

UV Absorption Measurements

Ultraviolet spectra were recorded on a CARY 15 spectrophotometer and uv melting temperature profiles were determined on a Beckman DB-G grating spectrophotometer connected to a Beckman Tm Analyzer.

The concentration of the DNA and polynucleotide solutions were calculated from solution absorbancies using the following analytical wavelengths and extinction coefficients (x10⁻³):

Native DNA¹₀ (259 nm, 6.6), denatured DNA¹₀ (259 nm, 8.0), poly A¹₀
(257 nm, 10.5), poly U\textsuperscript{10} (260 nm, 9.2), poly G\textsuperscript{11} (252 nm, 10.1),
poly C\textsuperscript{12} (268 nm 6.3), poly I\textsuperscript{13} (248 nm, 10.0), poly U-poly A\textsuperscript{14}
(260 nm, 6.8), poly U-2 poly A\textsuperscript{14} (260 nm, 5.9), poly G-poly C\textsuperscript{10}
(258, 8.2), and poly I-poly C\textsuperscript{15} (265 nm, 5.2 and 245 nm, 4.9).

**Spin-labeling of Ribosyl Homopolymers and Native and Denatured DNA**

The stock solutions of polynucleotides and DNA were made in HMP and stock solutions of IATMPO were in ethanol. The spin label solution was always freshly prepared and added to the polynucleotide solution. The following reactions, carried out at room temperature in the dark, were set up to determine the effect of time and concentration on the amount of spin label attached to the nucleotide polymers. a) Reaction solutions, 40% ethanol-HMP, containing IATMPO and polymer in a 5:1 ratio of spin label to nucleotide (final concentration 10\textsuperscript{-3} M nucleotide) were incubated for 7 days. b) Reaction solutions, 60% ethanol-HMP, containing IATMPO and polymer in a 10:1 ratio of spin-label to nucleotide (final concentration 10\textsuperscript{-3} M nucleotide).

At the end of the reactions the polymers were isolated, free of unreacted spin-label, in the following manner: sufficient 5M NaCl\textsubscript{0.4} and cold ethanol were added to yield a final mixture that was 0.1 - 0.15M in NaCl\textsubscript{0.4} and 75-80% ethanol. The mixture was cooled for one hour in ice and centrifuged at 15,000 RPM for 30 min. at 0°C; the supernatant removed by decantation. The precipitate was washed two times with ethanol and then dissolved in HMP-0.15M NaCl, pH 7.0. The solution was then exhaustively dialyzed, in the cold, against the same buffer. An aliquot of the final dialysis bath was always monitored by esr to establish the absence of detectable amounts of unreacted spin label.

The covalent nature of the spin label-nucleic acid linkage is indicated by the inability to remove the IATMPO from the nucleic acids by exhaustive dialysis or by extensive extraction with organic solvents even though IATMPO is substantially more soluble in non-aqueous solvents. In addition, this binding phenomenon is time-dependent, requiring 7-10 days to reach the present level. Furthermore, when poly C\textsubscript{s} is digested to monomers and the digest applied to a DEAE-sephadex column, less than 4% of the original spin label passed through the column without
binding. 53% of the spin label was recovered with polynucleotide digest with a spectrum indicative of a large rotational freedom. The remaining spin label could not be recovered in the DEAE-sephadex or in other fractions. This loss of spin label in the column chromatography was readily demonstrated by the repassage of the CMP associated spin label through the DEAE-sephadex column under similar conditions (2M ammonium bicarbonate, pH=8.5). A significant reduction of the CMP-attached spin label recovered from all the fractions was observed. Mixing of the IATMPO with the DEAE-sephadex causes no loss of esr signal, however. Since IATMPO and its hydrolyzed product do not bind to the DEAE-sephadex column, this data suggests that less than 4% of the spin label in the poly C can be associated with the polynucleotide through non-covalent binding.

In the case of the duplexes, a spin labeled polynucleotide was combined in the usual fashion with its unlabeled complimentary strand.

**Viscosity Experiment**

A 60% (w/w) solution of ribonuclease free sucrose was prepared. To this solution, varying amounts of polymer in HMP, pH=7 was added to make solutions of different viscosities. The viscosity of these carefully weighed sucrose solutions was obtained from the Handbook of Biochemistry. Viscosities at temperatures greater than 30°C were calculated from the empirical equations developed by Barber.

**RESULTS**

**Reaction**

Under the mild conditions employed (see Table I), IATMPO reacted well with poly A, poly C and denatured DNA. The extent of binding was enhanced by increasing the time of the reaction and the IATMPO to nucleotide ratio. The degree of binding appears to be a function of the secondary structure since double stranded (or multi-stranded) polynucleotides bound the IATMPO to a significantly lesser degree than did the single stranded polynucleotides.

The spin label/base ratio (24/10,000) was calculated from the relative base composition and the labeling efficiency of the corresponding ribohomopolynucleotides (e.g. .25 of .67%
The spectra of the polynucleotides at room temperature (Fig. 2) range from the totally immobilized poly G which is multi-stranded in solution to the weakly immobilized poly A. The spectra appear largely isotropic and, consequently, the formulation developed by Stone and co-workers for the calculation of the correlation time, $\tau$, at this range of values is probably valid.

$$
\tau = C \Delta H_0 \left\{ \left[ \frac{h(0)}{h(\pm 1)} \right]^\frac{1}{2} + \left[ \frac{h(0)}{h(-1)} \right]^\frac{1}{2} - 2 \right\}
$$

where $
\tau = \text{correlation time}$
$\Delta H_0 = \text{linewidth of central peak in gauss}$
$h = \text{height of peak in arbitrary units}$
$\pm 1, 0, -1 = \text{nuclear spin quantum number; +1 is the low field line; -1 is the high field line}$
$C = \text{constant which is a function of the anisotropy of the } g \text{ and } A \text{ tensors}$
Fig. 2 Representative spectra of spin labeled single and double stranded polynucleotides. Microwave power = 10 mW, modulation width = 2.0 G

Table II

<table>
<thead>
<tr>
<th>Polynucleotide</th>
<th>Correlation time (10^9 sec) at 25°C</th>
<th>Correlation time (10^9 sec) at 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>rA*</td>
<td>2.2</td>
<td>1.3</td>
</tr>
<tr>
<td>rG*</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>rA*-rU*</td>
<td>3.7</td>
<td>2.2</td>
</tr>
<tr>
<td>rA*-2rU*</td>
<td>3.3</td>
<td>2.3</td>
</tr>
<tr>
<td>rA*-rG*</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>rA*-2rG*</td>
<td>3.1</td>
<td>2.8</td>
</tr>
<tr>
<td>rG*</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>rC*</td>
<td>3.3</td>
<td>1.9</td>
</tr>
<tr>
<td>rC*</td>
<td>3.3</td>
<td>1.9</td>
</tr>
<tr>
<td>rU*</td>
<td>4.2</td>
<td>3.0</td>
</tr>
<tr>
<td>dRNA*</td>
<td>2.5</td>
<td>1.2</td>
</tr>
<tr>
<td>RNA*</td>
<td>Immobilized</td>
<td>Immobilized</td>
</tr>
</tbody>
</table>

Polynucleotides in double or multi-stranded complexes have larger correlation times (greater immobility) than those in single-stranded form (Table II). These values decrease with increasing temperature.
Temperature Dependence

Figure 3 shows the temperature dependence of the correlation times of poly (C₅), poly (I-C₅), poly (A₅), poly (A₅-U), poly (U₅), and denatured DNA. The esr temperature profiles of the double stranded complexes poly (A₅-U) and poly (I-C₅) show discontinuities at certain critical temperatures which reflect a sudden change in structure of the duplex. These critical temperatures for the two spin labeled duplexes are lower than the Tₘ values measured by uv absorbance. This may be the result of localized destabilization of the hydrogen bonding between complementary strands at the site of spin label attach-

![Graphs showing temperature dependence](image)

Fig. 3 Log₁₀ T (in nanoseconds) vs. inverse absolute temperature. Activation energies are calculated from the slopes of a least squares line fitted to the experimental points. In the case of the double stranded compounds, the slopes of the line before and after the transition are used.
ment. In such a case the esr will monitor the melting of the destabilized regions which are expected to melt at a lower temperature than the rest of the duplex.

Activation energies can be obtained from variations of correlation times with absolute temperature, expressed through the Arrhenius equation:

\[ \tau = \tau_0 \ e^{-\Delta E/kT} \]

where \( \Delta E \) is the energy of activation of the motion. The activation energy of the single stranded polynucleotides decrease in the following order (in Kcal/mole): poly C\textsuperscript{s} (11.81) > dDNA (9.0) = poly A\textsuperscript{s} (7.6) > poly U\textsuperscript{s} (6.2). This activation energy is a measure of the energy barrier which must be overcome to allow for the tumbling of the spin label. The order of energies for the single strands directly parallels their correlation times (Table II). Both the low correlation time and activation energy of poly U\textsuperscript{s} is probably a reflection of its known mobile secondary structure in aqueous medium. Activation energies for the duplexes in their double stranded state were found to be: poly (I\cdot C\textsuperscript{s}) (5.7) > poly (A\textsuperscript{s}\cdot U) (4.0).

The effect of temperature variation on the anisotropy of the esr line shapes of the spin label was also examined. The anisotropy factor \( \epsilon \) should approach zero and should have little temperature dependence in the isotropic case. Figure 4 is a plot of \( \epsilon \) versus temperature for the IATMPO, poly A\textsuperscript{s}, and poly (A\textsuperscript{s}\cdot U). This data indicates that although IATMPO free or attached to polynucleotides can be considered to be generally isotropic, it exhibits a small degree of anisotropy with a linear dependence of \( \epsilon \) on temperature.

**Viscosity**

The mobility of the spin label was also found to be affected by the viscosity of the medium. As shown in Figure 5 the correlation time of the free and attached spin labels is de-
Fig. 4 Relationship between the anisotropy factor, \( \varepsilon \), and temperature for Poly (A\(^8\)-U), Poly A\(^8\) and free IATMPO. The anisotropy factor is calculated according to the equation given in RESULTS.

Fig. 5 Correlation time vs. \( \log_{10} \) of viscosity. Solutions of the spin labeled polynucleotides and the free spin label in HMP buffer were made to different viscosities with sucrose. Correlation times were calculated at each viscosity at 29°C (solid lines) and some also at 40°C (broken lines). Each point is the average of two or three determinations.
scribed by the equation:

\[ \tau = a + b \log \eta \]

where \( a \) = constant
\( b \) = function of temperature

This finding is in contrast to the linear dependence of the correlation time with viscosity that is predicted by the Einstein-Stokes formulation. In addition, the difference in slopes at the two temperatures indicated in Figure 4 does not follow the reciprocal temperature dependence predicted by this formulation. It must be remembered, however, that the Einstein-Stokes equation assumes an ideal situation in which solvent-solute interactions are absent. Anomalies have been previously noticed, especially in aqueous solutions. Water is notorious for interacting with other molecules and it is not unreasonable to expect that the size and shape of the solvent cage influencing the tumbling of the spin label would vary both with sucrose concentration and with temperature.

Moreover, the spin label free or attached to single or double stranded polynucleotides have similar sensitivities to viscosity as measured by their slopes in Figure 5. This data suggests that the label is not rigidly attached to the nucleic acids and that the environment governing the motion of the spin label is affected similarly by the increase of sucrose concentration, regardless of whether the spin label is attached or free.

**Loss of Label**

Upon recooling the spin labeled polynucleotides, the esr spectra were consistently composed of two components, one of which manifested a high degree of mobility. After incubation of the spin labeled poly C\(^8\) for one hour at 70°C, only 46% of the spin label was recovered associated with the precipitate upon precipitation of the polymer with 0.3M ammonium acetate and ethanol at -20°C.

Release of spin label was also observed at 37°C and at room temperature when the spin labeled poly A and poly C were in phosphate buffer, but the spin label linkage to the polymers is stable in Tris, NaCl, and cacodylate buffers (Fig. 6).
Fig. 6 Spectra of Poly A$^5$ and Poly C$^5$ after 3 hours incubation at 37° C in 0.5M phosphate buffer pH 7.0. The arrows indicate the contributions of a highly mobile component in these compound spectra. Microwave power = 10 mW modulation width = 2.0 G.

In each of these experiments the buffers were sterilized by millipore filtration to insure against bacterial contamination. To assure that there was no enzymatic degradation, the polymers were examined in the analytical ultracentrifuge. Based on these observations, this instability of the label, at low temperatures in phosphate, cannot be attributed to bacterial or enzyme contamination but appears to be a unique reaction with phosphate. Comparative studies on the rate of loss of spin labeled material from poly A$^5$ and poly C$^5$ at 37° C in 0.5M sodium phosphate buffer over a six hour period indicate that the stability of the spin labeled poly A is greater than that of the spin labeled poly C.
The intrinsic reactivities of radioactive iodoacetate toward ribonucleotides have been determined both at the monomer level and with RNA which subsequently has been enzymatically degraded to mononucleotides for further separation and examination. GMP and AMP were found to be the most reactive, followed by CMP. This reactivity is highly dependent on secondary structure since native DNA and poly G which forms multi-stranded complexes were very poorly alkylated by IATMPO in comparison to denatured DNA or other less stacked or aggregated homopolymers. In our experiment, poly U was not labeled significantly by IATMPO as expected, since uridine shows little, if any, intrinsic reactivity towards alkylation by the iodoacetate. It is surprising that poly U was reported by Bobst to be labeled extensively by 4-(2-Iodoacetamide)-2,2,6,6-tetramethylpiperidinoxyl, a spin label that differs from ours only in the number of carbons in the nitroxide ring.

The instability of the spin label linked to the poly A and poly C at high temperature and in phosphate buffer was unexpected and has led us to reevaluate the potential usefulness of this spin label as a probe of nucleic acid conformation in biological systems. This important phenomenon has not been previously reported in literature. Nevertheless, it is stable in other buffers at room temperature and, consequently, its ability to probe many biochemical systems is not impaired. A detailed discussion of the causes of this instability in molecular terms must await a determination of the structure of the spin label-nucleic acid attachment.

The changes in rotational motion as a function of temperature and viscosity were studied in order to characterize the structural and conformational properties of nucleic acids by this technique. These characteristics can then be used to determine the properties of nucleic acid in complex biological media.

The temperature effect on the correlation time and anisotropy of the spin label on the polynucleotides clearly demonstrate the effect of the secondary structure on the response to the temperature perturbation. This observation is very useful.
in distinguishing the location of the spin label, whether it is on a mononucleotide, a single-stranded polynucleotide, or a helical duplex or aggregate. The changes in the correlation time and anisotropy of the spin label on these different compounds to the temperature variation, say from 20° - 40°, would be quite different.

We also anticipate that the effect of a change in viscosity of the medium would produce different responses in correlation times of the spin label attached to different compounds. This anticipation is based on the knowledge that the viscosity effects on the rotational frictional coefficients of molecules of different shapes and rigidity are quite different. However, this viscosity effect will only be observable if the spin label is rigidly attached to the polynucleotides. Apparently this situation is not yet achieved with the present spin label. As other spin labeled nucleic acids are developed, we expect that their sensitivity to viscosity would provide further characterization of the physical conformation of nucleic acids in complex systems.

REFERENCES

9. IATMPO = 3-(2-iodoacetamido)-2,2,5,5-tetra-methyl-1-pyrrolidinyl oxyl. Superscript "s" refers to a spin labeled polynucleotide, i.e. poly (I-C) is poly I annealed with spin labeled poly C.
12. Tazawa, I. and Ts'o, P. personal communication