Intracellular Plutonium: Removal by Liposome-Encapsulated Chelating Agent

Abstract. *Chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA) were successfully encapsulated within lipid spherules (that is, liposomes). Encapsulated [^{14}C]EDTA, given intravenously to mice, was retained longer in tissues than nonencapsulated [^{14}C]EDTA. Encapsulated DTPA, given to mice 3 days after plutonium injection, removed an additional fraction of plutonium in the liver, presumably intracellular, not available to nonencapsulated DTPA. It also further increased urinary excretion of plutonium. Introduction of chelating agents into cells by liposomal encapsulation is a promising new approach to the treatment of metal poisoning.*

The chelating agent, ethylenediaminetetraacetic acid (EDTA) has proved effective in removal of metals such as ^{90}Y, ^{144}Ce, ^{239}Pu, and Pb from animal tissues (*1*). Later a related compound, diethylenetriaminepentaacetic acid (DTPA) was shown to have greater effectiveness and a wider spectrum of action (*2; 3*, p. 139).

In the last 20 years, the use of polyaminopolycarboxylic acid chelating agents such as EDTA and DTPA in therapy of poisoning by various toxic metals has become widespread. The effectiveness of these chelating agents is based on their ability to form soluble, stable, and readily excretable complexes with metal molecules in situ. However, a serious limitation of this therapy is that these chelating agents are essentially unable to penetrate cellular membranes (*3*, p. 59; *4*).

Removal of intracellular deposits of toxic metals is of more than academic interest. The liver is recognized as one of the critical organs in the long-term radiation toxicity of radionuclides, including plutonium (Pu) (*5*). In dogs, Pu deposited in the liver, presumably within the cells, remains for most of the lifetime of the animal (*6*) and, under certain conditions, causes pathological changes and occasional tumors (*7*). In man, accidental exposure to Pu has also resulted in long-term retention of the radionuclide in the liver (*8*). Use of esterified polyaminopolycarboxylic acid chelating agents to increase penetration through cellular membranes has met with limited success (*9*, *10*), owing to insolubility (*9*) and toxicity (*10*).

We present here a preliminary report of a new approach to the removal of toxic metals by encapsulating the chelating agents within artificial lipid spherules called liposomes (*11*). Liposomes, when injected intravenously into rats, were found to be associated with lysosomes (*12*). Since colloidal Pu in the liver is also mainly, if not solely, localized in the lysosomes (*13*), we have tested the feasibility of removing intracellular Pu from mouse liver by intravenous administration of liposomes containing DTPA.

The liposomes were prepared with a mixture of 4.5 mg of egg lecithin (Schwarz/Mann, Orangeburg, N.Y.) and 1.5 mg of cholesterol (Applied Science Laboratories, Inc., State College, Pa.) dissolved in chloroform. This mixture was dried in a round bottom flask in a rotary evaporator. The flask was then placed in a 37°C water bath, and 1 ml of a 25 percent trisodium calcium DTPA solution (prepared by Geigy Chemical Company, Ardsley, N.Y.) was slowly added to the flask with immediate and constant stirring with a magnetic stirrer. The resultant suspension of liposomes containing DTPA was centrifuged in a Sorvall centrifuge (model GLC-1) at 2000 rev/min for 5 minutes. The supernatant was carefully pipetted off, and the liposome pellet was resuspended in normal saline. The same centrifugation and resuspension procedure was repeated five times to ensure the complete removal of the nonencapsulated DTPA solution. The liposomes were finally resuspended in saline for injection. The amount of DTPA encapsulated within liposomes was determined from at least three separate preparations, made with ^{45}Ca-labeled DTPA at the same time and in the same manner as the nonlabeled liposomal DTPA used for therapy.

Two experiments have been performed. In experiment 1, 25 female CF #1 (Carworth Farms) mice, 60 days of age with a mean weight of 25.7 g, were given a single intravenous injection of a midrange polymeric (*14*) plutonium preparation which was 24 percent ultrafiltrable (*15*). Each mouse received 0.4 μcurie of ^{239}Pu per kilogram of body weight. Separate groups of five

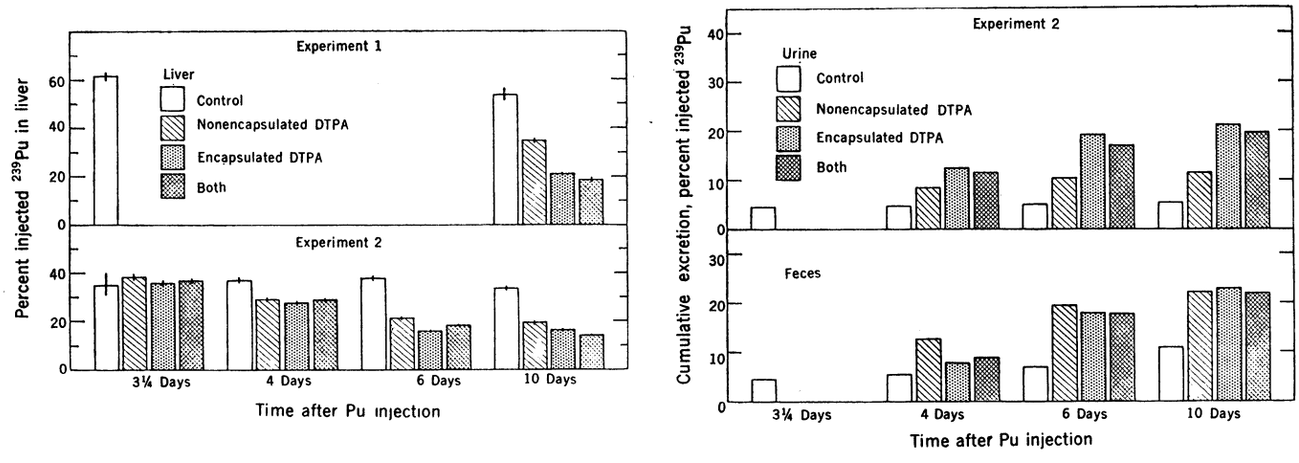

Fig. 1 (left). Plutonium in liver of mice after various treatments: (i) saline solution, (ii) nonencapsulated DTPA, (iii) liposome-encapsulated DTPA, and (iv) both encapsulated and nonencapsulated DTPA. In experiment 1, each treatment was given at 3 days and again at 6 days after injection of Pu. In experiment 2 treatment was given at 3 days only. The error bar indicates 2 standard errors of the mean. Fig. 2 (right). Cumulative excretion of Pu in the urine and feces of mice given various treatments once. 3 days after injection of Pu. Each mean value shown is derived from the two pairs of mice that were killed at the end of each collection period.

mice were treated at 3 and at 6 days after administration of Pu with one of the following: (i) saline solution, (ii) nonencapsulated DTPA, (iii) liposome-encapsulated DTPA, or (iv) both encapsulated and nonencapsulated DTPA. The mice were killed at day 10 after injection of Pu, that is, day 4 after the second therapy.

In experiment 2, 70 female $B6CF_1$ mice were used since CF # 1 mice were not available at that time. They were 82 days of age with a mean weight of 20.4 g, and were given a single intravenous injection of a midrange polymeric plutonium preparation which was 35 percent ultrafiltrable. Each mouse received 0.9 μcurie of ^{239}Pu per kilogram. Separate groups of mice were given the four different treatments used in experiment 1, but only once, at 3 days after injection of Pu. Groups of four or five mice were killed at the following intervals after treatment: 6 hours, 1 day, 3 days, and 7 days (that is, 3¼, 4, 6, and 10 days after administration of Pu). An additional group of five mice received liposomal DTPA twice, at both 3 and 6 days after Pu, and were killed at day 10.

Conventional nonencapsulated DTPA was given by intraperitoneal injection for convenience. It has been established that intraperitoneal and intravenous injections of DTPA give identical results in Pu therapy (*16*). A dose of 100 mg of DTPA per kilogram of body weight was given at each injection in both experiments 1 and 2. The liposomal DTPA was injected intravenously at a dose of 2 mg of DTPA per mouse (about 80 mg/kg in experiment 1 and 100 mg/kg in experiment 2). It was injected within 3 hours of its preparation in experiment 1 and within 1 to 2 days in experiment 2 (*17*). Tissues from individual mice and (in experiment 2 only) the separated urine and feces from pairs of mice were ashed and assayed for ^{239}Pu content (*18*).

The diameters of liposomes from separate preparations, roughly determined by dark-field photomicrography, were found to vary between 1 and 10 μm. When mixed in vitro with mouse blood, the liposomes were unchanged morphologically and did not aggregate for at least 2 hours. Injected intravenously into mice, [^{14}C]EDTA encapsulated in liposomes had a higher concentration and longer retention time in various tissues than nonencapsulated [^{14}C]EDTA. At 6 hours after a single injection of [^{14}C]EDTA liposomes, there was 41.6 percent of the injected radioactivity in the liver, 9.5 percent in the lungs, 11.8 percent in the spleen, and 0.26 percent in the brain; while after a single injection of nonencapsulated [^{14}C]EDTA, only 0.30, 0.025, 0.034 and 0.042 percent of the activity was in these respective tissues. At 24 hours after the injection of liposomal [^{14}C]-EDTA, 24.1, 7.1, 11.3, and 0.24 percent of the injected activity still remained in these tissues; but 0.30, 0.031, 0.045, and 0.033 percent of nonencapsulated [^{14}C]EDTA was found in the same tissues. All the above results are means from three mice.

The liposome-encapsulated DTPA, given alone, consistently reduced the level of Pu in the liver below that achieved by conventional nonencapsulated DTPA therapy (Fig. 1). It not only removed the essentially extracellular fraction of Pu, which can be removed by conventional DTPA, but it also removed an additional, presumably intracellular, fraction. In experiment 1, at 10 days after Pu, two injections of liposomal DTPA removed 37 percent of the "intracellular" Pu, while the combined therapy removed 45 percent. In experiment 2, the removal of intracellular Pu at 6 and 24 hours after a single injection of liposomal DTPA was not significant; but at 3 and 7 days after therapy, about 20 percent was removed. There was no additional removal of intracellular Pu in the group of five mice given two injections of liposomal DTPA. Therefore, the greater removal of intracellular Pu observed in experiment 1 was probably not due to the second injection of liposomal DTPA.

Analysis of urine and feces in experiment 2 (Fig. 2) showed that mice treated with liposomal DTPA had a significantly higher urinary excretion of Pu than mice receiving conventional DTPA therapy. The magnitude of this increase indicates that liposomal DTPA removed additional Pu from tissues other than liver. This additional urinary Pu appears to be from the skeleton. The Pu burden in the skeleton [calculated as the content of both femurs multiplied by a factor of 13 (*19*)] of mice receiving liposomal DTPA was lower by about 5 percent of the injected dose of Pu, compared to that of mice receiving conventional DTPA therapy. No unequivocal effect of the liposomal DTPA was observed in spleen or lung.

These results demonstrate that a chelating agent encapsulated within liposomes removes an additional fraction of Pu from the liver that is not removed by conventional DTPA therapy. Since Pu in the liver is found intracellularly in lysosomes (*13*) and injected liposomes are also associated with lysosomes of liver cells (*12*), we suggest that the liposomal DTPA has indeed chelated and successfully removed part of the intracellular Pu from the liver. The greater removal of Pu from the liver by liposomal DTPA in experiment 1 than in experiment 2 is probably due to a higher intracellular deposition of Pu. The mice in experiment 1 apparently received a more colloidal preparation of Pu than those in experiment 2, as judged from the greater amount in the liver. Colloidal Pu seems to be deposited intracellularly in the liver to a greater extent than noncolloidal Pu (*20*).

The encapsulation of chelating agents by liposomes and the resulting introduction of the drug into the cell should have potential use for therapy of poisoning by other radioactive and nonradioactive toxic metals. By manipulating the liposomal characteristics (such as size, surface potential, and lipid components) it should be possible to direct selected chelating agents toward specific intracellular loci to remove specific toxic metals.

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Hyaluronate Inhibition of Chondrogenesis: Antagonism of Thyroxine, Growth Hormone, and Calcitonin

Abstract. *The formation of cartilage-like aggregates in high-density stationary cultures of trypsinized chick embryo precartilage cells is blocked by low concentrations of hyaluronate. Thyroxine (and triiodothyronine), growth hormone, calcitonin, and adenosine 3',5'-monophosphate prevented this inhibition by hyaluronate, whereas other hormones tested did not.*

Hyaluronate turnover is a prominent feature of the early development of limb, vertebral skeleton (*1*), and cornea in the chick embryo (*2*) and of the regeneration blastema of the amputated newt (*3*). Hyaluronate synthesis is generally associated with the morphogenetic phase of cell migration and proliferation in these systems, and its removal by the action of a hyaluronidase accompanies subsequent differentiation, such as cartilage deposition. It was hypothesized that hyaluronate may inhibit precartilage cell aggregation consequently delaying differentiation, and thus, that its turnover may be involved in the timing of events leading to tissue formation. In a test of this idea hyaluronate, in concentrations from 1 ng/ml to 500 μg/ml, was shown to prevent cartilage nodule formation in vitro from chick embryonic precartilage cells (*4*). This phenomenon could not be mimicked by other polyanions—chondroitin sulfate, chondroitin sulfate–protein, DNA, RNA, heparin—or by the monosaccharide components of hyaluronate, namely *N*-acetylglucosamine and glucuronate. Thus the control of hyaluronate turnover and of its interaction with mesenchymal cells might be an important feature of morphogenesis in the above-mentioned developmental systems. The antagonism of some growth-promoting hormones toward the inhibition of chondrogenesis in vitro by hyaluronate is described below.

Table 1. Effect of hormone concentration on the prevention of hyaluronate (1 μg/ml) inhibition of chondrogenesis. Five to ten replicate dishes were used in each group.

Hyaluronate	Hormone	Aggregates/dish (mean ± S.D.)
None		18 ± 3
1 μg/ml		1 ± 1
	Thyroxine	
1 μg/ml	0.0001 μ*M*	5 ± 4
	0.01 μ*M*	16 ± 3
	0.1 μ*M*	16 ± 3
	1.0 μ*M*	23 ± 6
	Growth hormone	
1 μg/ml	0.001 μg/ml	8 ± 3
	0.01 μg/ml	15 ± 6
	0.1 μg/ml	14 ± 2
	1.0 μg/ml	10 ± 2
	Calcitonin	
1 μg/ml	0.001 μg/ml	0 ± 0
	0.1 μg/ml	14 ± 8
	1.0 μg/ml	13 ± 6

Stationary cultures of trypsinized 5-day (stage 26) chick embryo somite cells were used as described (*4*). Each dish (60-mm diameter) contained 2.5×10^6 to 5.0×10^6 cells, an amount which resulted in a cell density greater than a confluent monolayer and thus facilitated immediate cell-cell contact and the production of three-dimensional cartilage-like aggregates (*5*). The medium used was Ham's F12 containing fetal calf serum (10 percent), bovine serum albumin (1 percent), ascorbate (50 μg/ml), penicillin (100 units/ml) and streptomycin (100 μg/ml).

In the first series of experiments cultures were established in the presence of 0.1 μg of hyaluronate (*6*) with and without varying concentrations of hormones. Both hyaluronate and hormones were added to the medium before the trypsinized cell suspension was added. Thyroxine (and triiodothyronine), growth hormone, and calcitonin were consistently active in preventing the hyaluronate inhibition of cartilage nodule formation. Hydrocortisone was also active sporadically but only at the highest concentration tested, that is, 50 μ*M*. The following hormones were tested at two to four different concentrations in the ranges indicated in parentheses and found to be inactive: epinephrine, glucagon, and insulin (2.5 to 25 μ*M*); testosterone, progesterone, dexamethasone, and prednisolone (2.5 to 50 μ*M*); prolactin and adrenocorticotropic hormone (ACTH) (0.1 to 5 μg/ml). Thyroid stimulating hormone (0.4 unit/ml) was also inactive. Table 1 gives actual numbers of aggregates obtained in experiments with several concentrations of the active hormones. The ability of one of these hormones, thyroxine (1 μ*M*), to prevent the hyaluronate inhibition was tested at varying concentrations of polysaccharide and were effective up to 50 μg/ml under the conditions used (Table 2).

In various noncartilaginous tissues thyroxine (*7*), growth hormone (*8*), and calcitonin (*9*) have been shown to stimulate the production of cyclic AMP. If cyclic AMP mediates the action of these hormones in vivo it might be expected to duplicate their action in vitro as has been found to be the case for many other cell or tissue responses to hormones. Concentrations of cyclic AMP as low as 1 μ*M* were effective in preventing the hyaluronate inhibition. Dibutyryl cyclic AMP or theophylline

Table 2. Effect of hyaluronate concentration on chondrogenesis in the presence of thyroxine. Five replicate dishes were used in each group.

Hyaluronate (μg/ml)	Mean No. of aggregates per dish	
	1 μ*M* thyroxine	No thyroxine
0	12	20
0.001	27	1
0.1	18	3
10	25	1
50	22	2
100	5	1