Radiolabeling of Methylphosphonate and Phosphorothioate Oligonucleotides and Evaluation of Their Transport in Everted Rat Jejunum Sacs

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Purpose. The therapeutic use of antisense oligonucleotides will likely involve their administration over protracted periods of time. The oral route of drug dosing offers many advantages over other possible routes when chronic drug administration is necessary. However, little is known about the potential for oligonucleotide uptake from the gastrointestinal tract. This issue is addressed in the current work. ***Methods.*** We have developed a simple procedure for radiolabeling oligonucleotides by reductive alkylation with ^{14}C-formaldehyde. We have utilized this approach, as well as 5′ addition of fluorophores, to prepare labeled methylphosphonate and phosphorothioate oligonucleotides for use in intestinal transport studies. An everted rat gut sac model was employed to compare the transport of oligonucleotides to that of model compounds whose permeation properties are better understood. ***Results.*** We demonstrate that both methylphosphonate and phosphorothioate oligonucleotides are passively transported across the intestinal epithelium, probably by a paracellular route. The rates of transport for both types of oligonucleotides were similar, and were significantly greater than that of the very high MW polymer blue dextran, but were lower than the transport rate of valproic acid, a low MW compound known to have high oral availability. ***Conclusions.*** A significant degree of permeation of oligonucleotides across the gastrointestinal epithelium does occur, but it is still unclear whether this is sufficient to permit effective oral administration of oligonucleotides as drugs.

KEY WORDS: antisense; radiolabel; transport; gastrointestinal; methylphosphonate; phosphorothioate.

INTRODUCTION

Since the initial report of antisense oligonucleotide activity (1), there have been many demonstrations of specific inhibition of gene expression in tissue culture and in animals through use of antisense technology. For example, antisense compounds have shown efficacy in tissue culture models of malignancy and viral infections (2). An initial problem with antisense oligonucleotides was their lack of stability in the biological environment; however, stability of oligonucleotides can be improved through modifications in the connecting backbone (3). Two commonly used backbone modified oligonucleotides are the non-charged methylphosphonates (MP) and the charged phosphorothioates (PS). In these cases the non-bonding oxygen of the phosphate linkage is replaced with a methyl group or sulfur atom respectively.

Cellular membrane transport is another major problem for antisense therapeutics due to the polar nature and high molecular weights of oligonucleotides (4). To date, the majority of studies with oligonucleotides have been done in tissue culture systems. However, a few animal studies have evaluated the pharmacokinetics and tissue distribution of oligonucleotides after intravenous (IV) administration (5, 6). These initial studies demonstrated that oligonucleotides behave similarly to other macromolecular agents in regards to renal elimination. A ^{3}H-methylphosphonate (12 mer) oligonucleotide displayed biexponential disposition in serum after a single IV bolus dose in mice (5), with distribution and elimination half-lives of 6 min and 17 min, respectively. The kidney demonstrated the highest initial amount of radiolabel suggesting that this organ may play an important role in the elimination process. Studies with PS oligonucleotides demonstrate slightly different pharmacokinetics that are attributable to their high affinity for serum proteins (albumin and α_2-macroglobulin) (7).

In many therapeutic situations, antisense compounds may need to be given chronically in order to maintain the desired effect. Ideally, oral dosing is the best route for chronic therapies; however, little data exists evaluating oligonucleotides as possible candidates for oral administration (8). At first glance, the task of oral delivery of these macromolecules appears daunting due to many potential problems including metabolic degradation, poor intestinal absorption, and first pass effects. Similar problems have been encountered in the oral delivery of other macromolecular drugs such as peptides and proteins. There are no studies currently in the literature that investigated the stability of oligonucleotides when administered orally. However, backbone modified oligonucleotides (PS and MP) should offer substantial resistance to cleavage by intestinal enzymes; full length phosphorothioate oligonucleotides have been isolated from murine small intestine and liver 24 hours after IV or IP administration illustrating oligonucleotide resistance to metabolic enzymes (9). This potential of increased stability mitigates one of the problems facing the oral use of oligonucleotides. Little is known concerning the mechanism(s) of macromolecular transport across the small intestinal epithelium. It is clear that small peptide-type drugs (di- and tripeptide analogs) are actively transported through carrier mediated systems (10), while other peptides are transported predominately by paracellular mechanism (11). The manner and extent to which backbone modified oligonucleotides cross intestinal membranes are unknown.

The purpose of this investigation was to develop a simple method of radiolabeling commonly used backbone modified oligonucleotides, and to use these labeled compounds to examine the extent of intestinal transport of oligonucleotides. We chose to use reductive alkylation with ^{14}C-formaldehyde to label methylphosphonate and phospho-

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rothioate oligonucleotides. The trans-epithelial transport of these radiolabeled compounds was compared to fluorescent labeled compounds of the same base sequence. Oligonucleotide transport also was compared to the transport of an impermeant substance (blue dextran) and to that of a low MW compound known to be readily transported in the gut (valproic acid). A modest, but clearly demonstrable passive trans-epithelial flux of oligonucleotides was observed.

MATERIALS AND METHODS

The oligonucleotides used in these studies were complementary to the codon 12 activating mutation of Ki-Ras (12). HPLC purified methylphosphonate (26.2) (13) oligonucleotides (MP oligos) were supplied by Eric Wickstrom with the sequence 5′-TAC-GCC-AAC-AGC-TCC-3′. Phosphorothioate oligonucleotides of the same sequence were synthesized using β-cyanoethyl phosphoramidite chemistries. The syntheses were performed on an Applied Biosystems 380B automated synthesizer. The PS oligonucleotides were prepared via stepwise sulphurization of the phosphite linkages using tetraethylthiuram disulfide. A fluorescent label was sometimes attached to the terminal 5′ linkage group by the use of 5′-carboxyfluorescein phosphoramidite (FAM Amidite, Applied Biosystems). Oligonucleotide concentration was estimated based on the molar extinction coefficient for the oligonucleotide strand at 260 nm (ϵ = 154 units/μmol/cm). Blue dextran MW 2,000,000 was obtained from Sigma Biochemicals (St. Louis, MO) and fluorescein isothiocyante (FITC)-labeled dextran MW 3,000 and tetramethylrhodamine (TRITC)-labeled dextran MW 10,000 were obtained from Molecular Probes (Eugene, OR). ^{3}H-glucose (10 Ci/mmol) and ^{14}C-formaldehyde (1–3%) solution (11 mCi/mmol) were obtained from Amersham. ^{3}H-valproic acid (10 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Female Sprague-Dawley rats (250–300 gm) obtained from Charles River (Raleigh, NC) were maintained on a 12-hr light/dark cycle. Rats were housed in wire-mesh cages and allowed free access to food and water. Gut Buffer (GB) consisted of NaH_2PO_4 (1.5 mM), Na_2HPO_4 (2.5 mM), NaCl (140 mM), $CaCl_2$ (0.12 mM), KCl (4.75 mM) and glucose (5.8 mM) pH 7.2.

Radiolabeling of Oligonucleotides

Oligonucleotide exocyclic amine labeling was performed by reductive formylation using ^{14}C-formaldehyde. The procedure consisted of dissolving the oligonucleotide (200 nmol) in a 0.2M sodium phosphate buffer (250 μL) (pH 8.2). ^{14}C-formaldehyde (2–48 μCi) was added and the solution stirred at room temperature for 1 hour. Sodium cyanoborohydride (25 μl of a 100 mM solution) was added to the reaction and stirred for various times. Oligonucleotides were precipitated by adding 10 volumes of n-butyl alcohol (for methylphosphonates) or isopropyl alcohol (for phosphorothioates) and stored at −20°C for 1 hour. The oligonucleotide was pelleted by centrifugation at 4°C (10 min, 10,000 g). The supernatant was removed and the pellet washed (3×) with alcohol and dried. The oligonucleotide was dissolved in water and purified on Sureplate TLC plates (USB Biochemicals, Cleveland, OH). Sureplates were exposed to X-ray film (X-Omat AR; Kodak, Rochester, NY) overnight and developed. The TLC band corresponding to both the radioactivity detected on the X-ray film and oligonucleotide absorbance on the Sureplate was isolated, as per manufacturer's instructions. Variables tested in the labeling reactions included time of reaction and amount of ^{14}C-formaldehyde. The time course of the reaction was measured by adding constant amounts of the ^{14}C-formaldehyde (2 μCi) to oligonucleotide (50 nmol) and varying the time (1–25 hr) of the reaction. The ratio of ^{14}C-formaldehyde to oligonucleotide was evaluated by increasing amounts of ^{14}C-formaldehyde (2–48 μCi). Specific activity of the final product was determined by counting an aliquot of the labeling reaction and determining the absorbance of the labeled oligonucleotide at 260 nm. Specific activity is defined as μCi/μmol. After the oligonucleotides were labeled and purified they were maintained at − 80°C until their use.

Stability of the Label

The integrity of the label was tested by incubating the ^{14}C-oligonucleotides (100 nmol) in α-MEM tissue culture media (50 mL) with 10% fetal calf serum at 37°C, followed by testing the oligonucleotide for the retention of label at increasing times. Aliquots (100 μL) were removed in triplicate and precipitated with alcohol. The pellet was dissolved in water and transferred to Ecoscint H (5 mL) scintillation counting cocktail (National Diagnostics, Atlanta, GA). To test the stability of the ^{14}C and FITC labels during everted intestinal studies, aliquots (100 μL) of the luminal samples were collected, precipitated with alcohol, and analyzed using Sureplates. After development of the plate the lanes were isolated and divided into 1 cm sections; each section was analyzed for content of radioactive or fluorescent label and this was plotted against Rf values.

Everted Gut Studies

Sprague-Dawley rats were injected i.p. pentobarbital (50mg/kg). The small intestine was exposed through a midline abdominal incision. The small intestine was cannulated distal to the ampulla coli and the jejunal segment was perfused with phosphate buffered saline, pH 7.4 (PBS) at 4°C until all particulate matter was cleared. Intestinal segments from the animal were used immediately in order to prevent time-dependent deterioration of intestinal epithelial cells (14). The intestinal segments were excised and placed in ice cold, oxygenated GB immediately. Intestinal segments were everted over a glass Pasteur pipette and divided into 5-cm length sacs. The everted sac was tied at one end (#3 silk suture, American Cyanamid), filled with GB solution (1ml/cm/kg) and tied at the other end. The filled sacs were immersed in 37°C, oxygenated GB (75 mL) containing the tracers of interest (blue dextran 25mg, FITC-labeled dextran 500 μg, ^{14}C- or FITC- labeled oligonucleotide 50 nmol, ^{3}H-valproic acid 0.5 μCi). Everted sacs were removed at intervals (0–60 min), rinsed three times in GB and the contents removed. The intestinal liquid samples were centrifuged (10 min at 1,700 g) and the supernatant transferred to fresh tubes. Supernatant aliquots (500 μl) of the sacs were analyzed for appearance of tracer. It should be noted that transport into the gut sac in this model is equivalent to transport from the mucosal to serosal side in the physiological situation.

Transport at each time interval was defined as the % accumulation within the gut sac [(cpm of oligonucleotide luminal sample)/(cpm of aliquot oligonucleotide in GB) × 100]. The same type of formula was used for fluorescence analysis. Since the total accumulation of oligonucleotides during the experiment was small, little back flux of tracer would occur. Thus, the transport rates approximate initial unidirectional flux rates. Beta counting was done on a Beckman LS 6000SC liquid scintillation counter. Equal aliquots from the sacs were added to 5 ml of Ecoscint H scintillation fluid. For fluorescence measurements a Shimadzu RFU500 spectrofluorometer was used with an excitation wavelength of 495 nm and emission wavelength of 520 nm for FITC-labeled compound, while the rhodamine-labeled dextran was excited at 525 nm and emission monitored at 560 nm. Blue dextran concentration was monitored by visible absorption at 590 nm.

Active Transport

To examine the possibility of active transport process for oligonucleotides, ^{14}C-labeled oligonucleotides and ^{3}H-glucose (0.5 μCi) were added to the GB solution used in both the incubation medium and inside the sacs. The same concentration of the tracers was present in the external GB solution. The amount of radioactivity that accumulated with time in the gut sacs was monitored. The presence of ^{3}H-glucose allowed for testing the viability of tissues during the experiment, since glucose is actively transported by viable intestinal epithelium.

RESULTS

Oligonucleotide Labeling and Stability

The reductive-formylation-reaction scheme for exocylic amines is shown in Figure 1. There were 6 potential purine exocylic amine sites which could react with formaldehyde. Each individual exocylic amine site is likely to have its own reactivity and all amine sites might not react under the labeling conditions. Figure 2A depicts the time-course for labeling the methylphosphonate oligonucleotide. At room temperature the labeling reaction reached equilibrium in 4 hours after the addition of sodium cyanoborohyride. Figure 2B illustrates that formaldehyde amounts greater than 25 μCi did not increase the labeling. The specific activity of final products was 8 ± 2 μCi/μmol for both methylphosphonate and phosphorothioate oligonucleotides. Under the reaction conditions used (200 nmol of oligonucleotide, 25 μCi formaldehyde, and 4 hours reaction time), this equates to 11% of the possible total sites reacted with ^{14}C-formaldehyde. This will differ for various sequences of oligonucleotides and reaction conditions.

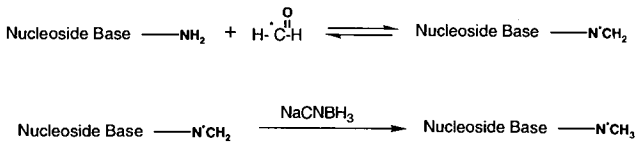

Fig. 1. The general scheme for radiolabeling exocylic amines of oligonucleotides.

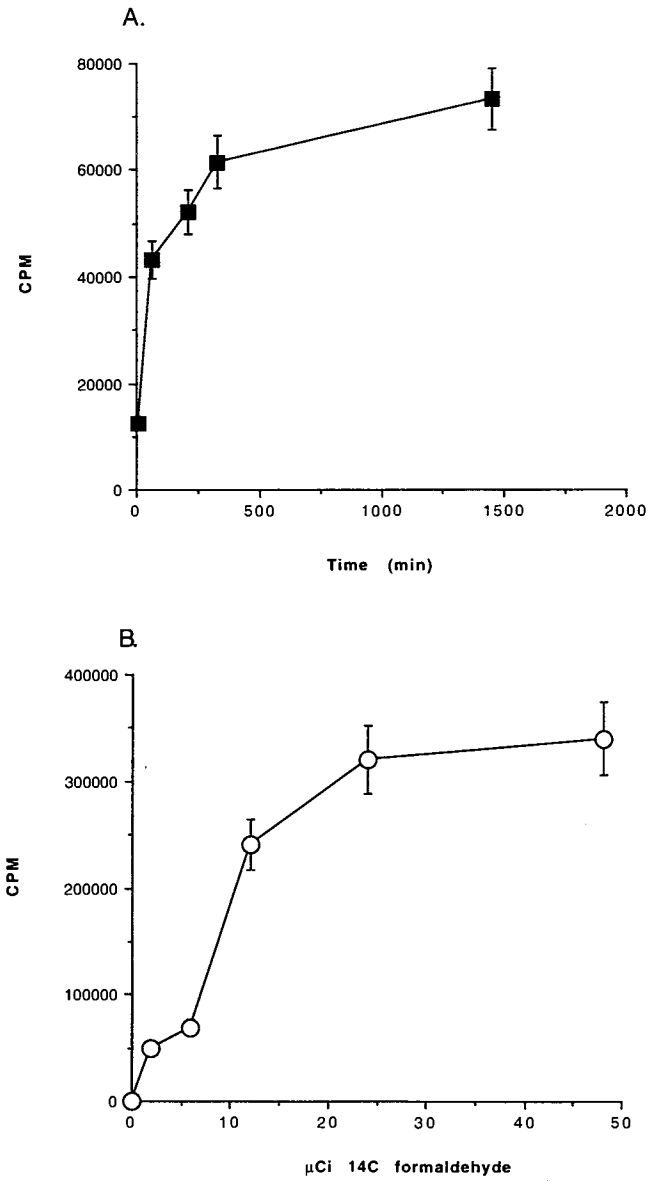

Fig. 2. Reductive alkylation of oligonucleotides. (A) Labeling versus time. MP oligonucleotide (50 nmol) was reacted with ^{14}C-formaldehyde (2 μCi) for increasing time intervals after the addition of sodium cyanoborohydride as described in Methods. The radiolabeled oligonucleotide was purified and the activity measured. (B) Labeling versus amount of radioactive label. A constant amount (100 nmol) of MP oligonucleotide was reacted with increasing amounts of ^{14}C-formaldehyde as described in Methods. The radiolabeled oligonucleotide was purified and the amount of radioactivity quantitated.

Acylation of nucleic acids often produces a labile base, as is the case with DNA foot printing experiments; however, either pH extremes or elevated temperature are required for depurination. In order to test the stability of the labeled oligonucleotide, an *in vitro* test system was used. Figure 3 illustrates the time-course of loss of the ^{14}C label for a phosphorothioate oligonucleotide in tissue culture media (pH 7.4) at 37 °C. Under the conditions tested, the label remains intact over the time-course subsequently used in transport studies. Approximately 13% of the label was lost over 100

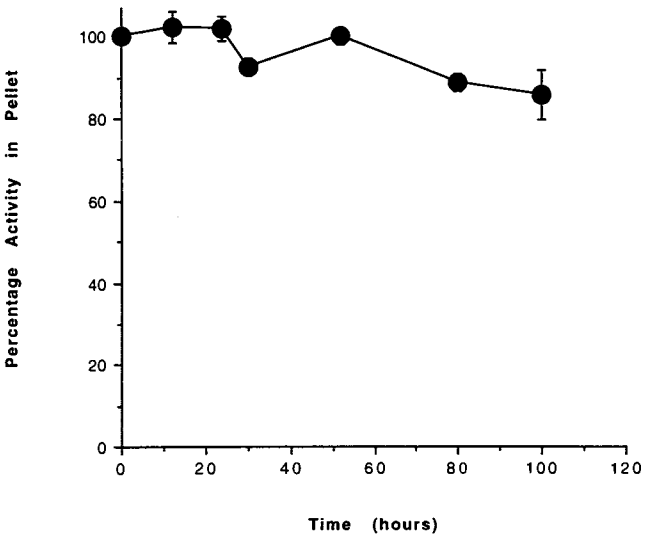

Fig. 3. Hydrolytic stability of ^{14}C-labeled PS oligonucleotide over time measured in tissue culture media at 37°C. The stability was measured by precipitating the oligonucleotide with isopropyl alcohol and determining the CPM within the pellet. The zero time point was considered 100% of the activity. Symbols represent mean ± standard deviation (s.d.) of three experiments.

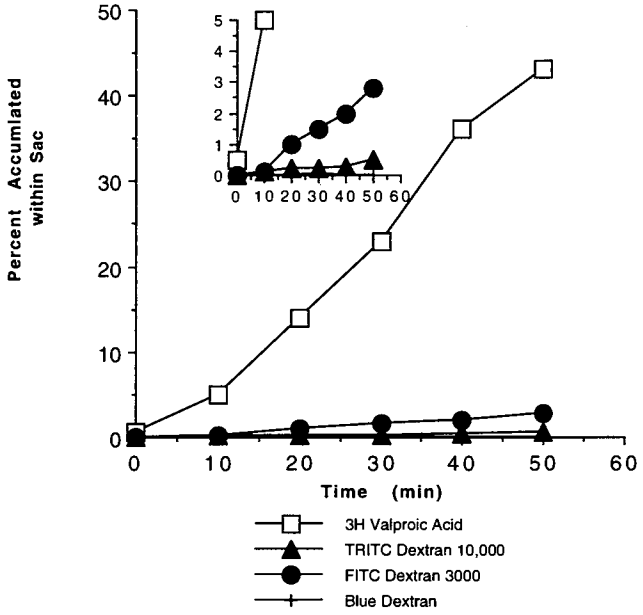

Fig. 4. Intestinal transport of model compounds. A representative study demonstrating oligonucleotide accumulation into everted jejunal sacs was monitored as described in Methods. The inset figure represents the same graph but with a condensed y-axis. The ^{3}H cpm, FITC fluorescence (corrected for quenching), and blue dextran absorbance were determined in each sample. Each point represents a single gut sac.

hours; similar percentages were also lost from MP oligonucleotides (data not shown).

Transepithelial Transport Studies

Intestinal transport studies with ^{14}C-radiolabeled and 5′-fluorescent oligonucleotides were conducted under unidirectional transport conditions where no tracers were present on the luminal side of the sac at the beginning of the experiment. Alternatively, tagged oligonucleotides (radiolabel or fluorescent label) were used to eliminate label-dependent transport phenomena. In all experiments conducted, high MW blue dextran, an impermeant substance, was used as a negative control to ascertain if major leaks were present in the intestinal sacs. The low MW drug ^{3}H-valproic acid (MW 144) served as a positive control, since it is known to be rapidly transported in the gut (15). In validation studies of the model, fluorescent labeled dextrans (MW 3,000 and 10,000) were used as tracers. Figure 4 illustrates the molecular weight dependency of transport through the everted intestinal sacs. The low molecular weight compound valproic acid penetrated very rapidly, while high molecular weight blue dextran was not transported to any significant degree. There was a distinct difference in the rate of transport of fluorescent labeled dextrans as a function of molecular size. This is consistent with previous oral dosing studies in rats demonstrating a molecular weight cut off of 20,000 for gut uptake of fluorescent dextrans (16). Thus the model used in these studies appears to mimic what normally occurs in the intact animal with regard to transport across the GI epithelium.

Intestinal transport of the backbone modified oligonucleotides is shown in Figure 5. This depicts a radiolabeled MP oligonucleotide co-incubated with a 5′-labeled FITC PS oligonucleotide of the same sequence, along with blue dextran as a control. As indicated, the internal concentration of oligonucleotides reached about 5% of the external concentration within 60 min. This is clearly a much slower rate of transport than that observed for valproic acid (Fig. 4), which is known to be readily available by the oral route. However, oligonucleotide transport is clearly more rapid than transport of high molecular weight blue dextran. A discrepancy was noted in the rate of transport for 5′- FITC-labeled and ^{14}C-labeled oligonucleotides, with the fluorescent label always indicating a slower rate of transport. This proved to be due to a measurement artefact, since the presence of lipids and cell debris within the gut sac tended to quench the fluorescent signal intensity. In mock experiments where identical

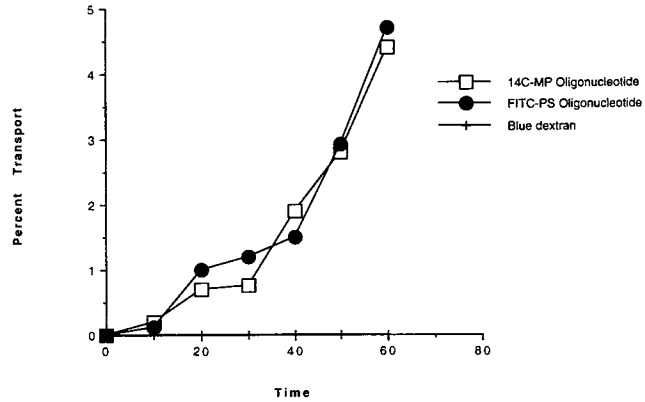

Fig. 5. Intestinal transport of oligonucleotides. A representative study demonstrating oligonucleotide accumulation in everted jejunal sacs was monitored as described in Methods. The ^{14}C cpm, FITC fluorescence (corrected for quenching), and blue dextran absorbance was determined in each sample. Each point represents a single gut segment.

amounts of 5'-FITC-labeled oligonucleotide were added to blank GB or the contents of a gut sac (collected after 60 min incubation with blank GB), the FITC conjugated oligonucleotide showed a 10 to 30% decrease in fluorescent signal in the gut sac contents; accordingly, the FITC data were corrected for quenching.

Table 1 summarizes individual oligonucleotide transport experiments, presented as the total percentage of label accumulated within the everted sac at the end of 60 min. For all oligonucleotides examined, 15% or less of the added tracer oligonucleotide accumulated within the sacs during 1 hr. This contrasts with 0–0.1% accumulation for blue dextran, and 42–65% accumulation for valproic acid. There were no consistent differences in the transport rates for MP-oligonucleotides and PS-oligonucleotides, indicating that charge of the oligonucleotides was not an important factor. The rates of transport of the MP-oligonucleotides and PS-oligonucleotides were similar to the transport of fluorescent labeled dextrans of comparable molecular weight (compare Figs. 4, 5).

Under the test conditions described above, the experiments would not differentiate between active or passive transport systems. To investigate the underlying transport mechanism, the model was evaluated under conditions of initial equilibrium (Figure 6). In this situation, active transport would be reflected as an increase in the amount of tracer within the gut sac. Under these conditions active accumulation of glucose was observed, as expected. However, no net accumulation was detected for the labeled oligonucleotides. The increase in glucose concentration is indicative of active transport of this nutrient in functional gut epithelium.

Oligonucleotide Stability During Transport

The stability of the labeled oligonucleotides was studied in the gut sac transport system. After the experiment, material was recovered from the internal contents of the gut sac. Ninety percent of the total accumulated radioactivity was precipitated by alcohol for both the methylphosphonate and phosphorothioate oligonucleotides. To ensure the label recovered from the gut sac remained associated with the oligonucleotide, the pellet was dissolved in water, applied to a TLC plate (Sureplate), and developed. As a control, one lane of the TLC plate contained the original labeled oligonucleotide. Figure 7 (A and B) illustrates the TLC patterns for the labeled oligonucleotides. The figure demonstrates that the original and recovered oligonucleotides had similar Rf values when either the ^{14}C or FITC labels were used. The TLC data indicates that the oligonucleotides (either PS or MP) did not suffer from backbone cleavage, since shorter oligonucleotides would be expected to have higher Rf values. The peaks tailed a small amount in the gut samples compared to the original labeled material. The reason for this tailing is not clear, but may be due to the presence of protein in the samples recovered from the gut sacs. Thus, most of the labeled material accumulating within the intestinal sacs represents high MW oligonucleotide rather than extensively degraded material.

Table 1. Accumulation of Oligonucleotides within Intestinal Sacs[a]

Sample Oligonucleotide	Percentage accumulated at 60 min		
	BD	VA	OND
^{14}C-Ki-Ras-MP	ND	45	12.0
^{14}C-Ki-Ras-MP	0.01	65	10.3
^{14}C-Ki-Ras-MP	0.10	43	8.0
^{14}C-Ki-Ras-MP	ND	NA	12.1
^{14}C-Ki-Ras-MP	0.05	NA	7.4
^{14}C-Ki-Ras-PS	0.04	47	11.8
^{14}C-Ki-Ras-PS	0.09	42	15.0
^{14}C-Ki-Ras-PS	ND	53	10.8
FITC-Ki-Ras-PS	0.05	NA	5.0
FITC-Ki-Ras-PS	0.02	NA	7.6
FITC-Ki-Ras PS	0.05	NA	6.6
FITC-Ki-Ras-PS	0.01	NA	7.8
^{14}C- & FITC-Ki-Ras-PS	0.1	NA	6.4^{14C} and 6.2^{FITC}

[a] BD = blue dextran, VA = valproic acid, ODN = oligonucleotide, ND = not detected, NA = not applicable.

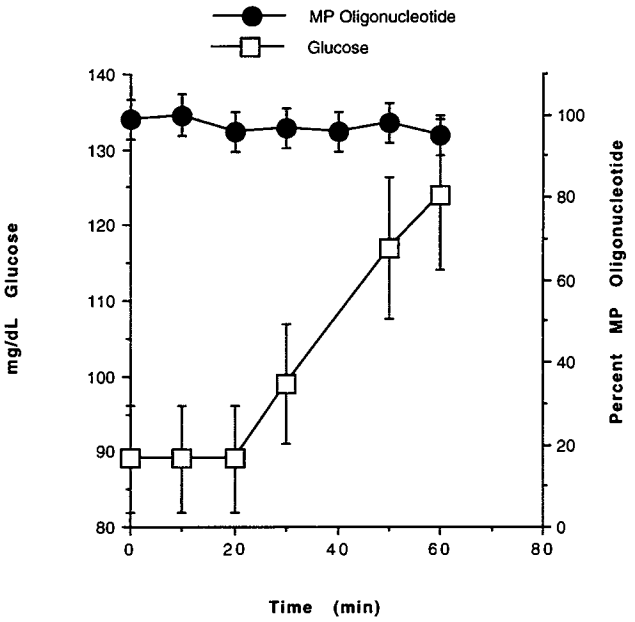

Fig. 6 Test for active transport. The time-course of accumulation within intestinal sacs under initial equilibrium conditions was measured for ^{14}C-labeled MP-oligonucleotide and ^{3}H-glucose. Symbols represent the mean ± s.d. of 4 experiments.

DISCUSSION

A stable radiolabeled oligonucleotide is important for efficient pursuit of biodistribution studies. Ideally, the labeling procedure should: (1) be generic in that it could label different backbone modified oligonucleotides; (2) result in a label that is stable to hydrolytic and enzymatic cleavage; (3) be simple and utilize readily available materials. Several methods have been reported for the introduction of radioisotopes into oligonucleotides. For naturally occurring phosphorodiester oligonucleotides, enzymatic systems can catalyze the phosphorylation of 5' (T4-kinase) or 3' ends (terminal transferase) using ^{32}P-triphosphate nucleotide donors. However, terminal phosphates undergo a rapid loss of label in *in vivo* systems due to enzymatic cleavage. Methylation of oligonucleotides with nucleic acid methylase enzymes (DAM, Hha1) and a ^{3}H-S-adenosyl methionine as the donor

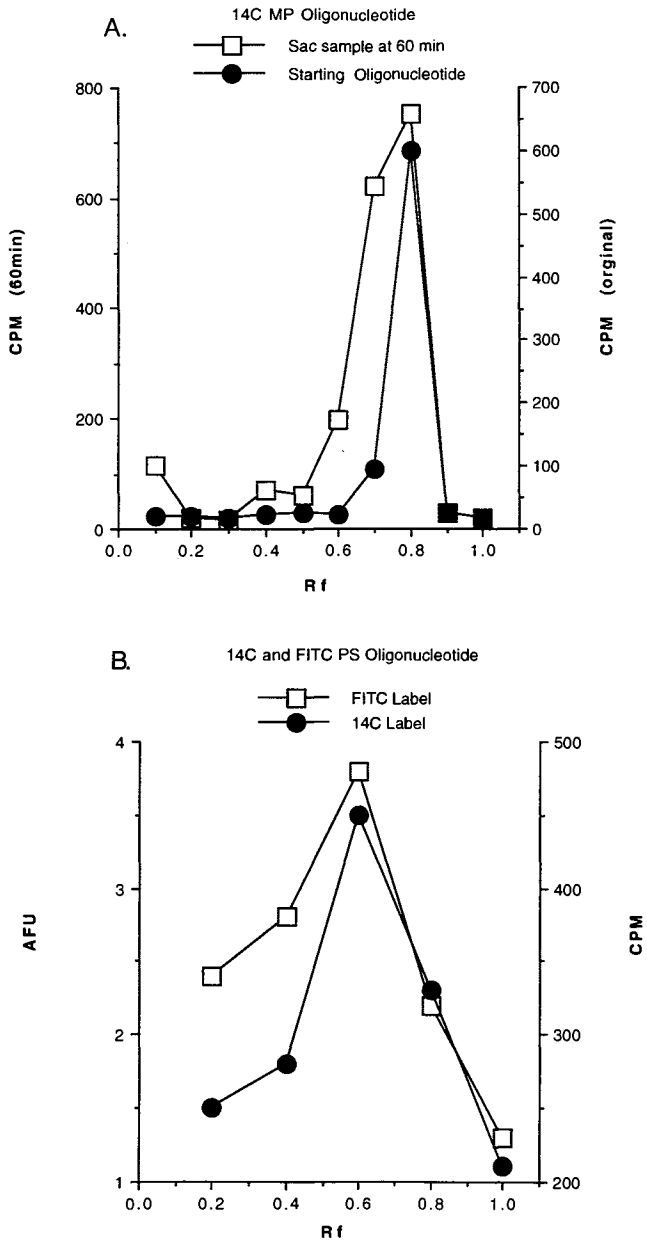

Fig. 7. Stability of labeled oligonucleotides in intestinal transport experiments. Graph A represents the TLC migration of original labeled oligonucleotide in gut sac sample contents from a 60-min transport study with ^{14}C-MP-oligonucleotide; the sample was precipitated with n-butyl alcohol and analyzed by TLC (Sureplates). Graph B illustrates the TLC migration of a dual labeled (^{14}C & FITC) PS oligonucleotide in gut sac sample contents from a 60-min transport study after precipitation with isopropyl alcohol.

source, is another method for labeling charged phosphorothioate and phosphodiester backbone oligonucleotides (17). However, these enzymes are very sequence specific and may not be applicable for many oligonucleotides; further, methylases do not work well on MP-oligonucleotides (S. Akhtar & R. Juliano, unpublished observations). Phosphorothioates are easily labeled in the backbone by using an ^{35}S-oxidizing agent (9,18) during the DNA synthesis cycle, but this requires introduction of high levels of radioactivity into the DNA synthesizer. Introduction of a radiolabeled synthon (7) is a very attractive method for labeling most oligonucleotides, but synthesis of the synthon often is a complex matter and is not feasible for many researchers. A tritium exchange labeling of thymidine residues is possible and applicable to all backbone modified oligonucleotides (19), but this technique requires special equipment and the ability to handle large amounts of radioactivity.

The labeling system described in this report is a very simple method that utilizes commercially available components and is applicable to most nucleoside base amines. Formaldehyde is known to react with purine amines to form a Schiff base that can be sequentially reduced by treatment with sodium cyanoborohydride. Endocylic imino groups of thymidine also have been reported to interact with formaldehyde (20). The specific activity obtained for methylphosphonate and phosphorothioate oligonucleotides were similar, demonstrating that the labeling reaction is independent of backbone linkage. The ^{14}C-labeled oligonucleotides remained intact under the experimental conditions used subsequently in transport studies. This ^{14}C labeling procedure would be expected to impair the biological action of oligonucleotides by interfering with Watson-Crick base pairing. Even with this limitation, ^{14}C-labeled oligonucleotides should serve as excellent tracers for biodistribution and transport studies.

Two major routes exist for the transport of drugs through intestinal barriers into the circulation assuming the drug reaches the intestinal membrane intact. Transcellular routes include passive diffusion and energy dependent transport systems. Small compounds (MW <500) usually undergo passive diffusion through the epithelial cell layer of intestines, and this is quite dependent on the lipophilicity of the compound. There are a number of active, receptor-mediated, transcellular pathways for nutrients (10). Alternatively, compounds may bypass cellular membranes and utilize a paracellular pathway. This is an aqueous, extracellular route across endothelia and epithelia for many polar substances, dependent on their size and charge. Under normal conditions, many exogenous macromolecules are restricted from transport via the paracellular route by tight junctions. Oral delivery strategies for peptides and proteins include approaches to alter the tight junctions in order to increase the bioavailability of the agent via the paracellular route (21).

Several systems are available for studying intestinal transport of drugs and macromolecules (10, 22, 23). However, some of these approaches require specialized techniques to obtain interpretable data. One of the first and easiest methods employed to study intestinal transport was the use of everted rat intestinal sacs (24); this model has been used to study the transport of many compounds (25, 26). Although the sacs may suffer from pathological changes after removal from the animal (27), if the tissue is treated carefully, many of the active transport mechanisms remain functional for quite some time (28). In our studies, D-glucose, which is transported by an active process, was used as a positive control for the viability of the everted intestinal sacs.

Oligonucleotides crossed rat intestinal epithelium by a passive process (Fig. 6), probably via a paracellular route. The rate of transport of oligonucleotides was far more rapid than that of high MW blue dextran, but was substantially less

than that for the low MW compound valproic acid. Dextrans of similar molecular weight were transported at a rate similar to that of the oligonucleotides (Figs. 4, 5); dextran serves as a model compound which is known to be transported by the paracellular pathway (29). The tight junctions of epithelial cells are likely to degrade with time after removal of tissue from the animal, but in our experiments the ability of the epithelium to exclude very high MW compounds such as blue dextran was maintained over the time course of the study (Fig 4). There are often different transport rates for charged and non-charged molecules through biological barriers (30). However, in this test system the charged (PS) and the uncharged (MP) oligonucleotides had similar transport rates. This suggests that both types of oligonucleotides are transported by the same mechanism(s), and that the MP oligonucleotides do not diffuse across the lipid bilayer membranes of the intestinal cells.

Many factors in addition to tissue transport influence the oral bioavailability of drugs, including acid degradation in the stomach, enzymatic cleavage in the intestine, and liver first-pass effects. Any of these factors could significantly alter the overall bioavailability of oligonucleotides; this study addressed only the issue of intestinal membrane transport. Further, the current study only evaluated the jejunum for transport; other regions of the small or large intestine could have different transport mechanisms or rates.

In summary, a simple and convenient method was developed for the radiolabeling of oligonucleotides. The method is sequence- and backbone-independent. The transport of ^{14}C- and FITC-labeled oligonucleotides was evaluated in everted rat intestinal sacs. Modest passive transport was observed for PS and MP oligonucleotides using this model. The goal of this study was to answer the question "can oligonucleotides cross intestinal membrane barriers ?" The answer to the question is—yes, but at relatively slow rates. The transport appears to follow a paracellular route, and resembles that of other polar compounds of similar size.

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