

Uptake Characteristics of Oligonucleotides in the Isolated Rat Liver Perfusion System

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ABSTRACT

The objective of this study was to examine the hepatic disposition characteristics of 20-mer model phosphodiester oligonucleotide (PO) and its partially phosphorothioated (PS₃) and fully phosphorothioated (PS) derivatives in the single-pass isolated rat liver perfusion system. [³²P]-labeled oligonucleotides were momentarily introduced into this system through the portal vein as a bolus input mode, and the venous outflow patterns were evaluated using statistical moment analysis. The apparent volumes of distribution of these oligonucleotides were greater than those of reference substances for vascular space (erythrocytes) and extracellular space (human serum albumin), indicating a significant interaction between oligonucleotides and the liver. Significant hepatic uptake of oligonucleotides was also observed. About 20%, 36%, and 52% of the injected dose (3 μg/rat) was taken up by the liver during a single passage after bolus injection of PO, PS₃, and PS, respectively. In the case of PS injection, slow efflux from the liver was observed in the latter phase of perfusion. This suggests that the hepatic uptake process of these oligonucleotides greatly depended on their types. The results of collagenase perfusion experiments suggest that PS₃ oligonucleotides were taken up by both liver parenchymal and non-parenchymal cells. The amount of total recovery in the liver decreased substantially by coadministration of polyinosinic acid, dextran sulfate, polycytidic acid and 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid. This suggests that PS₃ was taken up by the liver as an anionic molecule in a nonspecific manner.

INTRODUCTION

ANTISENSE OLIGONUCLEOTIDES have attracted special interest as a novel class of chemotherapeutic agents for the treatment of cancer, viral infections, and genetic disorders, as they can inhibit gene expression in a sequence-specific manner. However, unmodified phosphodiester oligonucleotides (PO) are primarily susceptible to nucleases (Zon, 1988; Crooke, 1995). To avoid this degradation, many derivatives of PO have been prepared; among them, phosphorothioated oligonucleotides (PS) are being investigated extensively (Crooke, 1991; Ghosh and Cohen, 1992). Although there are several articles demonstrating the usefulness of PS as antisense therapeutic agents (Bennett et al., 1994; Dean and McKay, 1994), there is growing evidence that this type of oligonucleotide may have problems of nonsequence-specific biologic activities (Ghosh et al., 1993; Stein and Cheng, 1993; Perez et al., 1994; Burgess et al., 1995; Barton and Lemoine, 1995) and toxicities (Galbraith et al., 1994; Sarmiento et al., 1994). Hence, it is desirable to develop delivery systems that can improve *in vivo* stability, cellular up-

take, and target selectivity of unmodified or minimally modified oligonucleotides as well as reduce their toxicity *in vivo*.

Following systemic administration, oligonucleotides are distributed to all major peripheral organs, with liver and kidney accumulating most of injected oligonucleotides (Agrawal et al., 1991; Cossum et al., 1993; Iversen et al., 1994; Saijo et al., 1994; Zhang et al., 1995a,b). Although the liver plays an important role in the disposition of oligonucleotides, their hepatic disposition characteristics are not yet fully understood. Hence, the objective of this study was to examine the hepatic disposition characteristics of 20-mer model antisense PO and its partially phosphorothioated (PS₃) and fully phosphorothioated (PS) derivatives in the isolated rat liver perfusion system.

MATERIALS AND METHODS

Chemicals

The 20-mer antisense oligonucleotides (3'-TACGGG-GAGTTGCAATCGAA-5') complementary to the human c-

myc mRNA, including the AUG translation initiation codon site, were purchased from Genosys Biotechnologies Inc., The Woodlands, TX. These oligonucleotides are PO, phosphoester oligonucleotides; PS₃ (3'-TsAsCsGGGGAGTTGCAATC-GAA-5') oligonucleotides, in which only three internucleotide phosphodiester linkages at the 3'-end are replaced by phosphorothioates; PS, fully phosphorothioated oligonucleotides (3'-TsAsCsGsGsGsGsGsAsGsTsTsGsCsAsAsTsCsGsAsA-5'). These oligonucleotides were obtained from the manufacturer as clear solutions with >99% purity and T_m of about 56–60°C. Polyinosinic acid (poly [I], mol wt 170,000–1,000,000), polycytidic acid (poly [C], mol wt 170,000–1,900,000), 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS, mol wt 498.5), and dextran sulfate (DS, mol wt 15,000) were purchased from Sigma Chemicals (St. Louis, MO). Bovine serum albumin (BSA) and [methoxy-¹⁴C]inulin (185 MBq/g) were purchased from Armour Pharmaceutical Co. (U.K.) and New England Nuclear (Boston, MA), respectively. Clear-sol I and T4 polynucleotide kinase (10 U/μl) were purchased from Nacalai Tesque (Kyoto, Japan) and Takara Biomedicals (Otsu, Japan), respectively. Adenosine triphosphate ([γ-³²P]ATP, ~6000 Ci/mmol), and Soluene-350 were obtained from Amersham Life Science (Tokyo, Japan) and Packard (The Netherlands), respectively. All other chemicals were obtained commercially as reagent-grade products.

Labeling of oligonucleotides

All oligonucleotides were labeled by adding γ-³²P to the 5'-end terminals using [γ-³²P]ATP and enzyme bacteriophage T4 polynucleotide kinase (Sambrook et al., 1989). Unincorporated label was removed from radiolabeled oligonucleotides by exclusion chromatography with NAP-10 columns (Pharmacia Biotech). Specific activity of the labeled oligonucleotides was about 6–9.5 × 10⁷ cpm/μg.

Liver perfusion experiment

Male Wistar rats (200–220 g) were obtained from Shizuoka Agricultural co-operative Association for Laboratory Animals (Shizuoka, Japan), and liver was perfused *in situ* as described previously (Nishida et al., 1989). The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg), the abdomen and chest were opened, and the portal vein and the inferior vena cava were cannulated with polyethylene tubing (PE-160). Freshly prepared and filtered (0.2 μm) albumin-free and erythrocyte-free Krebs-Ringer bicarbonate buffer (pH 7.4), supplemented with 10 mM glucose was oxygenated with 95% O₂:5% CO₂ and delivered to the portal vein catheter via a peristaltic pump at a constant flow rate of 13 ml/minute. The temperature of the perfusion cabinet and perfusion medium was thermostatically controlled at 37°C. The bile duct was cannulated with polyethylene tubing (PE-10), and bile was collected into the preweighed tubes at 10 minute interval for 1 hour. The bile flow rates for 37°C and 4°C experimental systems were 3.81 ± 1.31 and 0.61 ± 0.22 ml/minute, respectively.

An initial stabilization period of 30 minutes was allowed before introducing the mixture of [³²P]-labeled (0.15 × 10⁶ cpm/rat) and unlabeled oligonucleotides (0.03–30 μg as a total dose/rat) dissolved in the perfusion medium into the portal vein using a six-rotary valve injector. PS₃ was studied at doses of 0.03, 0.3, 3, and 30 μg/rat, whereas PO and PS were studied at

a dose of 3 μg/rat. Venous outflow samples were collected up to 1 minute into the preweighed tubes at 0.5–4 second intervals. The sample volumes collected were calculated from the gain in weight in the tube, assuming the density of the outflow perfusate to be 1.0. The sampling time was calculated from each sample volume, assuming a constant flow rate. After the perfusion experiment, the whole liver was excised, weighed, and homogenized. For the study at 4°C, all the experimental procedures were the same as those of the study at 37°C, except that the perfusion medium was kept on ice.

For determining the hepatic localization of oligonucleotides, the liver was perfused at 37°C with a perfusion medium containing 5 mM CaCl₂ and 0.05% (w/v) collagenase 15 minutes after [³²P]PS₃ (3 μg/ml) administration. Following perfusion, parenchymal cells (PC) and nonparenchymal cells (NPC) were separated by centrifugation, as described by Horiuchi et al. (1985). The cell viability was more than 90% as estimated by the trypan blue exclusion method.

Analytic methods

The radioactivity of the effluent perfusate and bile samples was measured using a liquid scintillation counter (LSA-500, Beckman, Tokyo, Japan) after addition of scintillation medium (Clear-sol I). The radioactivity in the homogenized liver or cell suspensions was measured in the same manner after dissolution with Soluene-350 through incubation overnight at 45°C and neutralization with 2 N HCl.

Pharmacokinetic analysis of outflow patterns

We previously described the detailed theoretical background of moment analysis for local injection (Kakutani et al., 1985) and its application to analyze the hepatic disposition of macromolecular and microparticulate carriers (Nishida et al., 1991; Takino et al., 1995). In the present study, outflow patterns were also analyzed by using statistical moment analysis. The statistical moment parameters for the outflow pattern are defined as follows.

$$AUC = \int_0^{\infty} C dt \quad (1)$$

$$\bar{t} = \frac{\int_0^{\infty} tC dt}{AUC} \quad (2)$$

where t is the time and C is the concentration of compounds normalized with respect to the percentage of dose per milliliter. AUC and \bar{t} denote the area under the concentration-time curve and mean transit time of the drug through the liver, respectively. The moments defined by Eqs. (1) and (2) can be calculated by numerical integration using a linear trapezoidal formula and extrapolation to infinite time based on a monoexponential equation (Yamaoka et al., 1978). The t values were corrected for the lag time of the catheter.

The hepatic disposition parameters of oligonucleotides, representing reversible and irreversible processes, were calculated using the following equations.

$$F = AUC \times Q/100 \quad (3)$$

$$V = \bar{t}/AUC \times 100 \quad (4)$$

$$E = 1 - F \quad (5)$$

$$t_{cor} = \bar{t}/F \quad (6)$$

$$k_{el} = E/\bar{t} \quad (7)$$

$$CL_{int} = V \times k_{el} \quad (8)$$

where F is the recovery ratio, V is the apparent volume of distribution, t_{cor} is the corrected mean transit time, E is the extraction ratio, k_{el} is the first-order elimination rate constant, CL_{int} is the intrinsic clearance, and Q is the perfusion flow rate. These parameters can be divided into three groups: parameters representing reversible (V and t_{cor}), irreversible (E , F , k_{el}) processes, and both (CL_{int}).

Inhibitory effect of polyanions on hepatic uptake of oligonucleotides

The mixtures of [32 P]-labeled (specific activity $\sim 9.5 \times 10^7$ cpm/ μ g) and unlabeled PS_3 (0.3 μ g/rat) as well as unlabeled polyanion (DS, poly [I], poly [C], 15 μ g/rat) or SITS (15 μ g/rat) were coinjected in the single-pass rat liver perfusion model. At 30 minutes after administration of oligonucleotides, the liver was harvested and subjected to radioactivity assay.

RESULTS

Outflow patterns after single-pass liver perfusion

Figure 1 shows the typical outflow concentration-time curves of [32 P]PO, PS_3 , and PS after bolus administration at a dose of 3 μ g/rat. The peak concentration of the outflow concentration-time curves was in the following order of magnitudes: PO (45%/ml) > PS_3 (35%/ml) > PS (10%/ml). Figure 2 shows the typical outflow concentration-time curves of [32 P] PS_3 after bolus administration at doses of 0.03, 0.30, 3.00, and 30 μ g/rat. The normalized peak concentration decreased as the dose de-

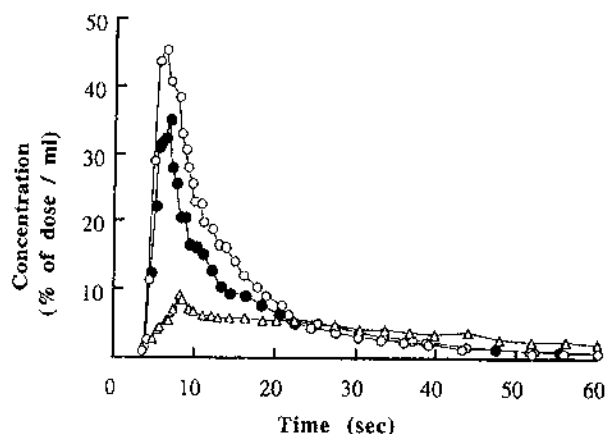


FIG. 1. Typical venous outflow patterns of [32 P]-labeled oligonucleotides in the single-pass rat liver perfusion system. \circ PO; \bullet PS_3 ; Δ PS.

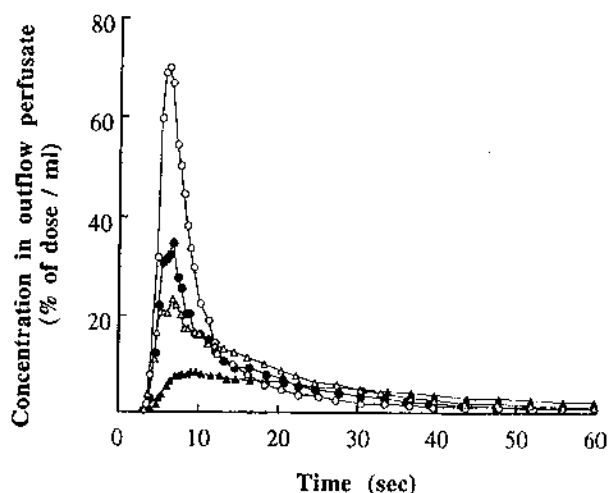


FIG. 2. Typical venous outflow patterns of [32 P] PS_3 in the single-pass rat liver perfusion system. Doses: \circ 30, \bullet 3, Δ 0.3, \blacktriangle 0.03 μ g/rat.

creased, suggesting a saturable process for hepatic uptake of oligonucleotides.

Pharmacokinetic analysis of outflow patterns

Table 1 summarizes the pharmacokinetic parameters derived from the concentration-time curves. The results for [51 Cr]RBC and [131 I]HSA were referred as vascular reference substances from our previous report (Nishida et al., 1989). The apparent volumes of distribution (V) for [51 Cr]RBC and [131 I]HSA correspond to the volume of the sinusoidal space (0.209 ml/g liver) and sinusoidal space plus the space of Disse (0.252 ml/g liver), respectively. The oligonucleotides showed a large distribution volume (0.70–1.55 ml/g), indicating that there is an interaction between oligonucleotides and the liver. The hepatic interaction of PS_3 was a saturable process because its V values decreased from 0.796 to 0.315 ml/g liver as the administration dose increased from 0.30 to 30 μ g/rat. The dose-dependent uptake mechanism of PS_3 in the liver was supported by the E_{∞} values, which decreased from 45.38 ± 8.46 to $3.00 \pm 0.84\%$.

Figure 3 compares the extraction ratio and liver accumulation of all three types of oligonucleotides. The former was estimated from the outflow patterns of 0–1 minute (i.e., E_1) following extrapolation to infinite time (i.e., E_{∞}), and the latter from the total radioactivity in the liver at 60 minutes after injection. The actual liver accumulation at 60 minutes after bolus administration of oligonucleotides at a dose of 3 μ g/rat was 16.86 ± 1.77 , 30.12 ± 3.53 , and $36.67 \pm 1.38\%$ of dose per rat for PO, PS_3 , and PS, respectively. No significant difference was found in the liver accumulation of PS_3 and PS at 60 minutes after bolus injection. The calculated E_{∞} values for PO, PS_3 , and PS samples (3 μ g/rat) were 15.33 ± 1.81 , 33.20 ± 3.07 , and $35.77 \pm 0.851\%$ of dose per rat, respectively. These data are in good agreement with the actual liver accumulation of these oligonucleotides. However, the E_1 value for the PS sample (3 μ g/rat) was $51.9 \pm 0.858\%$ of dose per rat, which is significantly different from its actual liver accumulation. At 4°C perfusion, the liver accumulation of PS_3 (3 μ g/rat) was 27.85 ± 1.74 (% of dose per rat), which was slightly lower than that of

TABLE 1. MOMENTS AND DISPOSITION PARAMETERS FOR OLIGONUCLEOTIDES AND REFERENCE COMPOUNDS IN SINGLE-PASS RAT LIVER PERFUSION SYSTEM

	Dose (mg/rat)	Moment parameters				Disposition parameters					
		AUC (% of dose. sec/ml)	t (sec)	V (ml/g)	t _{cor} (min)	E _∞ (%)	k _{el} (min ⁻¹)	CL _{int} (ml/min/g)			
[³² P]PO	3	402.42 ± 19.25 ^a	16.63 ± 1.45	0.691 ± 0.09	0.333 ± 0.02	15.33 ± 1.81	0.434 ± 0.17	0.214 ± 0.08			
[³² P]PS ₃	0.3	263.99 ± 27.16	20.82 ± 1.98	0.796 ± 0.02	0.408 ± 0.03	45.38 ± 8.46	1.446 ± 0.19	1.150 ± 0.85			
	3	320.32 ± 11.57	17.21 ± 8.82	0.746 ± 0.40	0.314 ± 0.03	33.20 ± 3.07	0.857 ± 0.56	0.786 ± 0.61			
	30	445.71 ± 5.83	12.51 ± 0.74	0.315 ± 0.01	0.214 ± 0.01	3.00 ± 0.84	0.162 ± 0.10	0.051 ± 0.03			
[³² P]PS	3	289.2 ± 5.09	41.69 ± 2.44	1.546 ± 0.14	1.081 ± 0.09	35.77 ± 0.85	0.516 ± 0.04	0.798 ± 0.01			
[⁵¹ Cr]RBC ^b	—	471.3	8.89	0.209	0.148	0	—	0			
[¹²⁵ I]HSA ^b	—	485.9	9.33	0.252	0.156	0	—	0			

^aResults are expressed as the mean ± SD (standard deviation) of three separate experiments.

^bPublished results (Nishida et al., 1989).

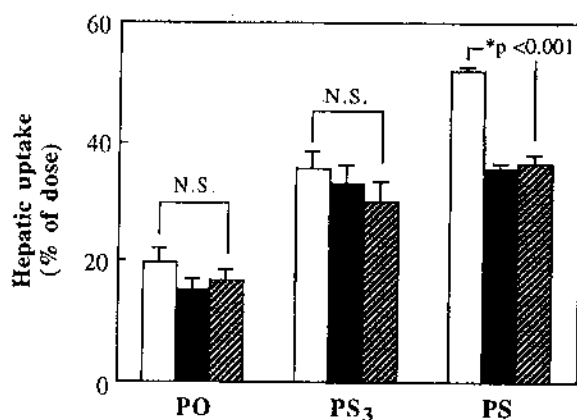


FIG. 3. Comparison between the liver accumulation and extraction ratio of oligonucleotides. Hepatic uptake was assessed at 60 minutes after injection, and the extraction ratio (E_1) was estimated from the out-flow patterns of 0–1 minute and extrapolated to infinite time (E_∞) in the single-pass rat liver perfusion system. □ E_1 ; ■ E_∞ ; ▨ liver accumulation.

the 37°C data. Moreover, these oligonucleotides were undetectable in the bile during the entire course of liver perfusion. Total recovery of the administered dose was almost 100%, as the sum of total radioactivity of the effluent perfusate and that of the liver was in the range of 98%–102% for these oligonucleotides. The extraction ratio and liver accumulation of these oligonucleotides were influenced by the number of sulfur atoms present in the oligonucleotide molecules, resulting in the following order of magnitudes: PO < PS₃ < PS.

Cellular localization of oligonucleotides

Figure 4 shows the results of collagenase liver perfusion experiments of [³²P]PS₃ administered at a dose of 3 μg/rat. Radioactivity was recovered in both PC and NPC as 7.85 ± 0.85% and 2.40 ± 0.25% of the dose/10⁹ cells, respectively.

Inhibitory effects of anionic compounds on hepatic uptake of PS₃

Coadministration of polyanions (DS, poly[I] and poly[C], each 15 μg/rat) or SITS (15 μg/rat) caused a significant de-

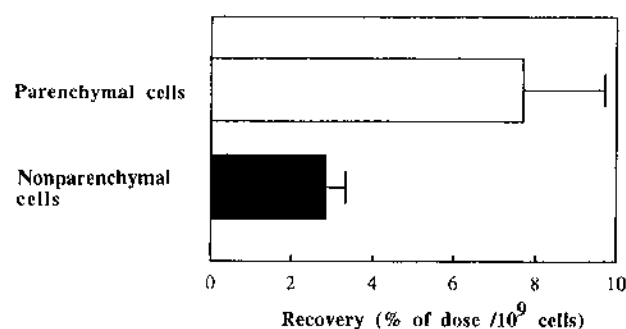


FIG. 4. Hepatic cellular localization of radioactivity of [³²P]PS₃ in the single-pass rat liver collagenase perfusion. The results are expressed as the mean ± SD of three separate experiments.

crease in the amount of total recovery of [³²P]PS₃ (0.3 μg/rat) in the liver (Fig. 5).

DISCUSSION

Systemic delivery of antisense oligonucleotides is promising for the treatment of both genetic and nongenetic disorders (Bayever et al., 1992). Rational delivery systems should be designed based on information on the disposition characteristics of naked oligonucleotides. Hence, as a first step, we previously studied the stability and pharmacokinetic properties of antisense oligonucleotides after intravenous injection into mice (Miyao et al., 1995). The oligonucleotides were rapidly eliminated from the plasma and accumulated mainly in the liver and kidney. In a subsequent study (Sawai et al., 1995), we also performed the kidney perfusion experiments to examine their renal disposition properties at organ level. In the present study, we investigated the hepatic disposition characteristics of three types of oligonucleotides at organ level using the isolated rat liver perfusion system.

The use of a buffer containing erythrocytes and serum albumin is good for maintaining better physiologic functions of the liver during perfusion. It is also better to use these blood components to correlate the results of liver perfusion experiments with *in vivo* results. However, erythrocytes were excluded from our experiments to avoid possible contamination of nucleases. Albumin-free perfusate was used to examine the direct interaction of oligonucleotides with the liver without the effect of protein binding. The concentration of [³²P]-end-labeled oligonucleotides in the effluent perfusates was monitored for a relatively short period to avoid the influence of possible degradation products in data analysis. Although the stability of [³²P]-labeled oligonucleotides was not confirmed in the present conditions, the labeled compounds were fairly stable in a 10% serum-containing medium and during passage through the perfused rat kidney (unpublished results).

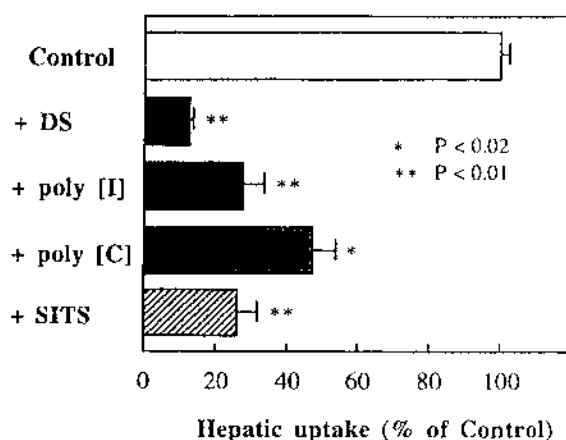


FIG. 5. Inhibitory effects of anionic compounds on hepatic uptake of PS₃ oligonucleotides in the single-pass rat liver perfusion system. [³²P]PS₃ (0.3 μg/rat) and inhibitory compounds (15 μg/rat) were coadministered to the portal vein. At 30 minutes after DNA administration, the liver was harvested and subjected to radioactivity assay. The results are expressed as the mean ± SD of three separate experiments.

The viability of the liver was checked with respect to both the bile flow and the glutamic oxaloacetic transaminase activity in the outflow. In all the experiments, perfused livers remained viable during the entire course of the study. The validity of the experimental conditions is supported by our previous series of studies on the hepatic uptake of macromolecules via nonspecific and receptor-mediated mechanisms (Nishida et al., 1991; Yoshida et al., 1996).

Our main purpose was to study the hepatic uptake characteristics of oligonucleotides at early phases, and, hence, the experimental doses (0.03–30 $\mu\text{g}/\text{rat}$) were chosen primarily based on pharmacokinetics. In this study, the peak concentration in the venous outflow perfusates was in the range of 3 ng/ml to 20 $\mu\text{g}/\text{ml}$ (Figs. 1 and 2), which approximately corresponds to the initial blood concentrations in the peripheral blood after intravenous injection in pharmacokinetic studies using rats.

Initial hepatic uptake processes of oligonucleotides were evaluated using the experimental system described, and the concentration-time curves of the perfusates were analyzed by moment analysis. All types of oligonucleotides were significantly taken up by the liver during a single passage, resulting in large volumes of distribution compared with those of the vascular reference substances, such as HSA and RBC (Table 1). For 3 μg PS_3/rat sample, there was no significant difference in hepatic uptake at 4°C and 37°C, suggesting that the apparent hepatic extraction will correspond to the hepatic binding of oligonucleotides. For 3 $\mu\text{g}/\text{rat}$ dose samples of PO and PS_3 , the hepatic extraction ratios (E_1) corresponded well to their actual hepatic accumulation (Fig. 3), validating this analysis. The discrepancy between E_1 and hepatic accumulation of PS (3 $\mu\text{g}/\text{rat}$) suggests its slow efflux from the liver in the latter phase of perfusion as characterized by a larger t_{cor} value. This suggests that the hepatic uptake process of PS greatly differs from those of PO and PS_3 . These results are supported by the fact that fully phosphorothioated oligonucleotides exhibit both increased cellular retention and increased nonspecific protein binding relative to unmodified and partially phosphorothioated oligonucleotides (Brown et al., 1994; Guvakova et al., 1995; Weidner et al., 1995). However, no significant difference in the actual liver accumulation was found between PS_3 and PS at 60 minutes after bolus injection. The oligonucleotides used in this study bound to BSA, and the extent of this binding was in the following order of magnitude: $\text{PS} > \text{PS}_3 > \text{PO}$ (unpublished data). Hence, the oligonucleotides must have bound nonspecifically to the hepatic cells, since protein-free perfusion medium was used in this study. Although serum-containing or albumin-containing buffer was not used in the present study, we speculate that the presence of serum or albumin is not likely to change the overall patterns of the hepatic uptake of protein-unbound oligonucleotides due to sulfur atom-dependent interaction of the oligonucleotides with the liver.

PS_3 was selected for further studies because partially phosphorothioated oligonucleotides may overcome problems associated with unmodified and fully phosphorothioated oligonucleotides. PS_3 was taken up by both PC and NPC (Fig. 4). Coadministration of polyanions (DS, poly [I], poly[C]) and SITS caused a substantial decrease in the amount of total recovery in the liver (Fig. 5). It is well known that poly[I] binds to the scavenger receptors, whereas poly[C] does not (Brown et al., 1980; Acton et al., 1993; Kawabata et al., 1995). This suggests

that PS_3 was nonspecifically taken up by the liver as an anionic compound, as both large and small anionic molecules inhibited its hepatic binding. In contrast to these results, Inagaki et al. (1992) reported that 1 hour after intravenous injection of ^{32}P -labeled 15-mer phosphorothioates into rats, the hepatic uptake of radioactivity was mainly by the nonparenchymal cells. Biessen et al. (1995) also demonstrated that the binding of ^{32}P -labeled 18-mer phosphodiester oligonucleotides to both Kupffer and endothelial cells *in vitro* was mediated by a specific receptor whose characteristics are similar to the scavenger receptor. However, the results reported by these authors cannot be directly correlated with our findings because of the difference in the types of oligonucleotides and the experimental procedures used.

To our knowledge, this is the first report of quantitative evaluation of hepatic uptake of oligonucleotides at organ level using the isolated rat liver perfusion system. The present findings suggest that oligonucleotides bind to the liver tissues in a nonspecific manner, and the introduction of sulfur atoms to the oligonucleotide internucleotides increases the interaction of oligonucleotides to the liver. The preferential uptake of oligonucleotides by the liver emphasizes the potential use of antisense therapy in liver diseases. However, the liver has been identified as one of the major target organs for dose-dependent and sulfur atom-dependent toxicity with antisense therapy (Sarmiento et al., 1994; Galbraith et al., 1994). Hence, to avoid both hepatic toxicity and target organs other than the liver, delivery systems need to be designed.

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