This report deals with the biliary concentration of a structurally diverse group of chemical compounds and indicates that the sampling of bile may be of potential use as an aid in monitoring water quality or as a diagnostic tool in the investigation of chemically related fish kills.

Rainbow trout (usually 10 g of biomass per liter) were placed in a glass tank that contained 50 liters of dechlorinated water (*p*H 7.2) and the ^{14}C-labeled compound or compounds. The tanks were aerated and kept at 12°C and the system was allowed to remain undisturbed for 24 hours. The concentrations of the compounds in the exposures were below the level of acute toxicity for the times indicated and had no observable effects on the fish during the 24-hour exposures. The concentrations were chosen for convenience in metabolite detection rather than to simulate environmental levels. The amount of ^{14}C in the tank water was determined by counting suitable portions in 15 ml of ACS scintillation mixture (Amersham/Searle) in a model 6872 (Searle Analytic) liquid scintillation counter. After exposure, the fish were killed by cervical dislocation and the bile was collected by gallbladder puncture. Portions of crude bile were then placed in the scintillation mixture for counting, and the remainder was pooled for metabolite identification. The pooled bile was diluted with water and passed over a bed (5 by 15 cm) of XAD-2 resin in a glass column and washed with two bed-volumes of distilled water. The radioactive materials were eluted from the columns with three bed-volumes of methanol. The methanol was then concentrated to 30 ml. Thin-layer chromatography was performed on 0.25-mm silica gel plates. The plates were scanned for radioactivity by scraping segments (1 by 2 cm) of silica gel from them from the origin to the solvent front and counting the gel in ACS scintillation mixture.

The data shown in Table 1 indicate that the ratios of ^{14}C in bile to ^{14}C in water after the 24-hour exposures to the indicated compounds range from a low value of 11 for 2,5,2′,5′-tetrachlorobiphenyl (TCB) to 10,000 for 2′,5-dichloro-4′-nitrosalicylanilide (Bayer 73). In most cases the ^{14}C in bile was associated with metabolites of the parent compounds and some of these biliary metabolites have been characterized (*1*, *2*). It is evident that the lowest bile-to-water ratios were associated with compounds (DDT and TCB) that have a high lipid solubility, and this may be due to a low rate of metabolism or conjugation related to the sequestration of these compounds by tissue lipids. Of great interest are the high bile-to-water ratios of the compounds that have comparatively lower lipid solubilities, since from a monitoring point of view, more polar compounds may have a low bioaccumulation potential (*6*). Although much attention has been given in the past several years to monitoring for chemicals that tend to accumulate in the food chain, there are few innovations in the area of monitoring for potentially hazardous chemicals that have lower bioaccumulation potentials, such as phenols and certain components of petroleum products. A recent report has suggested the use of liver benzopyrene hydroxylase activity as a monitor for petroleum pollution (*7*). The data in Table 1 concerning naphthalene and methylnaphthalene appear to be relevant, since both of these compounds are constituents of crude oil (*8*), and the appearance of metabolites of these compounds in bile in high concentrations suggests the possibility of using this technique as a tool in the monitoring of petroleum pollution.

Although more work needs to be done concerning the qualitative and quantitative aspects of the biliary concentrating system in the diverse species of fish and in the development of specific identification techniques for xenobiotic compounds and their metabolites, the value of capitalizing on this process is apparent. The careful design of monitoring methods based on bile collection from either captured fish or caged fish placed at a suspect site may well serve to provide increasingly needed environmental indices (*9*).

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Asparaginase Entrapped in Red Blood Cells: Action and Survival

Abstract. *As a strategy to avoid serious allergic reactions to the antitumor agent asparaginase, this enzyme was entrapped in autologous red blood cells before intravenous injection into monkeys. Additional advantages of this approach are prolonged enzyme half-life and targeting of this agent into the reticuloendothelial system.*

Enzymes are potential therapeutic agents. After isolation and purification, enzymes can be injected to replace a missing deficient enzyme (*1*) or to digest a thrombus (*2*). An injected enzyme may have antitumor action if it destroys a tissue nutrient that is essential for tumor growth. For example, the cancer cell of childhood leukemia characteristically is unable to synthesize asparagine (*3*). Intravenous injection of the enzyme asparaginase destroys this essential tumor nutrient and causes regression of the cancer tissue. Remission of this type of leukemia in response to asparaginase therapy has been demonstrated (*4*). Unfortunately, the remission is usually short-lived or incomplete. Further therapy with this enzyme is complicated by life-threatening allergic reactions (*5*), immunosuppressive activity of the enzyme (*6*), and occasional toxicity against normal tissues, primarily liver and pancreas.

As a strategy to counteract the problems of antigenicity and host proteolytic degradation of therapeutic enzymes and, more specifically, to achieve a safer, more effective method of injecting asparaginase, we entrapped this enzyme in resealed red blood cell (RBC) ghosts and studied the fate and enzymatic function of these modified cells after injection in the monkey. The asparaginase used (MSD E.C.2 lot L594269-0-51, a gift

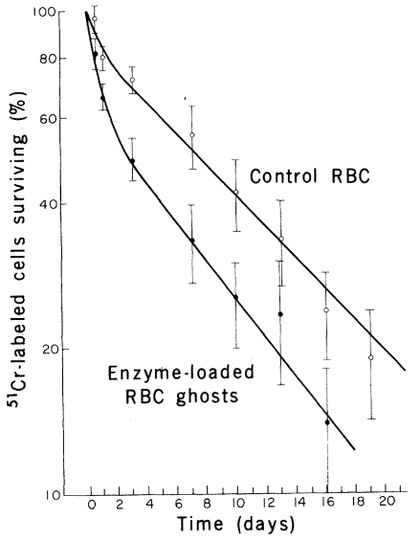

Fig. 1. Survival of enzyme-loaded and control ^{51}Cr-labeled RBC's. Open circles are data for control unlysed RBC's that were washed and incubated identically as the resealed ghost RBC's. Solid circles are data for enzyme-loaded RBC's with asparaginase concentration and activity of 3.9 mg and 780 I.U., respectively, per milliliter of RBC ghost volume. Each point represents blood samples drawn from three monkeys; brackets, 1 S.E.M.

from Merck Sharp & Dohme, Rahway, New Jersey) is one of two asparaginases produced by *Escherichia coli*.

The enzyme was entrapped in RBC's by resealing the cell ghosts by the methods of Bodemann and Passow (*7*) and Ihler *et al.* (*8*). Fresh heparin-treated whole blood (40 ml) was centrifuged at 1000*g* for 10 minutes; the plasma and buffy coat were removed, and the cells were washed three times in cold (6°C) Hanks basic salt solution (HBSS). The packed cells (hematocrit, 80 to 90 percent) were mixed rapidly in twice their volume of cold hemolysing solution consisting of distilled water containing the enzyme. After a 5-minute equlibration in the cold, sufficient concentrated cold HBSS was added to restore isotonicity. This suspension was warmed to 37°C and incubated at that temperature for 45 minutes. The resealed cells were collected by centrifugation at 1000*g* for 15 minutes and washed three times with isotonic HBSS to remove any untrapped enzyme.

The cells loaded by this method were analyzed with a type S Coulter counter (Coulter Electronics, Inc., Hileah, Florida). In comparison with normal washed RBC's, the cell volume of the loaded ghosts had decreased from 90 ± 4.0 μm^3 [mean ± standard error of mean (S.E.M.)] to 63 ± 4.0 μm^3. The corpuscular hemoglobin had reduced from 31 ± 0.5 pg per cell to 15 ± 0.5 pg per cell, and approximately 5 percent of the cells were lost during lysis, resealing, and washing.

Asparaginase was labeled with ^{125}I by modifying the procedures of Greenwood and Hunter (*9*). When the asparaginase concentration in the hemolysing media was 0.25 g/100 ml, 20 percent of the enzyme protein was entrapped in RBC's. The observed concentration of intracellular ^{125}I-labeled enzyme was 82.7 percent of the concentration expected if the enzyme had been distributed homogeneously both intracellularly and extracellularly before resealing. Enzyme activities (*10*) inside these asparaginase-loaded cells were 95 to 104 percent of those expected from ^{125}I determinations. Thus the entrapment process caused no apparent loss of enzyme activity. However, no enzyme activity was detected unless the asparaginase-loaded cells were relysed. The substrate of the entrapped enzyme, asparagine, cannot cross the cell membrane, probably because of its cationic charge.

Monkeys were injected with asparaginase (1850 I.U. per kilogram of body weight), either in free solution (three controls) or entrapped in RBC's (three experimental monkeys). The [^{125}I]asparaginase-loaded cells were labeled with ^{51}Cr (*11*) and returned by intravenous injection into each experimental monkey. Control monkeys were given injections

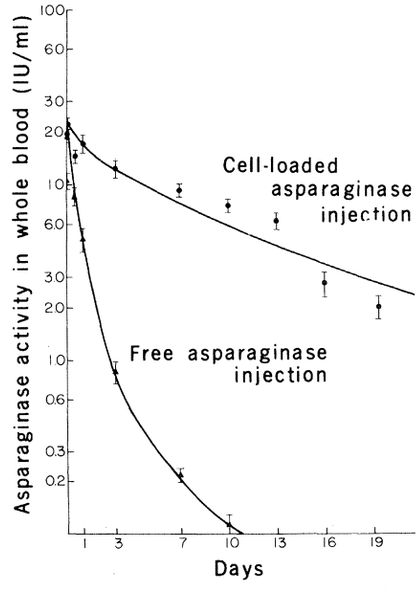

Fig. 2. Asparaginase concentration in blood. Circles are data for asparaginase injected entrapped in RBC's; triangles are data for asparaginase injected in HBSS. Each point represents the asparaginase activity in whole blood (plasma plus RBC's) drawn from three monkeys. Asparaginase activity was assayed by the method of Cooney (*12*).

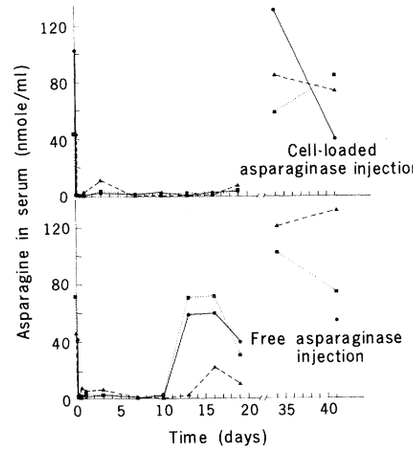

Fig. 3. Asparagine concentrations in monkey serum. Data are for each of six monkeys given a single intravenous injection of asparaginase (1850 I.U./kg), either entrapped in RBC's (top) or in free solution (bottom). Asparagine was assayed by the method of Cooney (*12*).

of free asparaginase in HBSS and ^{51}Cr-labeled control cells. The control RBC's were treated identically to the loaded cells except they were not lysed and resealed. Serial determinations of ^{125}I, ^{51}Cr, enzyme activity (*12*), and substrate concentration (*12*) in serum and whole blood were made over a 30-day period.

Survival data for ^{51}Cr-labeled resealed cells and control washed RBC's are shown in Fig. 1. A rapid loss of cells was followed by a slower, more gradual exponential loss. This gradual loss, which appears linear on the semilogarithmic graph, was used to estimate the half-life. A half-life of about 8 days was obtained for both enzyme-loaded and control RBC's. The initial more rapid loss of the resealed cells as compared to the controls is probably due to the presence of some crenated and leaky, unsealed cells. However, some initial rapid disappearance of ^{51}Cr is normal for monkeys as well as for humans (*13, 14*). Since the normal value for half-life of rhesus monkey RBC's is about 22 days (*14*), we believe that incubation and washing in our experiments significantly shortened the half-lives of both the enzyme-loaded and control RBC's.

After 10 days, the circulating asparaginase activity in monkeys injected with enzyme-loaded RBC's was two orders of magnitude higher than that in animals injected with asparaginase in solution (Fig. 2). Further, markedly depressed or undetectable levels of circulating asparagine lasted twice as long after a single injection of RBC-entrapped asparaginase than after a single injection of the free enzyme (Fig. 3). Monkeys receiving the RBC-loaded enzyme had lowered aspara-

gine concentrations for more than 19 days as compared to only 10 days for those receiving the free enzyme. We believe that in vivo lysis of the asparaginase-loaded cells slowly released the entrapped enzyme and maintained a higher enzyme concentration for the longer interval.

In summary, resealed RBC ghosts show promise as a slow-release vehicle for enzyme therapy. A single injection of asparaginase-loaded RBC's results in prolonged asparaginase activity which may produce safer and more effective therapeutic results than the conventional regimen of multiple injections of the enzyme. Substantial protection against degradation of the therapeutic enzyme by endogenous proteolytic enzyme or by antibody-complexing appears probable. For those therapeutic enzymes that should be targeted at the reticuloendothelial system, immobilization in RBC's may offer the additional advantage of release directly into this system. Partial uptake of the labeled RBC's was detected by gamma counter scans over the spleens and livers of monkeys used in these experiments.

The effect of slow release of the enzyme into the reticuloendothelial system is not yet clear. Tumor located in the reticuloendothelial system might be destroyed more effectively. Because the enzyme is released slowly at a site distant from the bronchial smooth muscle, this technique might also prevent life-threatening allergic reactions.

We conclude that entrapment of enzyme in RBC's offers a promising new strategy for enzyme therapy. This approach appears particularly advantageous for treatment of the enzyme deficiency–related storage diseases and the asparagine-dependent cancers of the reticuloendothelial system.

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Dopamine-Sensitive Adenylate Cyclase Occurs in a Region of Substantia Nigra Containing Dopaminergic Dendrites

Abstract. *The zona reticulata, the subdivision of the substantia nigra containing dendrites of the dopaminergic nigro-neostriatal neurons, contains dopamine-sensitive adenylate cyclase activity. This nigral dopamine receptor is similar to the striatal dopamine receptor. These and previous data suggest a physiological role (or roles) for dopamine in the substantia nigra.*

Dopamine has been implicated as a neurotransmitter in the mammalian nervous system (*1*). Also, dysfunctions in dopamine metabolism in the brain may be involved in the etiology of parkinsonism (*1*) and schizophrenia (*2*). Biochemical, physiological, and pharmacological evidence suggests that a postsynaptic receptor for dopamine regulates the formation of adenosine 3′,5′-monophosphate (cyclic AMP) (*3*). Thus, a dopamine-sensitive adenylate cyclase has been demonstrated in caudate nucleus, nucleus accumbens, and olfactory tubercle, regions of the brain containing the axonal terminals of dopaminergic neurons (*4–6*). Furthermore, lesions that destroyed the dopaminergic nerve terminals within the caudate nucleus did not diminish this dopamine-sensitive enzyme activity, thereby suggesting that this enzyme is located predominantly on postsynaptic structures (*7*).

Electrophysiological evidence suggests that dopamine receptors might also occur on the dopaminergic neurons located within the substantia nigra (*8*). Because of the possibility that a presynaptic dopamine receptor might exist in this region, we initiated a search for the biochemical model of this receptor. We now report a dopamine-sensitive adenylate cyclase activity in homogenates of the zona reticulata of the substantia nigra of the rat brain.

The substantia nigra is divided into three parts: the zona compacta, which contains the cell bodies of the nigro-neostriatal dopaminergic neurons; the zona reticulata, which contains the dendrites of these dopaminergic neurons; and the pars lateralis (*9*). These three areas were separated by a microdissection technique and the adenylate cyclase activity was determined (*10*). A dopamine-sensitive adenylate cyclase activity occurred in homogenates of the zona reticulata (Table 1); in this region, dopamine caused a 74 percent increase in enzyme activity. In homogenates of either the zona compacta or the pars lateralis the effect of dopamine was less pronounced

Table 1. Regional distribution of dopamine-sensitive adenylate cyclase in rat substantia nigra. Data represent mean ± S.E.M. of adenylate cyclase activity, which was determined in homogenates of the three subdivisions of the substantia nigra as described (*10*). *N* represents the number of individual tissue homogenates in which the effect of dopamine was determined; for each separate tissue homogenate, enzyme activity was measured in three to six replicate samples of the homogenate, both in the absence and in the presence of dopamine. The statistical significance of the data was determined with a two-tailed *t*-test.

Region	*N*	Adenylate cyclase activity (cyclic AMP per mg protein per minute)		
		No addition	Dopamine (100 μM)	Percent stimulation by dopamine
Zona reticulata	16	45.7 ± 2.5	79.9 ± 3.7*	74
Zona compacta	10	52.8 ± 5.6	72.1 ± 8.7†	36
Pars lateralis	9	44.5 ± 3.5	51.0 ± 3.6†	15

*P = .001 versus no addition. †Not significantly different from no addition.