Tritium labeling of antisense oligonucleotides by exchange with tritiated water

M.J. Graham, S.M. Freier, R.M. Crooke, D.J. Ecker, R.N. Maslova* and E.A. Lesnik*
ISIS Pharmaceuticals, 2292 Faraday Avenue, Carlsbad, CA 92008, USA and *V.A. Engelhardt Institute
of Molecular Biology, Russian Academy of Sciences, 32 Vavilov Street, Moscow 117984, Russia

Received May 3, 1993; Revised and Accepted July 6, 1993

ABSTRACT
We describe a simple, efficient, procedure for labeling oligonucleotides to high specific activity (> 1 x 10^6 cpm/µmol) by hydrogen exchange with tritiated water at the C8 positions of purines in the presence of β-mercaptoethanol, an effective radical scavenger. Approximately 90% of the starting material is recovered as intact, labeled oligonucleotide. The radiolabeled compounds are stable in biological systems; greater than 90% of the specific activity is retained after 72 hr incubation at 37°C in serum-containing media. Data obtained from in vitro cellular uptake experiments using oligonucleotides labeled by this method are similar to those obtained using 35S or 14C-labeled compounds. Because this protocol is solely dependent upon the existence of purine residues, it should be useful for radiolabeling modified as well as unmodified phosphodiester oligonucleotides.

INTRODUCTION
The evolution of antisense oligonucleotides as therapeutic modalities to specifically inhibit translation of a variety of viral and cellular mRNA targets (1–3) has created a need for development of assays to measure the uptake, metabolism, and distribution of these molecules both in vitro and in vivo. Although a variety of non-isotopic labeling techniques exist, the use of radiolabeled oligonucleotides to examine pharmacokinetics offers the distinct advantage of avoiding chemical modification of these compounds by addition of various reporter groups (1,4).

Unmodified and modified oligonucleotides may be radiolabeled at specific positions by a variety of methods (5–10). The most commonly used technique involves 5′ end labeling with 32P using bacteriophage T4 polynucleotide kinase (5–7). Another method, utilizing Klenow DNA polymerase, generates oligomers with internally labeled phosphates (8). While effective, each of these techniques has inherent limitations, including the short (2 week) half-life of 32P. A substantial amount of truncated sequences is routinely produced using the Klenow polymerase method. Additionally, 5′-chemically modified oligonucleotides are susceptible in biological systems to a variety of enzymatic activities that can rapidly remove the label (1,2).

Phosphorothioates may be uniformly labeled with 35S along their modified backbones using hydrogen phosphonate chemistry (9,10). This technique, while yielding high specific activity oligomers (greater than 1 x 10^6 cpm/µmol) with relatively longer half-lives (greater than 80 days), also has drawbacks. Significant amounts of oligonucleotide are lost during isolation and purification steps. Additionally, labeling efficiency may vary significantly as a function of reagent quality and oligonucleotide sequence (R. Crooke and M. Graham; unpublished results). This method of labeling is obviously limited to phosphorothioates and sulfuration of phosphorothioate oligonucleotides containing other chemical modifications could also prove to be difficult, if not impossible.

The heterocyclic bases represent an alternative site to the backbone for isotopic labeling of oligonucleotides. This alternative is particularly attractive for oligonucleotide analogs with non-phosphodiester backbones (11–13) which cannot be labeled with phosphorus or sulfur isotopes.

Recently our laboratory has devised a tritium exchange radiolabeling technique that circumvents many of the limitations associated with the procedures described above. Hydrogen exchange between purine C8H groups and water at elevated temperatures was first observed more than twenty years ago (14–17). Since that time, the mechanism and effects of pH, temperature, ionic composition, base modifications and secondary structure of nucleic acids and polynucleotides on exchange rates have been extensively studied (18–22). While this protocol produced high specific activity nucleic acids, radiolytic degradation of the resulting labeled polynucleotides precluded their use in biological assays (23–26). Along with backbone breakage, electron capture by pyrimidine bases is thought to be another pathway of radiation damage in DNA (27–30). We, therefore, evaluated a number of radical scavengers for their ability to prevent radiolytic degradation of nucleic acids. Based on these data and a knowledge of the reaction mechanism and kinetics, we optimized conditions for production of 3H-labeled oligonucleotides.

In this paper, we describe for the first time a tritium exchange procedure for phosphorothioate oligonucleotides in the presence of β-mercaptoethanol (β-ME), hybridization experiments to confirm the structure of the 3H-labeled phosphorothioates and,
finally, uptake and stability studies performed in vitro. Our data suggest the rate of hydrogen-tritium exchange between tritiated water (THO) and purines of single stranded phosphorothioate oligonucleotides is comparable to that of phosphodiester polynucleotides. This technique is an effective and invaluable means of radiolabeling backbone modified as well as unmodified compounds since exchange depends only on the presence of purine bases. Inclusion of an effective free radical scavenger allows a high yield of stable material with specific radioactivity that is sufficient for in vitro and in vivo pharmacokinetic experiments.

MATERIALS AND METHODS

Unlabeled oligodeoxynucleotides

Two phosphorothioate oligonucleotides, ISIS 1082 (5'-GCCG-AGGTCCATGTCGTACGC-3'), a 21-mer, and ISIS 2105 (5'-TTGCTTCCATCTTTCTCGTC-3'), a 20-mer, were synthesized using a Milligen 8800 automated DNA synthesizer and phosphoramidite chemistry. The thiation reagent was synthesized according to the procedure of Iyer et al (9).

Oligonucleotides were purified using reverse phase HPLC prior to removal of the 5' trityl protecting group. Analytical gel electrophoresis on a 20% polyacrylamide denaturing gel (PAGE) (31) and analytical reverse phase high performance liquid chromatography (HPLC) demonstrated oligonucleotides to be greater than 90% full length material. 31P nuclear magnetic resonance (NMR) demonstrated 99% phosphorothioate versus phosphodiester linkages. Oligonucleotide concentrations were determined from the absorbance at 260 nm and extinction coefficients calculated according to the method of Puglisi and Tinoco (32).

Synthesis of RNA oligonucleotides is described elsewhere (33,34).

Principles of hydrogen exchange at C8 position of purines

Hydrogen exchange reactions are pseudomonomolecular reactions whose rates are described by a first order rate equation (35). Half times for exchange of H-atoms from NH2-, NH- and OH- groups with water at room temperature are measured in minutes (for H-atoms participating in hydrogen bonding) and in seconds (for free H-atoms). Half times for slowly exchanging H-atoms of C8H groups of purines at 25°C are measured in months. Hydrogen exchange between water and C8H groups proceeds via an ylid mechanism. It has been shown that exchange rates for adenine, guanine and other purine derivatives in the pH range from 5 to 8, increase as a function of pK*, but are independent of pH. At pH values below the pK*, exchange rates decrease. At pH values higher than 8, exchange rates of purines in mono-and poly-nucleotides accelerate due to the onset of another mechanism of exchange (18,36). Exchange rates are also highly temperature dependent (19,37). The half times for exchange in single stranded poly A are approximately 4 hours at 90°C, 2 months at 37°C and extrapolated to be years at -20°C (37). This rapid exchange at 90°C, coupled with slow exchange at 37°C and immeasurable exchange at -20°C is ideal for efficient labeling at 90°C, pharmacokinetic experiments at 37°C and storage of unused material at -20°C.

Table 1. The effect of free radical scavengers on degradation of supercoiled plasmid DNA during incubation in tritiated water

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Linear ( Of total amount)</th>
<th>Nicked</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>68</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td>None</td>
<td>60</td>
<td>28</td>
<td>12</td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Tricine</td>
<td>19</td>
<td>55</td>
<td>4</td>
</tr>
<tr>
<td>DMSO</td>
<td>29</td>
<td>43</td>
<td>3</td>
</tr>
<tr>
<td>Acetone &amp; isopropanol</td>
<td>15</td>
<td>52</td>
<td>6</td>
</tr>
<tr>
<td>Thiourea</td>
<td>36</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>β-ME</td>
<td>35</td>
<td>32</td>
<td>10</td>
</tr>
</tbody>
</table>

Unincubated plasmid; 2plasmid incubated without THO; 3plasmid incubated in THO without scavenger.

Selection of oligonucleotide free radical degradation protector

High energy β^- particles, products of tritium disintegration in water, are proposed to produce significant amounts of highly reactive free radicals, atomic oxygen, solvated electrons, protons and peroxide (38,39) that break single and double stranded DNA (25,26). Five inhibitors of radical reactions (tricine, DMSO, β-ME, thiourea and a mixture of acetone and isopropanol) were examined for their ability to inhibit the radiolytic degradation of DNA and oligonucleotides. Supercoper plasmid (pUC19), whose intact supercoiled, linear and nicked forms are easily detected by electrophoresis, was used for quantitative testing of the protective effects of free radical scavengers. Plasmid DNA was incubated for 1 hour at 37°C in solutions containing highly radioactive THO (specific activity 3.3 Ci/gm), 100 mM NaCl, 10 mM phosphate buffer, pH 7.2, 0.1 mM EDTA and 0.3 M of each scavenger. After incubation followed by separation of bulk THO and rapidly exchangeable 3H atoms as described earlier (21), the fraction of native plasmid maintained in the presence of each scavenger was detected by electrophoresis in 0.8% agarose using TAE buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.0). Quantitative analysis of gels was run using a Molecular Dynamics 300 A computing densitometer (Table 1). Two protectors: β-ME and thiourea afforded the best protection with more than half the supercoiled plasmid remaining intact after incubation. Raising the β-ME concentration to 0.6 M increased the final fraction of supercoiled plasmid from 35% to 40%. 

Radiolabeling of oligonucleotides by hydrogen-tritium exchange in THO

For each labeling experiment, 12 mg of HPLC purified oligonucleotide were suspended in 200 μL of 50 mM sodium phosphate, 0.1 mM EDTA, pH 7.8 and lyophilized in a 2 mL gasketted microfuge tube. The dry oligonucleotide was resuspended in 200 μL of tritiated water (NEN, specific activity 5 Ci/gm) containing 8.3 μL β-ME and incubated six hours at 90°C. Following incubation, the sample was lyophilized to remove unexchanged THO. The sample was resuspended in 1 mL of water and held one hour at room temperature to allow rapidly exchangeable protons to dissociate. The room temperature incubation was followed by four cycles of lyophilization and subsequent resuspension in 0.5 mL of water. The sample was stored at -20°C until final purification. To remove final traces of tritiated water, labeled oligonucleotide was applied to a 1 x 10 cm column containing Sephadex G-10 (Pharmacia). Oligonucleotide was eluted with distilled water at a flow rate of 0.5 mL/min; 0.5 mL fractions were collected and stored at -20°C for further evaluation.
All fractions were initially analyzed by liquid scintillation counting (LSC) [Beckman LS5000TA] and UV absorption at 260 nm (Gilford Response). Counting efficiency was roughly 50%. Reaction products were additionally characterized using 20% PAGE and were detected using StainsAll (Sigma). (6). Autoradiography was also performed after pretreatment of gels with Amplify fluorographic fluid (Amersham).

Labeling of 2105 with $^{35}$S

Uniformly $^{35}$S labeled 2105 was prepared by Dr Patrick Iverson by synthetic incorporation of elemental $^{35}$S using H-phosphonate chemistry as described by Stein et al (10). Labeled oligonucleotide was purified by PAGE (6,31). Analytical gel electrophoresis of the purified product and detection by StainsAll (Sigma) or autoradiography demonstrated a single oligonucleotide product.

Preparation of $^{14}$C labeled 2105

$^{14}$C-ISIS 2105 was manually synthesized following standard phosphoramidite chemistry as described in the literature. The dA, dC and dG phosphoramidites were from either Milligen or Applied Biosystems. $^{14}$C was introduced by converting [2-$^{14}$C]-thymidine (Sigma) to 5'-O-(4,4'-dimethoxytrityl)-thymidine-3'-[2-cyanoethyl-N,N-diisopropylphosphoramidite in a two step synthetic process (H.Sasmor and D.Dellinger; manuscript in preparation). Solvents and additional synthesis reagents were purchased from available suppliers (Applied Biosystems, Baker Chemicals, and R.I.Chemicals).

The trityl-on synthetic product was purified by C18 reverse-phase HPLC. The column fractions were analyzed by PAGE and LSC. The collected trityl-on product peak was deprotected by acid hydrolysis and purified as the sodium salt by multiple ethanol precipitations from the aqueous solvent. The final product was resuspended in sterile water for injection and analyzed to determine specific activity and purity.

Melting experiments

Absorbance vs. temperature profiles (melting curves) were measured at 260 nm in 100 mM Na+, 10 mM phosphate, 0.1 mM EDTA, pH 7 as described by Freier et al (40). Thermodynamic parameters for duplex formation were obtained by fitting triplicate melting curves at 4 $\mu$M each strand to a modified two state model with linear sloping baselines (41).

Reverse hydrogen-tritium exchange under physiological conditions

Four optical units of $^{3}$H-ISIS 1082 were incubated at 37°C in 500 $\mu$L of 10 mM sodium phosphate (pH 7.2) containing 100 mM sodium chloride. After 1, 2, 3, 5 and 10 days of incubation, 90 $\mu$L aliquots were removed, lyophilized three times from water and stored at -20°C. At the end of the experiment, specific activities for each aliquot were determined. In addition, 1 $\mu$L of each sample was analyzed by 20% PAGE and autoradiography to determine the radiographic intensity of the full length material over time.

In a second set of experiments, 3 optical units of $^{3}$H-ISIS 1082 were incubated at 37°C in 400 $\mu$L of either 1 x PBS, Dulbecco's Modified Eagle's Media (DME) [Irvine Scientific] or DME containing 10% heat inactivated fetal bovine serum (FCS) [DME+] . After 24, 48 and 72 hours, 100 $\mu$L aliquots were removed from each sample; aliquots incubated without FCS were spiked with 10 $\mu$L FCS. Fetal calf serum was added to all samples prior to extraction to facilitate normalization of data since preliminary experiments revealed extractions carried out in the presence of serum proteins significantly altered the amount of material recovered and therefore, the accuracy of the specific activity determination. Protein was removed from the samples by phenol/chloroform extraction followed by ethanol precipitation (6,31). Pellets were resuspended in 100 $\mu$L of water, lyophilized three times from water and analyzed for specific activity and integrity as described for the first experiment.

Cell culture techniques

Cell line maintenance. HeLa (ATCC #CCL2), a human epithelioid carcinoma line, C127 (ATCC #CRL1616), a mouse mammary tumor line and normal human dermal fibroblast cells (NHDF) (Clonetics #CC2010) were maintained as monolayer cultures in low glucose DME containing 10% heat inactivated FCS and 100 units of penicillin G and 100 $\mu$g streptomycin sulfate per 500 mL. Cultures were incubated at 37°C in a humidified 5% CO$_2$ atmosphere.

Intracellular stability of $^{3}$H-1082 and $^{3}$H-2105. HeLa, C127 and NHDF cells were plated in six well tissue culture plates (5 x $10^4$ cells/well) in 1.5 mL 10% DME+ and allowed to adhere overnight. The next day, the media was aspirated and replaced with media containing 5 $\mu$L oligonucleotide. At the end of each incubation period (24-72 hours), media was aspirated from each well and adherent cells were washed once with 1 mL 1 x PBS and incubated at 4°C for 5 minutes in 1 mL of 0.2 M glycine to remove nonspecifically bound oligonucleotide. The supernatant was aspirated, cells were trypsinized and harvested with 10% DME+. Cell number and viability were determined by counting a 10 $\mu$L aliquot of cell suspension in the presence of trypan blue (viability at all time points exceeded 95%). Cells were pelleted by centrifugation and resuspended in 400 $\mu$L tris-EDTA, pH 8 (TE). Protein was removed from the samples by phenol/chloroform extraction followed by ethanol precipitation (6,31). Precipitates were resuspended in 10 $\mu$L TE and a 1 $\mu$L aliquot was removed for LSC determination of cell associated radioactivity. All time points were performed in triplicate.

Cellular uptake of $^{3}$H-, $^{35}$S- and $^{14}$C- radiolabeled 2105. Cellular uptake of 2105 radiolabeled by three techniques was determined in HeLa cells. Logarithmically growing HeLa cells were harvested from T-175 flasks by trypsinization, combined and washed twice with 10% DME+. After the second wash, cells were resuspended in medium containing 11% DME+ and diluted to a final concentration of 1.12 x $10^6$ cells per mL. Cells (450 $\mu$L) were then transferred to 15 mL screw cap centrifuge tubes and placed on ice prior to uptake experiments.

A 50 $\mu$L aliquot of radiolabeled ISIS 2105 (final concentration of 5 $\mu$L) was added to cells, and experiments were performed by placing the reaction mixture in a 37°C shaking water bath or on ice. Reactions were stopped by placing the tubes on ice and pelleting the cells by centrifugation at 4°C in a Beckman Tabletop centrifuge for 5 minutes at 1750 x g. Prior to the initial centrifugation, viability of cells was determined by removing a 10 $\mu$L aliquot of reaction mix and counting cells in the presence of trypan blue. After centrifugation, the supernatant was removed from the tubes, saved for counting and the cell pellet was washed first with ice-cold PBS and then with 0.2 M glycine to remove nonspecific cell-associated radioactivity. Integrity of radiolabeled ISIS 2105 in the media was determined by 20% PAGE as described above. Aliquots of the media, PBS and glycine washes
and the entire cell pellet were placed in vials and counted by LSC (Beckman LS5000TA).

RESULTS

Purification and characterization of \(^{3}\)H-oligonucleotides

After incubation in tritiated water, several cycles of lyophilization and resuspension, oligonucleotides were passed through Sephadex G-10 to separate all traces of tritiated water. To evaluate the efficiency of our separation technique, unlabeled ISIS 1082 was spiked with tritiated water and the mixture run on a Sephadex G-10 column as described in Materials and Methods. As shown in Figure 1A, oligonucleotide and tritiated water displayed different elution profiles, indicating this procedure is sufficient for complete resolution of oligonucleotides from traces of tritiated water. Figure 1B shows the radioactivity and absorbance profile of \(^{3}\)H-IS1082 at 260 nm during final purification on Sephadex G-10. Specific activity is uniform over the peak of absorbance. Analytical PAGE of each fraction demonstrated the \(A_{260}\) peak corresponds to full-length oligonucleotide. Fractions 5–10 were pooled and analyzed for specific activity and oligonucleotide integrity by analytical PAGE. The pooled product represented 90% of the starting material and ran as a single band with mobility corresponding to that of unlabeled, HPLC purified ISIS 1082 (Figure 2).

The specific activity of radiopure ISIS 1082, containing 10 purines, was \(3.9 \pm 0.8 \times 10^8 \text{cpm/\mu mol}\) while that of ISIS 2105, which contains only 3 purines, was \(1.3 \pm 0.1 \times 10^8 \text{cpm/\mu mol}\). These values demonstrate a direct correlation between purine content and amount of radioactivity incorporated into oligonucleotides during the labeling process. Per mole of purine, the average specific activities were \(3.9 \times 10^7\) (ISIS 1082) and \(4.3 \times 10^7 \text{cpm/\mu mol}\) (ISIS 2105). These values obtained for phosphorothioate oligonucleotides represent, respectively, 78% and 86% of the equilibrium value calculated from rate constants for purine nucleotides and correlate well with the observation of Shelton and Clark (24) who noted that exchange in natural DNA at 100°C reached only 62% of the expected value.

Thermodynamic melt analysis

Radiolysis of tritiated water results in production of radical and electron species (38) that could not only break the oligomer backbone but also introduce structural changes in the heterocyclic bases (27–30) resulting in a decreased ability of the compound to hybridize to its complementary sequence. To circumvent this
problem, β-ME was included in the labeling reaction mixture to quench radical species. This innovation resulted in 90% recovery of full length material. The integrity of radiolabeled ISIS 1082 and ISIS 2105 was further examined by evaluating hybridization of 3H-labeled and unlabeled oligonucleotides to complementary oligoribonucleotide targets. The hybridization characteristics are shown in Figure 3 which plots absorbance vs temperature profiles, and Table 2, which lists thermodynamic parameters obtained from fitting triplicate curves. Structural changes or mutation of a single base typically result in a decrease in Tₘ of 3–20°C (42). The Tₘ values and thermodynamic parameters of the oligomers are not affected by labeling. Therefore, if the labeled oligonucleotides are altered or degraded, it is at a level substantially less than one residue per oligonucleotide. Additionally, the widths of the derivative curves (dAbs/dT) are similar for duplexes with the labeled or unlabeled oligomers suggesting similar populations of oligonucleotides in both samples.

Stability of tritiated oligonucleotides in biological systems

The stability of the label in 3H-ISIS 1082 was examined under a variety of conditions used to mimic physiological conditions. Data in Figure 4 indicate greater than 90% of the specific activity of radiolabeled ISIS 1082 is retained after incubation for 72 hours at 37°C 100 mM NaCl, 10 mM sodium phosphate, pH 7.2. Even after 240 hours incubation, only a 25% decrease in specific activity was observed. In a second set of experiments (Figure 5), 3H-ISIS 1082 was incubated in various physiological solutions over a 72 hour period. The data again revealed only a minor loss (10%) of specific activity after 72 hour incubation in PBS, DME and DME containing 10% FCS.

Table 2. Thermodynamic parameters of duplex formation by 3H labeled and unlabeled oligomers with RNA targets

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>-ΔH° (kcal/mol)</th>
<th>-ΔS° (cal/mol/deg)</th>
<th>-ΔG° at 37°C (kcal/mol)</th>
<th>Tₘ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1082</td>
<td>105</td>
<td>288</td>
<td>15.7</td>
<td>61.2</td>
</tr>
<tr>
<td>3H-1082</td>
<td>106</td>
<td>291</td>
<td>15.6</td>
<td>60.6</td>
</tr>
<tr>
<td>2105</td>
<td>106</td>
<td>301</td>
<td>12.6</td>
<td>50.8</td>
</tr>
<tr>
<td>3H-2105</td>
<td>104</td>
<td>294</td>
<td>12.4</td>
<td>50.5</td>
</tr>
</tbody>
</table>

To determine whether cellular proteins would increase the rate of reverse tritium exchange from C8 position of oligonucleotide purines, 3H-ISIS 1082 and ISIS 2105 were incubated with three different cell lines over a 72 hour period. Oligonucleotides were extracted and analyzed as described in Materials and Methods. Analytical PAGE with detection of oligomers by StainsAll and autoradiography suggested specific activity of oligonucleotides did not change significantly in this cellular system over a 72 hour period. (Data not shown)

Cellular uptake of radiolabeled ISIS 2105

The pharmacokinetic parameters of a number of phosphorothioate oligonucleotides have been evaluated in our, as well as other,
laboratories primarily using $^{35}$S-oligomers (2,43). To demonstrate the validity of the $^3$H-oligomers in cellular systems, the uptake of ISIS 2105 radiolabeled with $^3$H, $^{35}$S or $^{14}$C was compared in HeLa cells. The cell association of ISIS 2105, as well as other oligonucleotides is time and temperature dependent (2,43). Approximately 1.75-2.25% of the total input radioactivity is associated with cells after 8 hours at 37°C (Figure 6). Most importantly, no significant differences in cell associated radioactivity and kinetics were observed using ISIS 2105 prepared by three different isotopic labeling procedures. Viability of cells under all conditions was greater than 95% during the course of the experiment.

**DISCUSSION**

The need for in vitro and in vivo pharmacokinetic analysis of chemically modified antisense oligonucleotides prompted our laboratory to develop methods for radiolabeling the bases of these novel therapeutic agents with tritium. Earlier attempts to use this technique for labeling of natural DNAs and phosphoester polynucleotides failed due to nucleic acid degradation by products of radiolysis (23-26). In the absence of a free radical scavenger, high specific activity could not be obtained without substantial loss of intact product (Table 3). The radiolytic breakage of DNA is a stochastic process; therefore the shorter a DNA chain is, the less probability of its damage. The number of single and double stranded breaks caused by tritium irradiation depends linearly on radiation dose (39). Calculations based on data of Yamamoto et al. (39) predict that in the absence of scavenger virtually all pUC19 plasmids (2686 b.ps.) would be damaged by single and double stranded breaks under the incubation conditions. Our data (Table 1) are consistent with this prediction and confirm the utilization of radical scavengers is crucial if long polynucleotides or high molecular weight DNA’s are labeled by tritium exchange. Correlation between single stranded breaks and irradiation dose was observed up to 500 Gy. Under our conditions of oligonucleotide labeling, irradiation dose reached $3.65 \times 10^3$ Gy, so it was difficult to predict the extent of degradation under these conditions. If a linear correlation is maintained over this wide range of irradiation dose we expect only 6% of the oligonucleotides would be damaged during the incubation. Nevertheless, we labeled short oligonucleotides in the presence of $\beta$-ME to prevent potential damage of both the backbone and the heterocyclic bases.

The procedure allowed us to prepare $^3$H oligonucleotides in more than 90% yield with specific activity approximately 80% that of the tritiated water used for labeling (Table 3). Analysis of the end product of the tritium exchange procedure on a denaturing polyacrylamide gel indicated that $^3$H-labeled oligonucleotide ran identically to unlabeled oligonucleotide. Also, thermodynamic melts revealed no significant difference in hybridization characteristics when tritiated oligomers were compared to their unlabeled progenitors. Consequently, we conclude that the labeling procedure results in intact labeled oligonucleotides.

To confirm that release of the tritium label is slow enough to support the use of $^3$H-oligonucleotides for biological studies, several experiments were performed to evaluate the relative stability of radiolabeled compounds under a variety of conditions. Incubation of $^3$H-oligomers in buffered salt solution revealed that after 10 days at 37°C, greater than 70% of the initial specific activity of the compound was retained. Even more encouraging was the observation that at 72 hours, which corresponds to the maximum time of incubation for our in vitro pharmacokinetic experiments, greater than 90% of the specific activity of the molecule remained. Additional experiments conducted in physiological solutions confirmed this result. At 37°C, in the presence of DME containing 10% FCS, which is standard tissue culture media, stability of both tritiated ISIS 1082 and ISIS 2105 was greater than 90% after 72 hours.

The final validation of the utility of $^3$H-oligomers involved performing experiments in in vitro cellular systems that had been used in our laboratories to investigate oligonucleotide pharmacokinetic parameters. Several important questions were addressed: 1) Do tritiated oligonucleotides associate with cells in a manner that is consisted with data obtained using more traditionally labeled oligomers, such as $^{35}$S?; 2) Are these compounds stable in extra- and intracellular milieu?; 3) Can we

---

**Table 3. Comparison of specific radioactivity and degradation of tritium labeled nucleic acids obtained during incubation in THO under different conditions**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Assay conditions (specific activity of THO, temperature, and time of incubation)</th>
<th>Specific activity of product$^1$</th>
<th>Data on degradation (change in median M.W.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present data</td>
<td>ISIS 1082, ISIS 2105 5 Ci/mL, 90°C, 6h, $\beta$-ME</td>
<td>$\sim 4 \times 10^8$ cpm/$\mu$M purines</td>
<td>80-90% full length material (according to denaturing PAGE)</td>
</tr>
<tr>
<td>K. Shelton, J. Clark (24)</td>
<td>DNA E. coli 5 Ci/mL, 100°C, 20 min</td>
<td>$\sim 2.6 \times 10^6$ cpm/$\mu$M purines</td>
<td>no data</td>
</tr>
<tr>
<td>U. Laushke et al. (26)</td>
<td>Poly (A), Poly (I) 5 Ci/mL, 80°C, 5h</td>
<td>$\sim 8.4 \times 10^8$ cpm/$\mu$M adenine</td>
<td>7.5-fold decrease in $s_{50}$ (~ 56-fold decrease in M.W.)</td>
</tr>
<tr>
<td>D. Searcy (23)</td>
<td>Salmon DNA 0.1 Ci/mL, 90°C, 48h</td>
<td>$\sim 2 \times 10^6$ cpm/$\mu$M purines</td>
<td>2-fold decrease in M.W.</td>
</tr>
<tr>
<td>F. Doppler-Bernardi &amp; G. Felsenfeld (25)</td>
<td>Calf Thymus DNA 0.1 Ci/mL, 83°C, 4h</td>
<td>$\sim 7.5 \times 10^6$ cpm/$\mu$M purines</td>
<td>16% decrease in $s_{50}$ (1.5 fold decrease in M.W.)</td>
</tr>
</tbody>
</table>

$^1$For convenience of comparison, specific activities of nucleic acids and oligonucleotides are expressed as cpm/$\mu$M purines.
detect tritiated oligomers within cells and biological fluids?; and finally 4) Are tritiated oligonucleotides more cytotoxic than $^{35}$S or $^{14}$C labeled congeners?

Previous pharmacokinetic experiments showed that $^{35}$S phosphorothioates, like ISIS 1082 and ISIS 2105, are taken up into HeLa cells in a time and temperature dependent fashion (43). Our preliminary data indicated that the cell association of $^{3}$H-ISIS 2105 in HeLa cells was also time and temperature dependent, and that the kinetic profile was similar using ISIS 2105 labeled with $^{3}$H, $^{35}$S or $^{14}$C. PAGE analysis and subsequent autoradiography confirmed integrity of both oligomer and tritium label in oligonucleotide recovered from cells. These data also show that $^{3}$H-2105 can readily be detected by LSC in both the incubation media and HeLa cell pellets. Finally, viability of all cells, as assessed by cell counts and trypan blue exclusion, was greater than 95% during the 8 hour incubation period of the experiment regardless of the label used, indicating no isotope specific cytotoxicities.

In summary, we applied a relatively simple exchange reaction using tritiated water for radiolabeling the C8 position of purine bases of phosphorothioate oligonucleotides and found an effective radical scavenger which can be used to label long oligo- and polynucleotides as well as natural DNA in highly radioactive tritiated water with reasonable yield of intact molecules. The adjusted method is easy and produces oligomers with relatively high specific activities (> $1 \times 10^{8}$ cpm/µmol oligonucleotide) and high product yield. There are several important advantages to this procedure. First, radiolabeling at purine residues within the molecule circumvents problems encountered in cells containing phosphatases and exonucleases and end-labeled molecules. Second, the internal isotopic label does not alter the chemical nature of the oligonucleotide. Third, because this technique does not involve the phosphate backbone, synthetic modifications to this region, introduced to improve stability or uptake of an antisense oligomer, should not reduce efficiency of labeling. Fourth, as the label is incorporated by a chemically benign procedure after completion of oligonucleotide synthesis, labeling should be compatible with most modified oligonucleotides. Finally, the relatively long half-life of $^{3}$H (12.33 years) compared to either $^{35}$S (80 days) or $^{14}$P (14 days) produces a stable, labeled molecule. In light of these results, we hope to use $^{3}$H-compounds to significantly advance our knowledge into the mechanisms of cellular uptake, distribution, and metabolism of modified and unmodified antisense oligonucleotides both in vitro and in vivo.

ACKNOWLEDGEMENTS

We thank Drs Henri Sasmor and Doug Dellinger for synthesis and purification of $^{14}$C-ISIS 2105, Dr Patrick Iversen for the synthesis of $^{35}$S-ISIS 2105, Drs Stanley Cooke and Christopher Mirabelli for critical evaluation of the manuscript, and Ms Carol Collins for her excellent secretarial help.

REFERENCES