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Cellular Pharmacology of Phosphorothioate Homooligodeoxynucleotides in Human Cells

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SUMMARY

The uptake and distribution of phosphorothioate oligodeoxynucleotides by human cells were studied using ^{35}S-labeled 28-mer phosphorothioate oligodeoxycytidine [S-$(dC)_{28}$]. Accumulation of intracellular S-$(dC)_{28}$ was found to be higher in the carcinoma cells (grown in monolayers) than in the leukemia cells (grown in suspension culture). A hepatoma cell line transfected with hepatitis B virus, 2215, was chosen for further studies. The uptake of S-$(dC)_{28}$ was partially dependent on temperature and energy. The intracellular concentration was significantly higher than that in the medium and the amount accumulated was dependent on the extracellular concentration. It appears that the uptake of S-$(dC)_{28}$ involves mechanisms of both fluid-phase pinocytosis and adsorptive endocytosis. Neither oligonucleotides nor 5′-phosphorylated nucleotides inhibited S-$(dC)_{28}$ uptake. Unlike horseradish peroxidase, which was primarily associated with endosomes once it was taken into the cell, S-$(dC)_{28}$ was found to be present in both nuclear and cytoplasmic fractions. Efflux of S-$(dC)_{28}$ from the cell was multiphasic; a trapping mechanism that could be due to a potent interaction of S-$(dC)_{28}$ with cellular proteins was implicated. This trapping mechanism could be responsible for the lack of biological activity such as cytotoxicity and antisense activity of phosphorothioate oligodeoxynucleotides in some human cells.

A new approach for chemotherapy has been developing based on the fact that addition of defined oligos (antisense inhibitors) to cell cultures could block specific gene expression (1–3). S-oligos have been reported to be some of the most potent inhibitors in antisense technology (4, 5). To date, mechanisms for the transport of S-oligos into living cells remain unclear; however, some indirect information from studies of fluorescently labeled oligos has been advanced by other laboratories (6, 7). These oligos are taken up by cells in a saturable and temperature-dependent manner that may be compatible with adsorptive endocytosis (6). One of the critical proofs of this hypothesis is that the uptake of oligos should be against a concentration gradient; however, such information is currently lacking, and data from studies of 5′-^{32}P-labeled oligos are unreliable because of the undefined stability of these compounds in cell cultures. Little information is available on the intracellular distribution and release of S-oligos. Experiments performed with fluorescently labeled oligos suggest that their distribution in HL-60 cells is restricted to cytoplasm (6). Studies by Chin *et al.* (7) suggest that when fluorescent oligos are injected into cytoplasm they diffuse directly into the nucleus, whereas when they are taken up by the cells they are trapped into endosomes distributed in the cytoplasm perinuclearly. This should prevent intranuclear action by S-oligos; however, studies by Matsukura *et al.* (3) showed that a 28-mer antisense S-oligo complementary to human immunodeficiency virus 1 *rev* gene, when added to the medium of chronically infected cells, could reduce the unspliced virus mRNA transcripts but had little effect on the spliced viral transcripts. These results suggest that the major action of antisense S-oligo occurs in the nucleus. Because the fluorescent oligo carries a highly hydrophobic group, its distribution could be different from that of S-oligo. Furthermore, our previous studies demonstrated that S-$(dC)_{28}$, but not its oxygen congener $(dC)_{28}$, is an inhibitor of human DNA polymerase α, γ, and δ as well as of RNase H *in vitro*, with K_i values in the nanomolar range (8). In cell culture studies, however, we have not found profound cytotoxicity of S-$(dC)_{28}$. In addition, antisense S-oligos targeted at the 5′ leading sequences of various RNAs of hepatitis B virus did not appear to suppress their expression in cell cultures. These results indicate that either cellular accumulation of S-$(dC)_{28}$ is very low or intracellular compartmentation occurs that may prevent the interactions of S-oligos with their targets.

In view of the undefined transport of S-oligo across the cell membrane and the disparity of its cellular distribution between

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ABBREVIATIONS: oligo(s), oligonucleotide(s); S-oligo(s), phosphorothioate oligodeoxynucleotide(s); S-$(dC)_{28}$, 28-mer phosphorothioate oligodeoxycytidine; HPLC, high performance liquid chromatography; TEA, triethanolamine; $(dC)_{28}$, 28-mer oligodeoxycytidine.

TABLE 1

Accumulation and distribution of S-(dC)$_{28}$ in human cells

Cells were incubated with 0.5 μM ^{35}S-(dC)$_{28}$ for 48 hr at 37°, followed by removal of the medium and three washes with ice-cold Krebs-Ringer buffer, pH 7.5. The membrane-bound ligands were eluted in 10 min with a solution of 0.2 M acetic acid and 0.5 M sodium chloride at 4°. After the acid-salt treatment, nuclei were purified by the citric acid-Nonidet P-40 procedure (11). The radioactivity associated with different fractions was examined. Data represent the mean ± standard deviation from two experiments with triplicates in each experiment.

Cell line	S-(dC)$_{28}$			
	Cell-associated	Nucleus	Cytoplasm	Nucleus/Cytoplasm ratio
	pmol/1 × 10^6 cells			
HeLa	11.6 ± 1.0	5.2 ± 0.5	6.2 ± 0.3	0.8
KB	13.0 ± 1.1	2.8 ± 0.2	9.5 ± 0.9	0.3
Hep PLC/PRF/5	35.7 ± 3.2	8.5 ± 1.0	26.9 ± 2.8	0.3
Hep G2	18.8 ± 1.2	3.3 ± 0.3	15.3 ± 0.9	0.2
Hep 2215	42.4 ± 3.8	11.2 ± 1.7	30.1 ± 2.2	0.4
CEM	8.1 ± 0.5	4.6 ± 0.4	3.2 ± 0.1	1.4
H9	10.2 ± 0.6	6.2 ± 0.2	3.8 ± 0.1	1.6
Raji	5.7 ± 0.3	3.4 ± 0.2	2.1 ± 0.1	1.6
P3HR-1 (H1)	5.1 ± 0.4	3.6 ± 0.2	1.2 ± 0.1	3.0
P3HR-1 (L5)	12.6 ± 0.7	9.7 ± 0.1	2.5 ± 0.1	3.8
HL-60	6.4 ± 0.5	3.9 ± 0.2	2.2 ± 0.1	1.8
K-562	6.7 ± 0.4	3.2 ± 0.1	3.3 ± 0.2	1.0

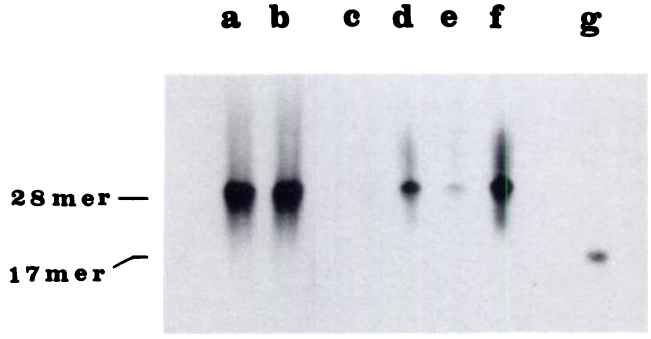


Fig. 1. Stability of ^{35}S-(dC)$_{28}$ in hepatoma cell culture. Hep G2 and 2215 cells were incubated with 0.5 μM ^{35}S-(dC)$_{28}$ for 72 hr at 37°, followed by separation of the nuclei from cytoplasm (11). Samples were desalted and electrophoresed on 15% polyacrylamide-urea gels. Metabolites of ^{35}S-(dC)$_{28}$ from the medium of Hep G2 (*lane a*) and 2215 (*lane b*) cell cultures and from Hep G2 cells (1 × 10^7 cells; *lane c*, nuclei; *lane d*, cytoplasm) and Hep 2215 cells (1 × 10^7 cells; *lane e*, nuclei; *lane f*, cytoplasm) were examined. *Lane g*, 17-mer oligodeoxynucleotide marker.

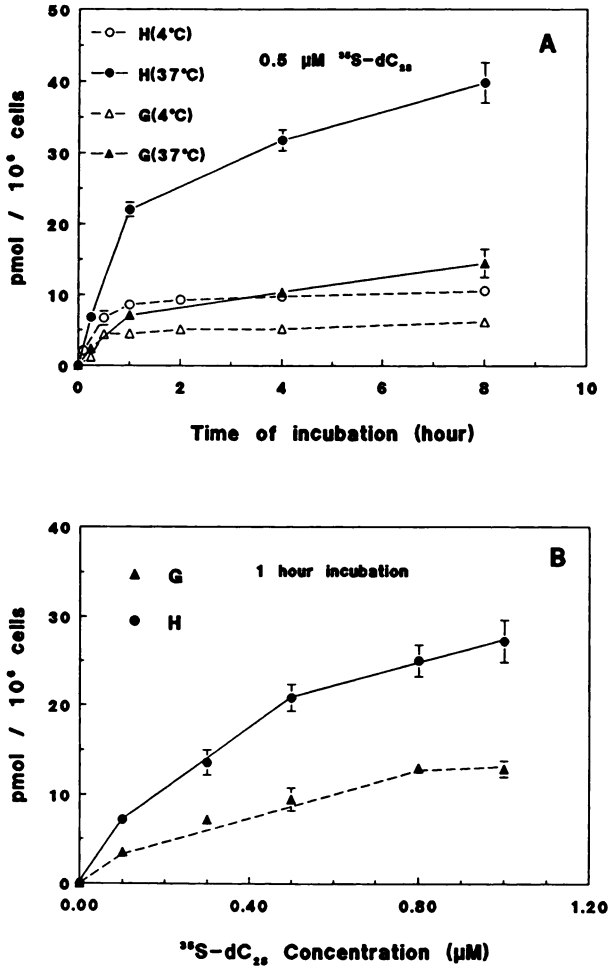


Fig. 2. Kinetics of accumulation of S-(dC)$_{28}$ by Hep G2 and 2215 cells. A, Hep G2 (*G*) and 2215 (*H*) cells were incubated with 0.5 μM ^{35}S-(dC)$_{28}$ at 4° or 37° for specified periods. Cell layers were washed three times with ice-cold Krebs-Ringer buffer. Cell membrane-bound ligands were removed by the acid-salt elution method at 4°. Intracellular radioactivity was measured and interpreted as the intracellular ^{35}S-(dC)$_{28}$. B, Uptake of ^{35}S-(dC)$_{28}$ by hepatoma cells depended on the extracellular concentration of ^{35}S-(dC)$_{28}$. Data represent the means ± standard deviations from two experiments, with triplicates in each experiment.

fluorescently labeled oligo and S-oligo, we undertook an investigation of the cellular uptake and distribution of S-(dC)$_{28}$. Our results show that the uptake of S-(dC)$_{28}$ may involve both fluid-phase pinocytosis and adsorptive endocytosis. S-(dC)$_{28}$, once it enters the cell, is distributed in both nucleus and cytoplasm. This compound binds to cellular proteins including small nuclear proteins (10 to 20 kDa). These results may have implications for the design of improved antisense inhibitors.

Experimental Procedures

Cell and cell culture. The human hepatoma 2215 cell line (subclone of Hep G2 transfected with a plasmid containing hepatitis B virus DNA) was kindly provided by G. Acs (Mount Sinai Medical Center, New York, NY). Cellular accumulation of S-(dC)$_{28}$ was measured in the following human cell lines: the T lymphoblastoid cell line CEM; the T cell line H9; the Burkitt lymphoma cell lines Raji; P3HR-1 subclones H1 and L5; the myelogenous cell line K-562; the cervical carcinoma cell line HeLa S3; the epidermoid carcinoma cell line KB; and the hepatoma cell lines G2, PLC/PRF/5, and 2215 (Hep 2215). Cells were maintained under 5% CO_2/95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hazleton Research Products, Lenexa, KS).

^{35}S-(dC)$_{28}$ uptake. ^{35}S-(dC)$_{28}$ (100 Ci/mmol) was synthesized and purified as described previously (9, 10). Cellular uptake of ^{35}S-(dC)$_{28}$ (50 mCi/mmol) was measured using the procedure described previously (11). Briefly, cell surface binding and the internalization of ^{35}S-(dC)$_{28}$ were determined by an acid-salt elution method, followed by isolation of ligands associated with the nuclei from those of cytoplasm by using a citric acid-Nonidet P-40 procedure (11).

Endosome labeling and subcellular fractionation. Endosomes were selectively labeled with the endocytic tracer horseradish peroxidase (type II; Sigma, St. Louis, MO) (12). Briefly, Hep 2215 cells were incubated with horseradish peroxidase (10 mg/ml in serum-free minimal essential medium) for 1 hr at 37°. After removal of the horseradish peroxidase-containing medium, the cells were washed six times with ice-cold serum-free medium. After washing, all manipulations were carried out at 0–4°. The cells were disrupted by passage through a tight-fitting Dounce homogenizer in TEA-sucrose buffer (0.25 M sucrose, 3 mM $CaCl_2$, 10 mM TEA, 1 mM EDTA, 10 mM acetic acid, pH 7.4). Homogenization was monitored by microscopy and the homoge-

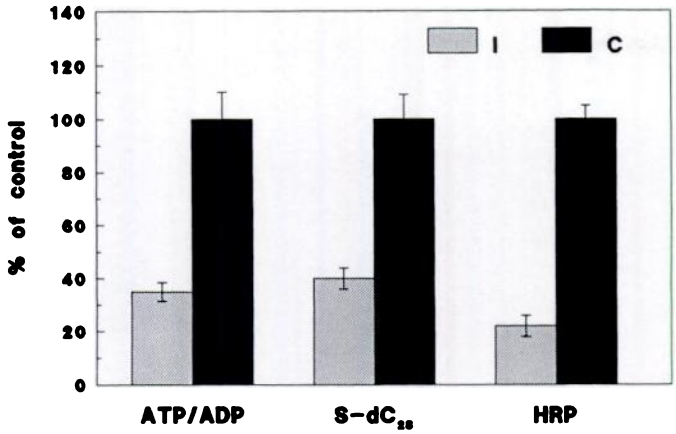


Fig. 3. Effect of energy depletion on cellular uptake of S-(dC)$_{28}$. Hep 2215 cells were preincubated with 20 mM 2-deoxy-D-glucose and 10 mM NaN_3 at 37° for 1 hr. Cellular uptake of S-(dC)$_{28}$ and horseradish peroxidase (*HRP*) was measured after a 15-min incubation in the presence of the aforementioned inhibitors. The ATP/ADP ratio was examined by HPLC. *I*, In the presence of deoxyglucose and NaN_3; *C*, in the absence of deoxyglucose and NaN_3.

nates were centrifuged at 700 × *g* for 15 min. The pellet was resuspended in a small volume of Nonidet P-40 solution (0.5% Nonidet P-40, 0.25 M sucrose, 1.5% citric acid) and was incubated at 4° for 10 min, followed by centrifugation at the same speed to yield a nuclear pellet and a nuclei-wash fraction. The TEA supernatants were centrifuged at 1300 × *g* for 10 min to obtain an intermediate pellet. The supernatants were centrifuged at 3200 × *g* for 10 min to obtain a mitochondrial pellet. The postmitochondrial supernatants were centrifuged at 200,000 × *g* (Beckman TL 100) for 30 min to obtain a microsomal pellet and a cytoplasm supernatant. For comparison, the cells were incubated with 0.5 μM ^{35}S-(dC)$_{28}$ (50 mCi/mmol) for 1 hr at 37° before the fractionation.

Plasma membranes were isolated as described previously by Rodriguez and Edelman (13). Briefly, the postnuclear supernatants were applied to a 12-ml continuous (13–37%) sucrose gradient and centrifuged (100,000 × *g* for 6 min) in a Beckman TL 100 ultracentrifuge. The first six fractions (3.6 ml) were pooled, diluted with TEA-sucrose solution, and centrifuged at 100,000 × *g* for 1 hr. Radioactivity associated with the pellet was interpreted as S-(dC)$_{28}$ binding to the plasma membrane.

Horseradish peroxidase activity in subcellular fractions was measured with a spectrophotometer, using the pyrogallol procedure provided by Sigma. The relative enrichment of each fraction was calculated by normalizing each value to that of homogenate.

Determination of ATP and ADP. Hep 2215 cells were preincubated with 20 mM 2-deoxy-D-glucose and 10 mM NaN_3 at 37° for 1 hr, followed by a 15-min incubation in the presence or absence of S-(dC)$_{28}$. The cells were washed three times with cold phosphate-buffered saline and harvested. The cell pellets were extracted with 1.5 M perchloric acid at 0° for 30 min, and the perchloric acid-soluble fractions were neutralized with KOH and KPO_4 buffer, pH 7.4. ATP/ADP ratio was determined by HPLC analysis, using a Partisil 10-SAX column with a 0.03–0.3 M KPO_4 gradient, pH 6.6.

DNA-binding protein assay. Hep 2215 cells were fractionated by a differential centrifugation procedure as described above. Nuclear and cytoplamic fractions were sonicated and extracted in a buffer containing 0.5 M KPO_4, pH 7.5, 4 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 2 mM EDTA, and 20% glycerol, followed by separation on a 12% sodium dodecyl sulfate-polyacrylamide gel. The gels were washed in 500 ml of renaturing buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 4 M urea) for 30 min at room temperature, with one change of buffer after 30 min. The proteins were then transferred to nitrocellulose filters by Western blotting in the renaturing buffer at 4° for 36 hr. The filters were washed two times (15 min each time) with

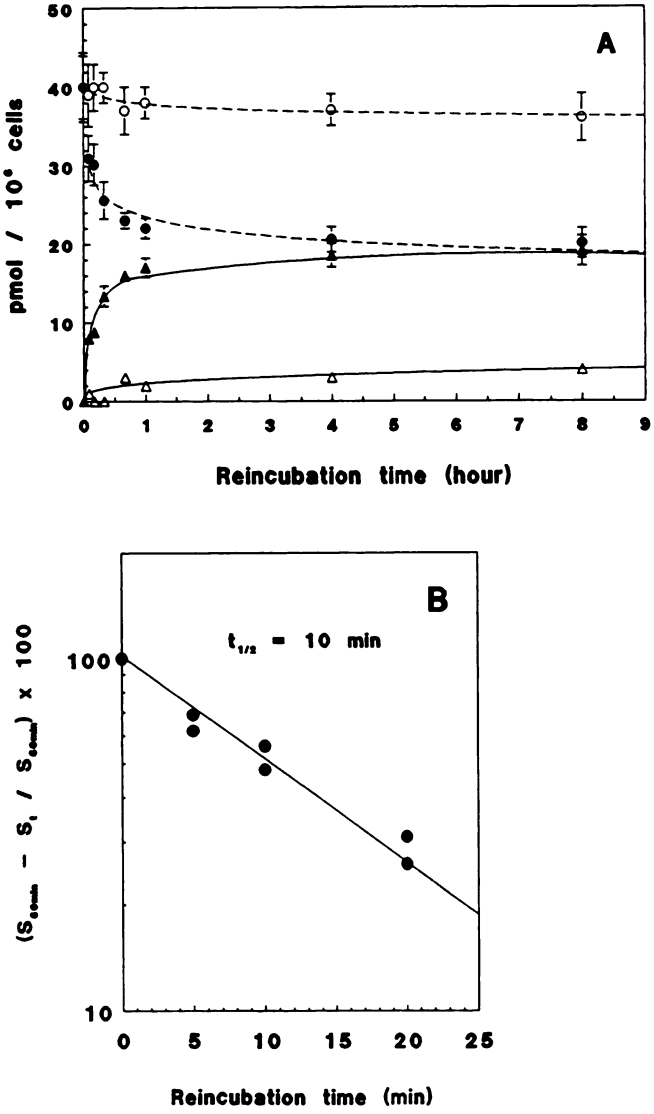
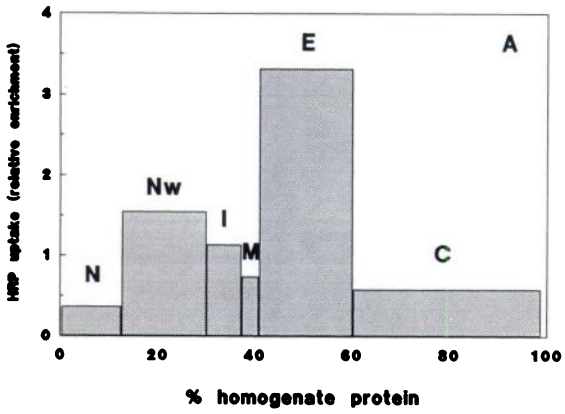


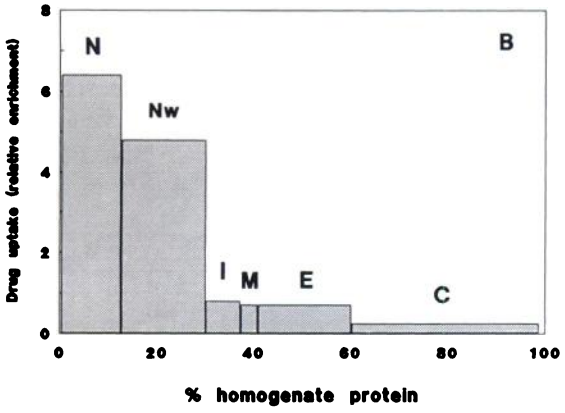
Fig. 4. Time course of exocytosis of S-(dC)$_{28}$. A, Hep 2215 cells were preloaded with 0.5 μM ^{35}S-(dC)$_{28}$ for 1 hr at 37°. Release of ^{35}S-(dC)$_{28}$ was examined at 4° and 37°. ——, Released radioactivity in the medium at 37° (▲) and 4° (△). – – –, Cell-associated radioactivity at 37° (●) and 4° (○). Data represent means ± standard deviations from two experiments, with duplicates in each experiment. B, Release of S-(dC)$_{28}$ was a function of release time. $S_{60\ min}$, amount of S-(dC)$_{28}$ released by 60 min; S_t, amount of S-(dC)$_{28}$ released by time *t*. The *r* value for the calculated line is 0.9.

binding buffer (10 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll-400), followed by quenching with 3% bovine serum albumin in binding buffer. The filters were incubated with 0.5 nM (0.3 Ci/mmol) 5′-^{32}P-end labeled oligomers in binding buffer for 1 hr and then washed with the same buffer at room temperature.

Results

Accumulation of S-(dC)$_{28}$ in human leukemia and carcinoma cells. To distinguish the internalized S-(dC)$_{28}$ from that bound to the cell surface, an acid-salt elution method was adapted from studies of receptor-mediated endocytosis (11). S-(dC)$_{28}$, ^{35}S-labeled at each internucleotide linkage, was incubated with cells for 48 hr. As seen in Table 1, the leukemia





C

Relative Ratio of 28-mer S-dC / HRP Activity

N	Nw	I	M	E	C
100	18	3	4	1	0.9

Fig. 5. Comparison of intracellular distribution of S-$(dC)_{28}$ with that of horseradish peroxidase. Hep 2215 cells were preincubated with either 10 mg/ml horseradish peroxidase (*HRP*) or 0.5 μM ^{35}S-$(dC)_{28}$ at 37° for 1 hr. Cell fractions were prepared and assayed as described in Experimental Procedures. Horseradish peroxidase activity was primarily associated with the endosome fraction (A), whereas ^{35}S-$(dC)_{28}$ was associated with the nuclei and nuclei-wash fractions (B). Relative ratio of ^{35}S-$(dC)_{28}$ versus horseradish peroxidase is with respect to the endosome value of 1.0 (C). *N*, Nuclei; *Nw*, nuclei-wash; *I*, intermediate; *M*, mitochondria; *E*, endosome; *C*, cytoplasm. Relative enrichment is with respect to the homogenate value of 1.0. Data represent the means from three experiments.

cells (grown in suspension culture) had a low capacity for accumulation, whereas the carcinoma cells (grown in monolayers), especially Hep 2215 cells, had a high capacity. The Hep 2215 cells were slightly larger than leukemia cells, as examined by Coulter Channelyzer (Coulter Corp., Hialeah, FL) (data not shown); however, the capacity for accumulation of S-$(dC)_{28}$ in Hep 2215 cells was 4–8-fold higher than that in leukemia cells (Table 1). The apparent intracellular concentration of S-$(dC)_{28}$ was 30 μM (approximately 1.2 μl/1 × 10^6 Hep 2215 cells), which was 60-fold higher than the extracellular concentration (Table 1). Membrane binding in these studies was found to be <5% of the internalized ligand (data not shown). After removal of the plasma membrane binding, nuclei were isolated from cytoplasm by the citric acid-Nonidet P-40 procedure. The majority of intracellular S-dC_{28} in the carcinoma cells was found to associate with the cytoplasm, whereas in the leukemia cells it associated with the nucleus. The L5 (subclone) of P3HR-1 cells was found to have the highest ratio of nucleus/cytoplasm distribution of all the cell lines examined (Table 1). The reason for the leukemia cells having a higher ratio of nuclear distribution is unclear; one explanation could be that the relative volume of nucleus versus cytoplasm in leukemia cells is higher. These results were confirmed by separation of the organelles using differential centrifugation in TEA-sucrose buffer. The integrity of internalized ^{35}S-$(dC)_{28}$ was examined on 15% polyacrylamide-urea gels. More than 90% of the compound in the cells and in the culture medium was found to be intact after a 72-hr incubation at 37° (Fig. 1). The cell viability was examined using the method of trypan blue exclusion and was routinely 90–99% through the experimental time period (data not shown).

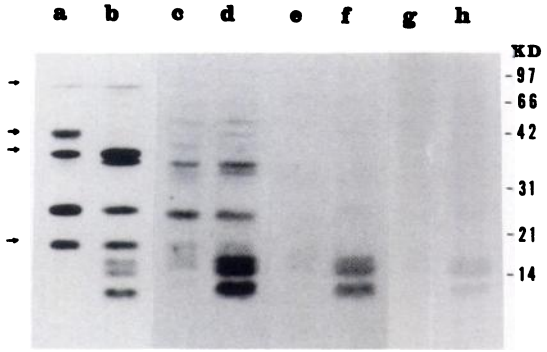


Fig. 6. Detection of S-$(dC)_{28}$ protein binding by Western blot. Hep 2215 cells were fractionated by differential centrifugation. Cellular proteins were extracted from cytoplasmic (*lanes a, c, e,* and *g*) and nuclear (*lanes b, d, f,* and *h*) fractions and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel. The proteins were then renatured and transfered to nitrocellulose filters, which were incubated with 5′-^{32}P-labeled S-$(dC)_{28}$ or $(dC)_{28}$. The profile of $(dC)_{28}$ protein binding (*lanes a* and *b*) and the profile of S-$(dC)_{28}$ protein binding (*lanes c* and *d*) are shown. In the competition studies, $(dC)_{28}$ at 1000-fold higher concentration did not inhibit S-$(dC)_{28}$ binding to small nuclear proteins (*lanes e* and *f*). The cellular proteins were stained with amido black (*lanes g* and *h*). Arrows indicate different binding proteins of $(dC)_{28}$ compared with those of S-$(dC)_{28}$.

Partial dependence of the cellular uptake of S-$(dC)_{28}$ on temperature and energy. Effects of temperature on the uptake of S-$(dC)_{28}$ were studied using hepatoma cells. To equilibrate the temperatures, the cells were preincubated at 4° or 37° for 1 hr, followed by incubation with 0.5 μM S-$(dC)_{28}$ for various time periods. As shown in Fig. 2A, the rate of cellular uptake was temperature dependent, with higher rates at 37°. The low temperature (4°), however, did not completely inhibit cellular uptake and the temperature-insensitive accumulation after 1 hr at 4° was 40% of that at 37° (Fig. 2A).

The internalization of S-$(dC)_{28}$ was found to be nonlinear with incubation time at both 4° and 37°. A rapid initial phase continued for approximately 1 hr, followed by a slower second phase. In the initial phase, internalization of S-$(dC)_{28}$ was a function of extracellular concentrations at 37° (Fig. 2B). In the slow phase, the rate of the uptake decreased with time at 37°, whereas little change in the uptake was found at 4° (Fig. 2A).

Effects of energy depletion on the uptake of S-$(dC)_{28}$ were studied further. Hep 2215 cells were preincubated with both 10 mM NaN_3 (a cytochrome oxidase inhibitor) and 20 mM 2-deoxyglucose (a glycolytic inhibitor) for 1 hr, and drug uptake was then measured in the initial phase (15 min). This treatment could deplete the ATP content (ATP/ADP ratio) by 70%, as determined by HPLC. Under these conditions, S-$(dC)_{28}$ uptake was inhibited by 60%, whereas uptake of horseradish peroxidase, the endocytic marker, was inhibited by 80% (Fig. 3). Increasing the concentration of NaN_3 to 20 mM and of 2-deoxyglucose to 40 mM did not cause further inhibition of S-$(dC)_{28}$ and horseradish peroxidase uptake or further depletion of cellular ATP content (data not shown).

Multiphasic and temperature-dependent release of S-$(dC)_{28}$. The kinetics of ^{35}S-$(dC)_{28}$ efflux are shown in Fig. 4. Hep 2215 cells were incubated with ^{35}S-$(dC)_{28}$ for 1 hr (loading time) at 37° and washed with phosphate-buffered saline. The cells were then reincubated in isotope-free medium at 4° or 37°. As shown in Fig. 4A, the release of S-$(dC)_{28}$ depended on the temperature, with 95% inhibition at 4° (Fig. 4A). Efflux of S-$(dC)_{28}$ appeared to have at least two exponential phases at 37°. There was a rapid efflux in the initial hour ($t_{1/2}$ = 10 min) (Fig. 4B), followed by a much slower efflux. Fifty percent of the intracellular ^{35}S-$(dC)_{28}$ remained trapped in the cells after 8 hr of incubation (Fig. 4A). Increasing the loading time (up to 8 hr) could increase the amount of ^{35}S-$(dC)_{28}$ exocytosed; however, it did not change the multiphasic pattern (data not shown).

Intracellular distribution and binding. Studies were performed to examine the intracellular distribution of S-$(dC)_{28}$ and to compare it with that of horseradish peroxidase. As shown in Fig. 5, the horseradish peroxidase activity was primarily associated with the endosomal fraction, whereas ^{35}S-$(dC)_{28}$ was distributed in both the nuclear and nuclei-wash fractions. The nuclei-wash fraction could represent the late endosomes and lysosomes in perinuclear cytoplasm. The relative ratio of ^{35}S-$(dC)_{28}$ to peroxidase activity in the nuclear fraction was 100-fold higher than that in the endosome (Fig. 5C). These results indicate that the radioactivity in the nuclear fraction did not come from endosomal contamination and that the nucleus could, therefore, be one of the major intracellular compartments for S-$(dC)_{28}$, although the localization of S-$(dC)_{28}$ in nucleus is not clear. The redistribution of S-$(dC)_{28}$ during the nuclei isolation procedure was <10% (data not shown). This value was estimated by mixing non-drug-exposed nuclei with cytoplasm or homogenization buffer containing ^{35}S-dC_{28} at 4°, and vice versa, followed by isolation of nuclei from cytoplasm using the same procedure and examination of radioactivity redistribution between nuclei and cytoplasm. Binding of S-$(dC)_{28}$ to the inner plasma membrane was also examined. Hep 2215 cells were incubated with ^{35}S-$(dC)_{28}$ at 37° and the cell surface binding was removed. The plasma membranes were isolated through 13–37% sucrose gradients at 4° (13). The radioactivity associated with the isolated plasma membrane was approximately 5% of the total internalized compound (data not shown); however, we do not rule out the possibility of redistribution during the isolation procedure.

Because thioate modification changes several biological properties of oligos, it is of interest to know whether this modification changes the properties of binding to cellular proteins. This question was addressed by renaturing cellular proteins after electrophoresis and examining their interactions with oligos. As shown in Fig. 6, there were distinct DNA-binding proteins in the cytoplasm (Fig. 6, *lane a*) and in the nucleus (Fig. 6, *lane b*), which could bind to $(dC)_{28}$. The binding pattern of $(dC)_{28}$ was apparently different from that of S-$(dC)_{28}$ (Fig. 6, *lanes c* and *d*). They differed not only in the numbers of binding proteins (as indicated by the *arrows* in Fig. 6) but also in the binding affinities (as indicated by the density of the bands). In addition, the densities of the bands for $(dC)_{28}$ and S-$(dC)_{28}$ were, respectively, distinct from and similar to those for cellular proteins (Fig. 6, *lanes g* and *h*). Of all the proteins, only small nuclear proteins (10–20 kDa) exhibited an extremely high affinity for S-$(dC)_{28}$. A 1000-fold higher concentration of $(dC)_{28}$ did not inhibit S-$(dC)_{28}$ binding to these proteins (Fig. 6, *lane f*) but did inhibit binding to the cytoplasmic proteins (Fig. 6, *lane e*).

Discussion

This study demonstrated that the uptake of S-$(dC)_{28}$ by hepatoma cells partially depends on temperature and energy and that the intracellular S-$(dC)_{28}$ concentrations are significantly higher than those in the medium (Table 1). The cellular uptake of antisense S-oligos, therefore, involves a mechanism of active transportation, such as adsorptive endocytosis (6); however, the adsorptive endocytosis is not likely to be the sole mechanism of S-$(dC)_{28}$ uptake and pinocytosis may also be involved. It has been demonstrated that pinocytosis is concentration dependent and relatively insensitive to energy and temperature changes (14–16). These results are consistent with our observations shown in Fig. 2B. The internalization of S-$(dC)_{28}$ by hepatoma cells depended on the extracellular concentrations, and neither energy depletion nor low temperature could completely inhibit S-$(dC)_{28}$ uptake (Figs. 2 and 3), when adsorptive endocytosis should have been totally inhibited (16, 17). Furthermore, leukemia cells were reported by others to have low levels of pinocytosis, whereas carcinoma cells (grown in monolayers) have a high capacity (15). The accumulation of S-$(dC)_{28}$ in the leukemia cells was found to be lower than that in the carcinoma cells (Table 1). Together, these results suggest that internalization of S-$(dC)_{28}$ may involve two distinct endocytotic pathways; a fluid-phase pinocytosis and an adsorptive endocytosis. In the former, S-$(dC)_{28}$ could be pinocytosed directly as part of the medium without prior adsorption to the cell surface. The molecular basis of the internalization process requires further investigation. The inhibition studies indicated that neither oligos nor 5′-phosphorylated nucleotides at 4000-fold higher concentrations inhibit the uptake of S-$(dC)_{28}$ (data not shown). These results suggest that the mechanisms of internalization of S-$(dC)_{28}$ could be different from those for $(dC)_{28}$.

Distinct multiphasic kinetics of the exocytosis of S-$(dC)_{28}$ were observed. When hepatoma cells were pulse-labeled with ^{35}S-$(dC)_{28}$, about 50% of the internalized compound disappeared within 60 min of reincubation in radioactivity-free medium, after which little decrease of cell-associated ^{35}S-$(dC)_{28}$ was seen. In addition, a rapid increase of ^{35}S-$(dC)_{28}$ in the reincubation medium occurred within 60 min, after which no further increase took place (Fig. 4). These results indicate that a large portion of internalized ^{35}S-$(dC)_{28}$ is rapidly exocytosed, whereas the rest becomes sequestered within the cells. Release of plasma membrane binding is an unlikely explanation for the

rapid efflux, because the amount of membrane-bound ^{35}S-$(dC)_{28}$ was found to be insignificant. The biphasic exocytosis supports a generalized two-compartment model for the uptake of S-$(dC)_{28}$, with one compartment turning over rapidly ($t_{1/4}$ = 10 min) and the other compartment turning over very slowly. The former could be early endosomes (pinosomes), because shortly after their formation they could fuse with the plasma membrane and release their contents to the medium (16, 18, 19), whereas the latter may involve an intracellular trapping mechanism. Our studies show that the internalized S-$(dC)_{28}$ was distributed in both nucleus and cytoplasm. The profile of S-$(dC)_{28}$ binding to proteins was different from that of $(dC)_{28}$. The former had a significantly higher level of binding to small nuclear proteins, which are abundant, than did the latter. The oxygen congener, $(dC)_{28}$, at 100-fold higher concentrations did not efficiently compete with S-$(dC)_{28}$ (Fig. 6, *lane f*), whereas S-$(dC)_{28}$ did efficiently compete with $(dC)_{28}$ (data not shown). The differential behavior of S-$(dC)_{28}$ and $(dC)_{28}$ binding to proteins could be due to differences in the amount of binding proteins or in the binding affinity of those proteins. Nevertheless, these results indicated that intracellular S-$(dC)_{28}$ could bind extensively to the proteins, which may be responsible for the long retention time and the lack of biological activity of this type of compound. It should be noted that there could be additional proteins that interact with S-$(dC)_{28}$. These proteins might not be detected due to low protein concentration or a lack of the ability to renature after denaturation. Because interaction of S-oligo with cellular components involves the phosphorothioate backbone and the base moiety, the mode of action of hetero-S-oligo may not be the same as that of homo-S-oligo. The biological significance of this difference remains unclear. Based on our previous studies of the structural activity of S-oligo *in vitro* and in cell cultures, we believe that the principles described in this study are applicable to oligomers containing phosphorothioate linkages but heterosequences.

In conclusion, cellular uptake of S-$(dC)_{28}$ partially depends on temperature and energy and may involve mechanisms of pinocytosis as well as of adsorptive endocytosis. Once S-$(dC)_{28}$ enters cells, it can be transported into the nuclei, as well as interacting with cellular proteins present in both nuclei and cytoplasm. This interaction with cellular proteins could prevent S-oligos from reaching their targets and could be responsible for poor biological activity and cytotoxicity of antisense S-oligos in certain types of cells. It is conceivable that shorter phosphorothioate hetero-oligomers may have less interaction with cellular proteins and may serve better as antisense molecules (20, 21); however, these interactions require further investigation.

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