

# Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice

(antisense inhibition of gene expression/antiviral therapy/oligodeoxynucleotide uptake/human immunodeficiency virus)

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Communicated by Paul Zamecnik, June 7, 1991

**ABSTRACT** We describe preliminary studies of the pharmacokinetics, biodistribution, and excretion of an oligodeoxynucleotide phosphorothioate ([S]oligonucleotide) in mice. After either intravenous or intraperitoneal administration of a single dose (30 mg/kg of body weight), [S]oligonucleotide (<sup>35</sup>S-labeled at each internucleotide linkage) was found in most of the tissues for up to 48 hr. About 30% of the dose was excreted in urine within 24 hr, irrespective of the mode of administration; the excreted [S]oligonucleotide was found to be extensively degraded. In plasma, stomach, heart, and intestine, the [S]oligonucleotide was degraded by only 15%, whereas in the kidney and liver degradation was about 50% in 48 hr. The surprising observation was made that chain length extension of administered [S]oligonucleotide occurred in kidney, liver, and intestine. These results provide an initial definition of parameters for the pharmaceutical development of antisense oligonucleotides.

Previous studies demonstrated that replication of human immunodeficiency virus (HIV) could be inhibited by normal phosphodiester oligonucleotides complementary to HIV RNA (1–3). However, the relatively short half-life of normal oligonucleotides in serum and in cells, due to the presence of nucleases, limits their potential usefulness *in vivo* (4). To overcome this limitation, oligonucleotides with modified internucleoside phosphate backbones, such as methylphosphonates (5, 6), phosphorothioates, and several phosphoramidate analogues (3, 7–12), have been studied. These analogues of oligodeoxynucleotides are more resistant to nucleases than their unmodified counterparts, and they should therefore exhibit longer survival times *in vivo*. Oligodeoxynucleotide phosphorothioates ([S]oligonucleotides) are effective in inhibiting HIV replication in tissue culture, with ID<sub>50</sub> values (inhibitory dose causing 50% suppression of virus) as low as 10–100 nM (3).

Our continued interest in developing antisense oligonucleotides as possible chemotherapeutic agents led us to investigate the pharmacokinetics, biodistribution, and half-life of [S]oligonucleotides in mice.

## EXPERIMENTAL PROCEDURES

**Synthesis of Oligodeoxynucleotides.** [S]Oligonucleotides were synthesized on an automated synthesizer (model 8700; Milligen/Biosearch, Novato, CA) using H-phosphonate chemistry on controlled-pore glass, followed by oxidation with 0.2 M sulfur in carbon disulfide/pyridine/triethylamine (9:9:1, vol/vol). The syntheses were carried out on a 5- to 10- $\mu$ mol scale. The [S]oligonucleotides were purified by low-pressure ion-exchange chromatography (DEAE-cellulose, DE-52; Whatman) followed by reverse-phase chromatogra-

phy (C<sub>18</sub>) and finally dialysis (10). The [S]oligonucleotide synthesized for the present study had the base sequence 5'-ACA-CCC-AAT-TCT-GAA-AAT-GG-3', which is complementary to the HIV *tat* splice acceptor site (nucleotides 5349–5368).

**<sup>35</sup>S-Labeled [S]Oligonucleotide.** Five milligrams of oligodeoxynucleoside H-phosphonate (20-mer, same sequence as above) bound to controlled-pore glass was oxidized with a mixture of <sup>35</sup>S<sub>8</sub> (5 mCi, 1 Ci/mg, Amersham; 1 Ci = 37 GBq) in 40  $\mu$ l of carbon disulfide/pyridine/triethylamine (9:9:1). After 30 min, 100  $\mu$ l of 7% (wt/vol) unlabeled S<sub>8</sub> in the same solvent mixture was added and the reaction continued for another 60 min. The solution was removed and the support was washed with carbon disulfide (3  $\times$  500  $\mu$ l) and with acetonitrile (3  $\times$  700  $\mu$ l). The product was deprotected in concentrated ammonia (55°C, 14 hr), evaporated, and desalted with a Sep-Pak C<sub>18</sub> column (Waters). The resultant product was purified by 20% polyacrylamide gel/7 M urea electrophoresis. The desired band was excised under UV illumination and the [S]oligonucleotide was extracted from the gel and desalted. The yield was five A<sub>260</sub> units (150  $\mu$ g; specific activity, 5  $\times$  10<sup>9</sup> cpm/ $\mu$ mol; 0.44  $\mu$ Ci/ $\mu$ g). Purified <sup>35</sup>S-labeled [S]oligonucleotide 20-mer contained  $\approx$ 2% 19-mer.

**Mice.** Male CD2F1 mice (average weight, 20 g) were used. The [S]oligonucleotide was dissolved in sterile water. The concentration of the solution was adjusted so that 200  $\mu$ l administered to a 20-g mouse resulted in a dose of 30 mg/kg of body weight (specific activity, 8.02  $\mu$ Ci/mg). <sup>35</sup>S-labeled [S]oligonucleotide was mixed with unlabeled [S]oligonucleotide to obtain the desired concentration.

The dose was administered intravenously via the tail vein in one group of mice and intraperitoneally in another group. At 5 min, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 6 hr, 9 hr, 12 hr, 24 hr, and 48 hr following intravenous administration, one test animal was sacrificed and blood and organs were removed. At 1, 2, 3, 6, 9, 12, 24, and 48 hr following intraperitoneal administration one test animal was sacrificed and blood and organs were removed for determination of total radioactivity. The organs were homogenized (in deionized water, 1:10 dilution) prior to the determination of total radioactivity. The homogenates were kept at –20°C until extractions.

The urine and feces were collected in metabolism cages for total radioactivity determination from each of three animals following both intravenous and intraperitoneal routes of administration. Samples were collected from 0 to 6 hr, 6 to 12 hr, and 12 to 24 hr.

**Extractions of [S]Oligonucleotides from Tissue Homogenates.** Homogenized tissue (200  $\mu$ l) was treated with proteinase K (2 mg/ml) in extraction buffer (0.5% SDS/10 mM

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Abbreviations: HIV, human immunodeficiency virus; [S]oligonucleotide, oligodeoxynucleotide phosphorothioate.

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NaCl/20 mM Tris-HCl, pH 7.6/10 mM EDTA) for 2 hr at 37°C. The samples were then extracted twice with phenol/chloroform (1:1, vol/vol) and once with chloroform. After ethanol precipitation, the oligonucleotides were analyzed by electrophoresis in 20% polyacrylamide gels containing 7 M urea. The gels were fixed in 10% acetic acid/10% methanol solution before autoradiography.

## RESULTS

**Excretion and Status of [S]Oligonucleotide in Urine.** Following a dose of 30 mg/kg given either intravenously or intraperitoneally,  $\approx 30\%$  of the administered dose was excreted in the urine over 24 hr (Table 1). In a separate experiment, three mice were injected intravenously with [S]oligonucleotide as in Table 1 and 36% was again found to be excreted in the urine over the first 24 hr. From 24 to 48 hr, urine was collected by catheterization, which revealed an additional 8.7% excretion (average) of [S]oligonucleotide (data not shown). The excretion in urine was found to be independent of the route of administration. The dose excreted in feces was  $<1\%$  in the first 24 hr, followed by 15% between 24 and 48 hr (data not shown).

The urine was analyzed by gel electrophoresis to check the status of the [S]oligonucleotide. For the intravenous route of administration, there was no degradation of [S]oligonucleotide in urine collected between 0 and 6 hr; about 5% degradation was observed in 6–12 hr and 15% in 12–24 hr. In contrast, for the intraperitoneal route, 75% of the [S]oligonucleotide in urine collected between 0 and 6 hr was degraded and by 12–24 hr degradation was about 90% (Fig. 1).

**Bioavailability and Status of [S]Oligonucleotide in Plasma and Tissues.** Plasma levels of [S]oligonucleotide in mice were rapidly depleted after either intravenous or intraperitoneal administration with a concomitant accumulation of oligonucleotide in the tissues (Figs. 2 and 3). As expected, higher plasma concentrations were found after intravenous administration than after intraperitoneal administration.

[S]Oligonucleotide concentration in the kidney and liver was markedly higher than in other tissues after either route of administration. The [S]oligonucleotide accumulated with time in these two organs up to 48 hr, the end point of the present study. In the other tissues studied there was an elimination of the [S]oligonucleotide with time from the initial higher concentration. The highest concentrations were found in the kidney. The [S]oligonucleotide did not appear in the brain in significant amounts.

Gel electrophoresis of [S]oligonucleotide recovered from plasma indicated that the circulating material remained quite intact over 24 hr (Fig. 4), although obviously decreasing in total amount (see Fig. 3). The same overall result was obtained with [S]oligonucleotide recovered from small and large intestine (Fig. 5). In kidney (Fig. 6) and liver (data not shown), a much larger fraction of the [S]oligonucleotide recovered at each time point was present as degraded frag-

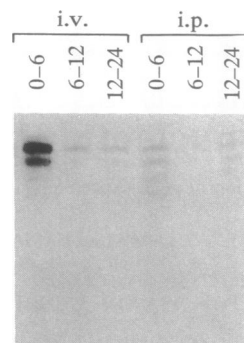


FIG. 1. Polyacrylamide gel electrophoresis of  $^{35}\text{S}$ -labeled [S]oligonucleotide in urine excreted from mice receiving a dose of 30 mg/kg administered intravenously (i.v.; mouse IV3) or intraperitoneally (i.p.; mouse IP3). The urine was collected during periods 0–6 hr, 6–12 hr, and 12–24 hr after injection.

ments, approaching 30–40% of the total [S]oligonucleotide at 24 hr and 50% at 48 hr (Fig. 7).

The gel electrophoretic analysis of the [S]oligonucleotide recovered from kidney displayed the progressive appearance at 6 hr of two bands of slower mobility than the initially administered oligonucleotide (Fig. 6). From the mobility of these two bands it appears that they might represent the addition of nucleotides to the [S]oligonucleotide. At 24 hr,  $\approx 10\%$  of the [S]oligonucleotide was in this form.

In the case of intestine, a more pronounced alteration of [S]oligonucleotide electrophoretic mobility was observed. A

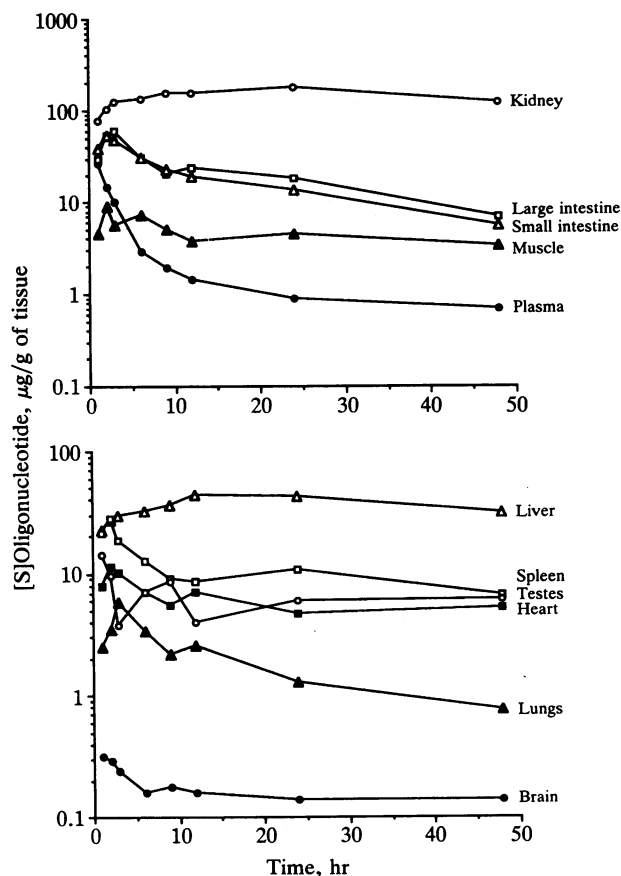


FIG. 2. Tissue levels of [S]oligonucleotide in mice after 30 mg/kg was administered intraperitoneally. One mouse was killed at each time point and the total radioactivity was determined in various organs. The tissue concentrations of oligonucleotide presented here are based on the radioactivity determination.

Table 1. Urinary excretion of [S]oligonucleotide

Mouse	% dose recovered in urine			Total
	0–6 hr	6–12 hr	12–24 hr	
Intravenous administration (30 mg/kg)				
IV1	18.2	4.39	7.51	30.1
IV2	6.76	16.4	9.64	32.8
IV3	28.2	4.65	9.44	42.3
Intraperitoneal administration (30 mg/kg)				
IP1	9.11	2.23	17.6	28.9
IP2	6.35	8.09	12.6	27.0
IP3	10.1	7.35	13.1	30.6

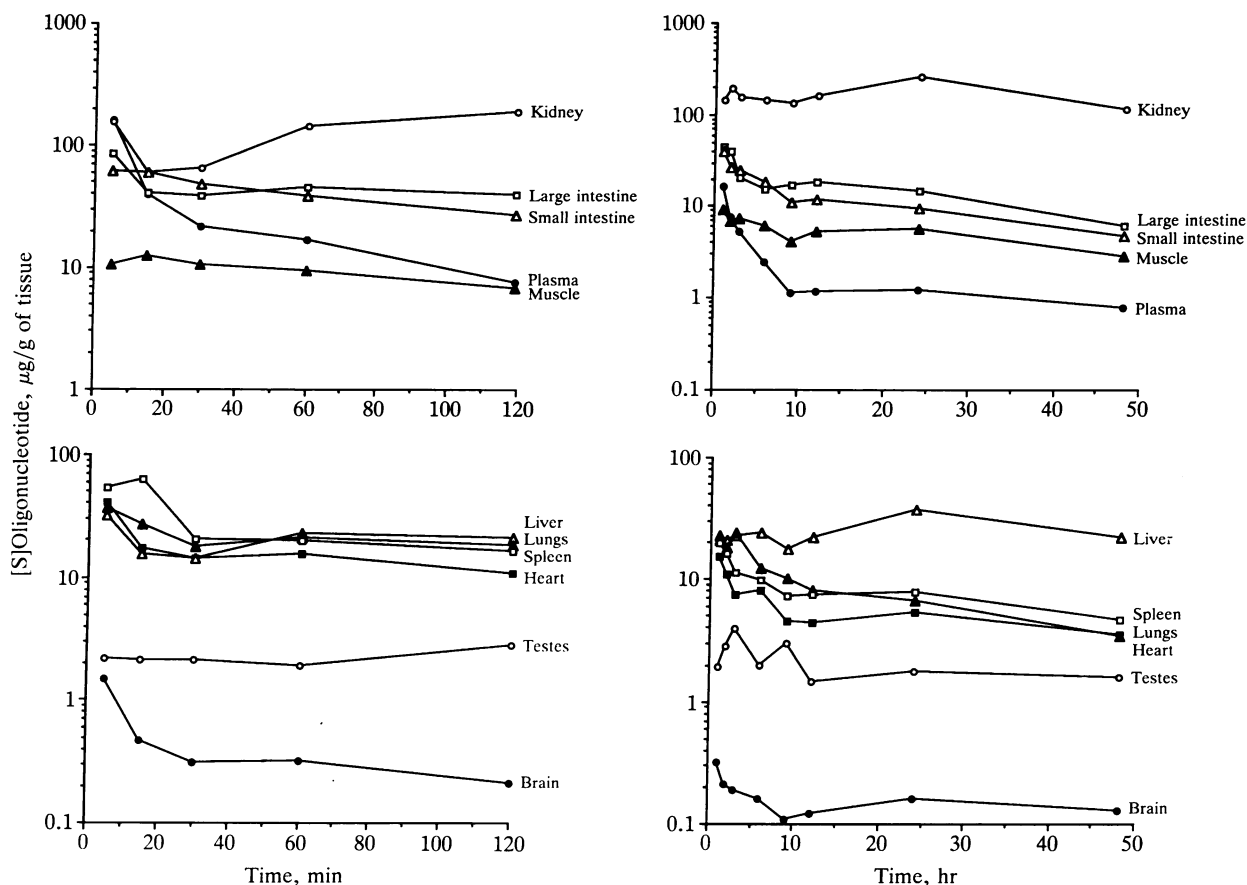


FIG. 3. Tissue levels of [S]oligonucleotide in mice 5–120 min (Left) or 1–48 hr (Right) after 30 mg/kg was administered intravenously. The concentrations of oligonucleotide in tissues were calculated from the total <sup>35</sup>S radioactivity.

portion of the <sup>35</sup>S radioactivity migrated much slower than the injected [S]oligonucleotide (Fig. 5). In the case of large intestine (Fig. 5 Upper), at only 5 min postinjection there was a series of bands that migrated behind the initial [S]oligonucleotide. These bands appeared to represent an oligomeric series. There were also some very high molecular weight bands that barely entered the gel (see 30-min, 1-hr, 3-hr, and 9-hr lanes). In small intestine (Fig. 5 Lower) these high molecular weight bands were more evident, whereas bands of intermediate mobility were less apparent. In both large and small intestine, there was no evidence of bands migrating immediately behind the initial oligonucleotide as was observed in kidney (Fig. 6).

**DISCUSSION**

[S]oligonucleotides at concentrations of 10–100 nM are effective in inhibiting HIV replication in cell culture (3). The

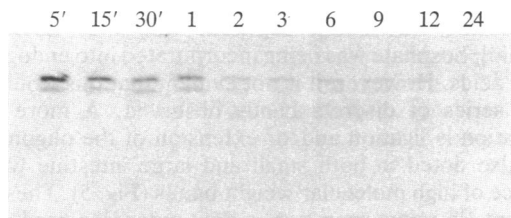


FIG. 4. Stability of [S]oligonucleotide in plasma after intravenous administration to mice. The <sup>35</sup>S-labeled [S]oligonucleotide injected was a 20-mer with a trace of 19-mer. At times indicated above the lanes [5 min (5') to 24 hr], the mice were sacrificed and the oligonucleotide was extracted from plasma and analyzed by gel electrophoresis followed by autoradiography.

inhibition of HIV replication in chronically infected cells is sequence-dependent, and only complementary [S]oligonucleotides are effective (9, 10). A preliminary toxicity study of [S]oligonucleotides in mice and rats has shown that a dose of 150 mg/kg of body weight is nontoxic if given intraperitoneally (3). Daily administration, either intraperitoneally or subcutaneously of [S]oligonucleotide at 100 mg/kg of body weight for 14 days causes no observable toxicity or mortality in mice (unpublished data).

The present study shows that [S]oligonucleotide administered either intravenously or intraperitoneally to mice are initially distributed in most of the tissues and then are depleted. About 30% of the [S]oligonucleotide was excreted in urine after 24 hr regardless of the route of administration or the level of [S]oligonucleotide in plasma. Less than 1% of the [S]oligonucleotide was excreted in feces during the first 24 hr, and an additional average 15% was excreted between 24 and 48 hr. In a parallel study, an oligonucleotide (20-mer) containing 15 phosphodiester linkages and 4 phosphorothioate linkages (contiguous at the 5' end) was excreted in urine to about 75% of the administered dose (30 mg/kg) in 12 hr (data not shown).

The [S]oligonucleotide was quite stable in most of the tissues except kidney and liver. Only 10–15% degradation was observed. The tissues from which [S]oligonucleotide was extracted and analyzed by gel electrophoresis included plasma, heart, spleen, large and small intestine, and stomach. In kidney and liver, [S]oligonucleotide was degraded with time, most probably by exonucleases (as suggested from the pattern of degradation). The rate of degradation was found to be almost linear with time, and about 50% was degraded in 48 hr.

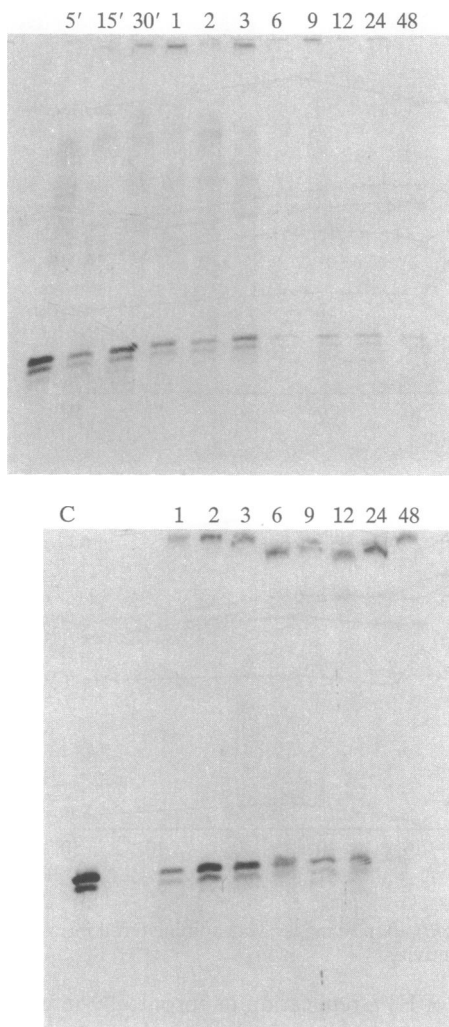


FIG. 5. Status of the  $^{35}\text{S}$ -labeled [S]oligonucleotide (20-mer) in large intestine after intravenous administration (*Upper*) and in small intestine after intraperitoneal administration (*Lower*). Mice were killed at the times indicated [5 min (5') to 48 hr], and intestine was removed, homogenized, and extracted. Lane C shows the [S]oligonucleotide that was administered. Bands moving more slowly than the 20-mer were observed within 5 min.

In kidney and liver, new bands appeared on gel electrophoresis at the 1-hr time point, moving more slowly than the 20-mer band (Fig. 6). The intensity of this band increased

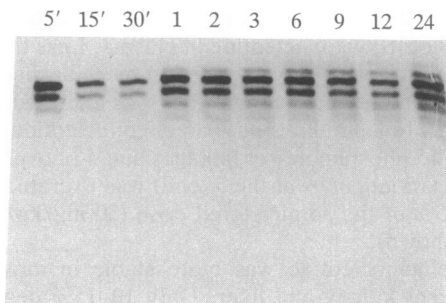


FIG. 6. Stability of [S]oligonucleotide in kidney after intravenous administration to mice. Mice were killed at the times indicated [5 min (5') to 24 hr], and kidneys were removed, homogenized, and extracted. Degradation was observed within 5 min and increased with time. By 24 hr, about 50% of the [S]oligonucleotide was degraded. Bands moving more slowly than the 20-mer could be observed by 1 hr. Similar results were obtained when kidney samples were from mice receiving [S]oligonucleotide intraperitoneally.

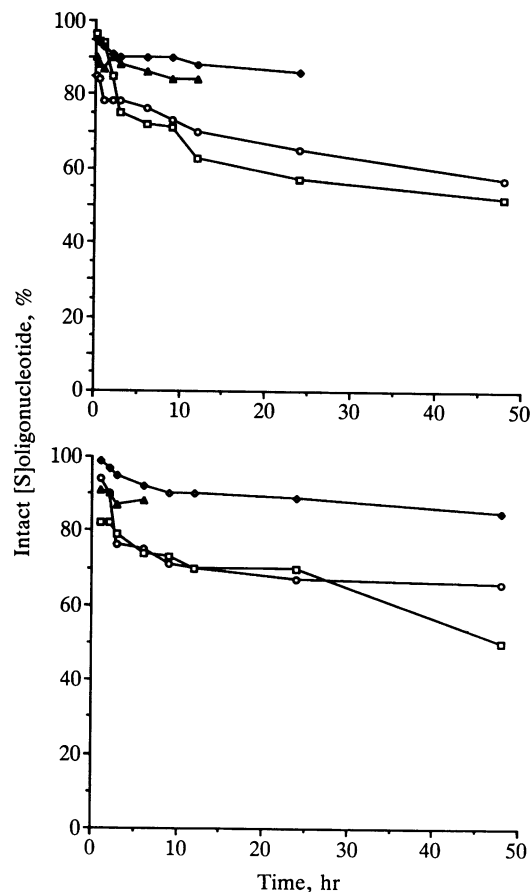


FIG. 7. Percentage of intact [S]oligonucleotide (20-mer) in liver (O), kidney (□), plasma (▲), and stomach (◆) after intravenous (*Upper*) and intraperitoneal (*Lower*) administration. The percentage is based on measurement of total radioactivity in tissues, followed by gel electrophoresis and autoradiography. The autoradiograph was scanned by a densitometer. In plasma, exact percentage of intact oligonucleotide at all time points could not be measured because of low radioactivity.

with time after injection, and by 6 hr an additional band appeared. By 24 hr, three bands moving more slowly than the administered 20-mer were observed. The mobilities of these additional bands are suggestive of the presence of extra nucleotides. A similar phenomenon was observed with [S]oligonucleotide recovered from liver (data not shown). The presence of the slower-mobility bands in both kidney and liver was observed with both intraperitoneal and intravenous routes of administration.

In the case of oligonucleotide extracted from large and small intestine, following either intravenous or intraperitoneal administration, a series of bands moving more slowly than 20-mer was observed (Fig. 5). It is possible that [S]oligonucleotide was being degraded and that nucleoside [ $^{35}\text{S}$ ]thio]phosphate was being incorporated into endogenous nucleic acids. However, it is not evident that this would lead to the series of discrete bands observed. A more likely explanation is ligation and/or extension of the oligonucleotide. Also noted in both small and large intestine was the presence of high molecular weight bands (Fig. 5). These may represent  $^{35}\text{S}$  reincorporation, a limit extension product, or, conceivably, ligation of the [S]oligonucleotide to an endogenous macromolecule.

In summary, a pharmacokinetic study of [S]oligonucleotide in mice has demonstrated that the [S]oligonucleotide is distributed in most of the tissues and that up to 30% is excreted in urine in 24 hr and an additional 10% in 24–48 hr.

The oligonucleotide analogue appears to be degraded mostly by exonucleases. In some organs, such as kidney, liver, and intestine, it is extended in length.

We are indebted to Paul C. Zamecnik for encouragement, continuous guidance, and support in this work. We are also indebted to Dr. Thoru Pederson for critical review of the manuscript and comments. We gratefully acknowledge the advice and cooperation of Dr. Wayne Galbraith (APD Co., Alexandria, VA). The assistance of Dr. Salab El Dareer and Kathy Tillery of the Southern Research Institute (Birmingham, AL) in evaluating the tissue oligonucleotide concentrations is gratefully acknowledged. We thank Ms. Rebecca Bell and Mrs. Carol Tuttle for processing the manuscript and for expert secretarial assistance. This work was supported in part by a grant from the National Institute of Allergy and Infectious Diseases (U01 124846) and financial assistance from Hybridon, Inc.

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