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Encapsulation of Phosphotriesterase within Murine Erythrocytes

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A new conceptual approach was employed to antagonize organophosphorus intoxication by using resealed carrier erythrocytes containing a recombinant phosphotriesterase. This enzyme has been reported to hydrolyze many organophosphorus compounds, including paraoxon, a potent cholinesterase inhibitor. Paraoxon is rapidly hydrolyzed by this enzyme to *p*-nitrophenol and diethylphosphate. Incorporation of phosphotriesterase within resealed murine erythrocytes was accomplished by hypotonic dialysis. The properties of this enzyme within these resealed erythrocytes were investigated. Addition of paraoxon to reaction mixtures containing these resealed erythrocytes loaded with phosphotriesterase resulted in the rapid hydrolysis of paraoxon. Hydrolysis of paraoxon did not occur when these carrier erythrocytes contained no phosphotriesterase. These *in vitro* studies suggest that carrier erythrocytes may be developed as an approach for the prophylactic and therapeutic antagonism of organophosphorus intoxication.

Organophosphorus intoxication has occurred from occupational exposures of insecticides (Hayes and Vaugh, 1977; Namba *et al.*, 1971). The availability of a prophylactic and/or therapeutic agent against organophosphate poisoning would be desirable. Although the use of atropine and pralidoxime (2-PAM) is an effective antidotal combination under clinical situations, this antidotal combination is not always successful because oximes, in certain circumstances, are not efficient reactivators and do not reverse the toxicologic effect (Willems *et al.*, 1992). This may be partly attributed to the fact that the mechanisms of action of these antagonists, e.g., atropine (O'Brien, 1960), 2-PAM (Wilson and Ginburg, 1955), toxigonin (Heilbronn and Tolagen, 1965), TMB-4 (Karlog, 1967), and HI-6 (Clement and Lockwood, 1982), do not actually involve destroying the toxicant. An anticholinesterase organophosphorus antagonist which exerts its effect by destroying the toxicant should be developed. Enzymes may be useful in such an approach, particularly if they are placed in a protected, stable environment. Recently, phosphotriesterase[1] has been cloned (McDaniel *et al.*, 1988) and purified from *Pseudomonas diminuta* (Dumas *et al.*, 1990) and *Flavobacterium* (Mulbry and Karns, 1989). This enzyme has been reported to hydrolyze paraoxon to the less toxic 4-nitrophenol and diethylphosphate (Dumas *et al.*, 1989, 1990); moreover, it has broad substrate specificity, as it can hydrolyze parathion, DFP, sarin, soman, diazinon, and many other organophosphorus compounds. The broad substrate specificity of this enzyme would be invaluable in developing an antidote to antagonize organophosphorus intoxication.

The use of exogenous free enzymes as an organophosphorus antidote to reverse organophosphorus intoxication was first reported by Cohen and Warringa (1957) and subsequently by Ashani *et al.* (1991) and Broomfield (1992). When these purified enzymes are administered directly into the blood, they may undergo rapid enzymatic degradation and inactivation as well as renal excretion. The potential therapeutic value of such free enzymes as antidotes was quite limited until Ihler *et al.* (1973) first reported that enzymes and drugs can be successfully entrapped within resealed erythrocytes by hypotonic dialysis. In the past two decades resealed erythrocytes have been used successfully in clinical therapy (DeLoach, 1983), e.g., iron chelation during iron overload (Green *et al.*, 1980), trapping enzymes for prolonged antitumor therapy (Updike and Wakamiya, 1983), and enzyme (glucocerebrosidase) replacement therapy for inborn errors of metabolism in Gaucher's disease (Beutler *et al.*, 1977). Recent reports by Leung *et al.* (1986, 1991) using thiosulfate:cyanide sulfurtransferase trapped within erythrocytes in cyanide antagonism indicate that erythrocytes loaded with enzymes can be successfully used as a carrier system to detoxify compounds. These studies

[1] Synonyms: organophosphorus acid anhydrase, paraoxonase, parathion hydrolase, parathion aryl esterase.

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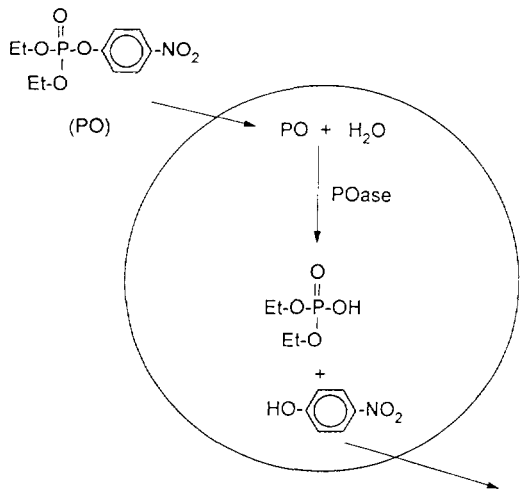

FIG. 1. Mechanism of paraoxon (PO) hydrolysis by phosphotriesterase (POase) entrapped within erythrocytes.

suggest that erythrocytes encapsulated with phosphotriesterase may be an effective carrier system in organophosphorus antagonism and warrant further investigation.

Parathion is one of the most widely used insecticides and it is metabolized to paraoxon for its predominant anticholinesterase activity. This toxic intermediate can be hydrolyzed *in vitro* by phosphotriesterase to the relatively nontoxic *p*-nitrophenol and diethylphosphate (Dumas *et al.*, 1990). However, in most animals paraoxon is not rapidly hydrolyzed. If there were a mechanism to more rapidly hydrolyze paraoxon, then parathion would be much less toxic. It is with this thought in mind that a new conceptual approach was developed in an attempt to more rapidly hydrolyze paraoxon to render parathion less toxic. This approach involves the encapsulation of highly purified recombinant enzymes into a carrier cell, in this case, erythrocytes. Since paraoxon is a hydrophobic compound that readily crosses cell membranes to get to cytosol, it can be rapidly hydrolyzed by phosphotriesterase encapsulated within the erythrocytes to its metabolites which subsequently can diffuse out of the erythrocytes (Fig. 1). This provides the basis to explore stabilizing this enzyme in a protected environment and to examine its antidotal properties as an organophosphorus antagonist. Encapsulation of a phosphotriesterase into carrier cells may represent a new conceptual approach for the prophylaxis and treatment of organophosphorus intoxication. This study describes the encapsulation of phosphotriesterase within murine resealed erythrocytes and the properties of these carrier erythrocytes to hydrolyze paraoxon.

MATERIALS AND METHODS

Animals. Swiss–Webster mice (Harlan Sprague–Dawley, Inc., Indianapolis, IN) weighing between 18 and 30 g were housed in light (12-hr intervals)-and temperature (22–24°C)-controlled rooms. Food and water were provided *ad libitum*.

Chemicals. Paraoxon and adenosine were purchased from Sigma (St. Louis, MO). Dichloromethane, sodium carbonate, sodium chloride, potassium monobasic phosphate, and potassium dibasic phosphate were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Dextrose and magnesium chloride were purchased from J. T. Baker Chemicals (Phillipsburg, NJ). All other chemicals were certified ACS grade. Commercially available paraoxon was found to be about 90% pure. Purification of paraoxon was conducted by dissolving 0.20 ml of paraoxon in 20.0 ml dichloromethane. This solution was extracted with 20-ml aliquots of 0.1 M sodium carbonate until the solution was colorless. Dichloromethane was evaporated under nitrogen and the clean paraoxon was dissolved in water to a total volume of 100.0 ml. This stock solution was stored in aliquots (5.0 ml) at −20°C and used as needed. The concentration of the paraoxon stock solution was determined by completely hydrolyzing these samples with the phosphotriesterase and measuring absorbance at 400 nm.

Purification of the phosphotriesterase. Phosphotriesterase was purified by the method of Omburo *et al.* (1992) with minor modifications. The enzyme was purified from the *Escherichia coli* expression system using plasmid pKJ33 originally obtained from Dr. Jeffrey Karns. These bacteria were grown at 30°C in a medium containing 12 g/liter Bactotryptone, 24 g/liter Bactoyeast, 80 mM K_2HPO_4, 20 mM KH_2PO_4, and 4 ml/liter glycerol. The microorganisms were incubated for 40 hr and harvested by centrifugation and the wet cell paste was stored at −80°C. All purification steps hereafter were conducted at 2–4°C in 50 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer at pH 8.5. These cells were suspended in the buffer and lysed with an ultrasonic processor. The lysed cells then were centrifuged at 13,000*g* for 15 min; the supernatant was collected. Subsequently, 2% protamine sulfate (v/v) was added to 0.4% concentration, and this suspension was centrifuged. The supernatant fluid was collected and subjected to ammonium sulfate fractionation to 45% saturation. The residue was collected by centrifugation at 13,000*g* and then dissolved in 40 ml of buffer. This fraction then was placed on an Ultrogel AcA 54 (IBF, Columbia, MD) gel filtration column (5.0 × 150 cm) and was developed at a flow rate of 1.0 ml/min. The fractions were collected, concentrated, and applied to a DEAE Sephadex A-25 anion exchange column (5 × 25 cm) and further developed. Purity of the enzyme was determined by measuring the specific activity and analyzing fractions with a sodium dodecyl sulfate–polyacrylamide gel electrophoresis employing a 12.5% denaturing gel.

Preparation of carrier erythrocytes. Carrier erythrocytes were prepared by hypotonic dialysis as reported by Leung *et al.* (1986). Erythrocytes were dialyzed against a hypotonic buffer solution (50 mOsm/kg, 1–2°C) for 30 to 40 min. Osmolality of the erythrocytes was determined periodically during dialysis. The dialysis was continued until the RBC osmolality was 130 to 150 mOsm. Hematologic parameters such as RBC counts, RBC volume, and hematocrit (HCT) were determined throughout these procedures. These studies were conducted with a Coulter Counter (Coulter Counter ZM, Coulter Electronics Inc., Hialeah, FL) in combination with a MCV/HCT/RBC computer. Size distribution analysis of normal and carrier erythrocytes was performed with a particle size analyzer. Mean corpuscular hemoglobin content (in picograms) of the cell was determined by dividing the amount of hemoglobin per unit volume of cells by the number of cells in that volume. The rate of cell recovery was calculated from actual cell counts after encapsulation divided by the actual cell counts in the initial dialysis mixture and expressed as a percentage. Efficiency of encapsulation was calculated by the amount encapsulated divided by the amount added (as a percentage).

Enzyme activity determination. Enzymatic activity was measured at 25°C by determining the increase in 4-nitrophenol concentration with time in the presence of excess paraoxon. The standard solution used to determine phosphotriesterase activity encapsulated within erythrocytes

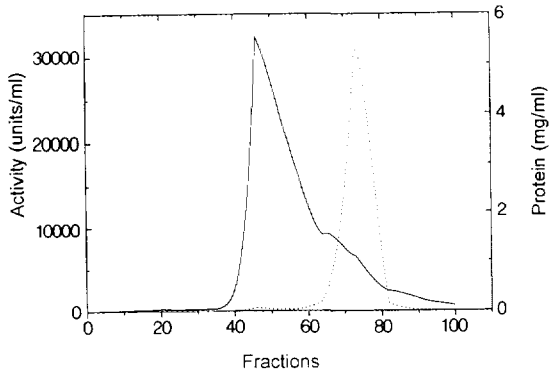

FIG. 2. Elution profile of the phosphotriesterase from an Ultrogel AcA 54 column. All the enzyme activity (---) is associated with a single peak and is separated from other major proteins (—) when eluted with Hepes buffer at pH 8.5.

(CRBCs) was isotonic (290.0 mOsm). It contained 1.0 ml of phosphate buffer (15.0 mM sodium phosphate, 216.0 mM NaCl, 3.0 mM $MgCl_2$, and 0.08 mM $ZnCl_2$, pH 7.8), 0.20 ml of paraoxon standard (6.0 mM), and varying amounts of CRBCs, depending on the particular experiment. Water was added to make the total volume 1.50 ml. Leakage of phosphotriesterase from CRBC was determined as the following: Suspension of CRBCs in isotonic phosphate buffer (no paraoxon) was incubated at 25°C. At a different time interval, fractions of the sample were taken to determine total enzymatic activity and buffer enzymatic activity, which reflects enzyme leakage. The buffer enzymatic activity was determined after CRBCs were removed by centrifugation. The reaction was started with addition of paraoxon. This enzymatic reaction was terminated by the addition of 0.04 ml of 2 N HCl. To measure 4-nitrophenol concentration, samples were extracted with dichloromethane by the following method: Dichloromethane (1.0 ml) was added to each sample, mixed, and allowed to stand for 5 min on ice. The organic layer was separated by centrifugation at 300*g* at 4°C for 10 min, and the extraction was repeated. The two organic fractions were pooled and evaporated under a stream of nitrogen, and the residues were redissolved in 1.5 ml phosphate buffer (pH 7.8). Absorbance of the solution was measured at 400 nm with a Beckman spectrophotometer (DU-8). To quantify the extraction procedure, *p*-nitrophenol standard was added to samples of washed red blood cells, extracted, and resuspended as described above. Efficiency of the extraction was determined by measuring the recovery of 4-nitrophenol from these control samples. The millimolar absorbitivity (molecular extinction coefficient) of 4-nitrophenol in phosphate buffer at pH 7.8 was determined using 4-nitrophenol standards. Statistical analysis of ANOVA was performed on Microsoft Excel 4.0 program. Two straight-line comparisons were conducted as described by Kleinbaum and Kupper (1978).

RESULTS

The phosphotriesterase from an *E. coli* expression system was purified to homogeneity in four steps. The purification procedure involved the following: protamine sulfate (to remove nucleic acids), ammonium sulfate fractionations, gel filtration (Ultrogel AcA 54), and anion exchange chromatography (DEAE-Sephadex A-25). The elution profile from the fractionation of the gel filtration at pH 8.5 is shown in Fig. 2. This column separated the enzyme from most of the other proteins and further fractionation with DEAE-Sephadex column resulted ultimately in a 1600-fold purification. After the two column chromatographic procedures, a single band of protein was observed in a sodium dodecyl sulfate gel electrophoresis and this enzyme preparation had a specific activity of 2.1 μmol/min/mg using paraoxon as the substrate.

Hydrolysis of paraoxon is linear to the amount of enzyme added to the samples as determined by *p*-nitrophenol formation. Velocity of *p*-nitrophenol formation varies as a function of paraoxon concentration in the reaction mixture. This enzyme was essentially saturated when paraoxon concentration reached 0.1 mM. A double-reciprocal plot of paraoxon hydrolysis by the phosphotriesterase is shown in Fig. 3. The V_{max} and K_m of the enzyme were determined to be 2.1 mmol/mg/min and 0.02 mM, respectively. Spectral interference of hemoglobin on the enzymatic activity determination was successfully eliminated by extracting samples with dichloromethane. Spectral data of *p*-nitrophenol, paraoxon, hemoglobin, and phosphotriesterase, either alone or in combination after extraction and resuspension in phosphate buffer, are shown in Fig. 4. After dichloromethane extraction, the absorbance of the RBC sample is essentially zero at 400 nm (Fig. 4), and no other chromophores are detectable in this region. When a known amount of *p*-nitrophenol was added to the samples, recovery of 4-nitrophenol was linear to the amount of the chemical in the sample ($r^2 = 0.98$, $p < 0.05$). The recovery rate of 4-nitrophenol was 85.0 ± 3.0%.

The effect of the encapsulation procedure itself on the cell was studied by using the hypotonic buffer solution to promote cell swelling resulting in encapsulation and by using isotonic buffer solution where no cell swelling and no encapsulation occurred. These results are summarized in Table 1, where hypotonic buffer solution (dialyzed) is compared with isotonic buffer solution (undialyzed) in the encapsulating procedure. Essentially no encapsulation occurred when isotonic buffers were employed, whereas ap-

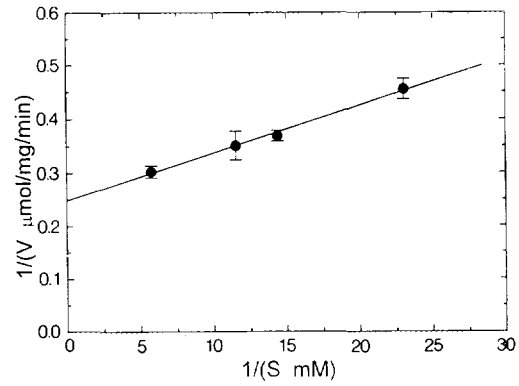

FIG. 3. A double-reciprocal plot of the velocity of paraoxon hydrolysis by phosphotriesterase as a function of paraoxon concentration.

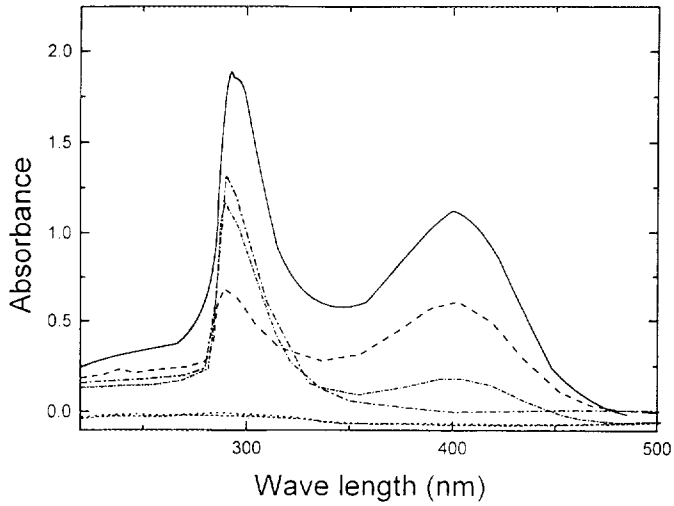

FIG. 4. Spectra of reaction mixture (—), 84 μM paraoxon (- · -), 84 μM paraoxon and phosphotriesterase (- · · ·) 100 μM 4-nitrophenol (– –), phosphotriesterase (· · ·) and RBC (---) after dichloromethane extraction.

proximately 30% encapsulation occurred when hypotonic buffer solutions were used. The cell recovery was approximately 77% when conducted by hypotonic dialysis and all the cells were recovered with the isotonic buffer. The mean cellular volume and mean cellular hemoglobin content were reduced with encapsulation. In most hypotonic dialysis procedures there is usually a decrease in cell volume, accompanied by a decrease in hemoglobin content.

Phosphotriesterase entrapped in the resealed erythrocytes was capable of rapidly hydrolyzing paraoxon (Fig. 5). Formation of *p*-nitrophenol was used as an index of paraoxon hydrolysis. The amount of *p*-nitrophenol formed was found to be linear to incubation time when the reaction was allowed to proceed with phosphotriesterase-containing RBCs (Fig. 5; $r^2 = 0.98$, $p < 0.05$). No increase in *p*-nitrophenol concentration was detected when RBCs did not contain any enzyme. When phosphotriesterase was encapsulated into the erythrocyte, 4-nitrophenol formation was found to be directly related to the amount of RBC (Fig. 6). There was a direct linear relationship between the amount of CRBC (phosphotriesterase) and the amount and rate of *p*-nitrophenol formed in the reaction mixture ($r^2 = 0.99$, p

TABLE 1
Encapsulation of Phosphotriesterase in Murine Erythrocytes

	Erythrocytes	
	Dialyzed	"Control" undialyzed
Encapsulation (%)	29.2 ± 1.23	0.78 ± 0.21
Cell recovery (%)	77.2 ± 6.96	98.2 ± 2.14
Mean cellular volume (fl)	42.93 ± 0.57	44.57 ± 1.56
Mean cellular Hb (pg)	6.53 ± 1.46	8.89 ± 0.09

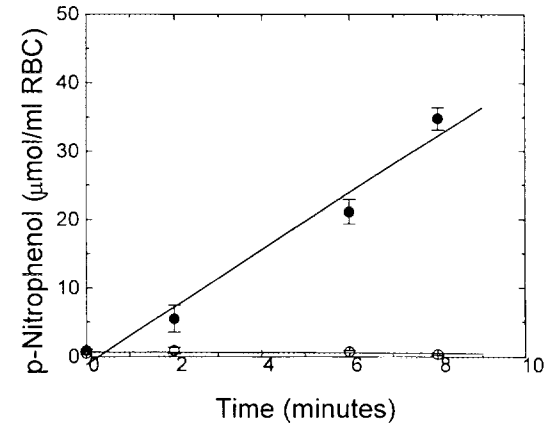

FIG. 5. Paraoxon hydrolysis by carrier erythrocytes as a function of time: with phosphotriesterase (●) and without phosphotriesterase (○).

< 0.05). The isotonically washed RBCs and those hypotonically dialyzed RBCs in the absence of enzyme showed little or no detectable enzymatic activity. Under these *in vitro* conditions, the amount of leakage of encapsulated enzyme was negligible as indicated by almost nondetectable enzyme activity in the buffer fraction (Fig. 7), especially during the first 1.5 hr of incubation. Almost all the enzyme activity existed in the cell fraction. Only a small percentage of enzyme could be detected if the incubation period was prolonged. These results indicated that the phosphotriesterase was successfully encapsulated into the carrier erythrocytes and that once encapsulated, the enzyme can also hydrolyze paraoxon rapidly. The encapsulated enzyme remained active within this protected environment at 4°C for 2 weeks with full activity.

DISCUSSION

Use of carrier cells in the antagonism of toxic compounds represents a new conceptual approach in the prophylaxis

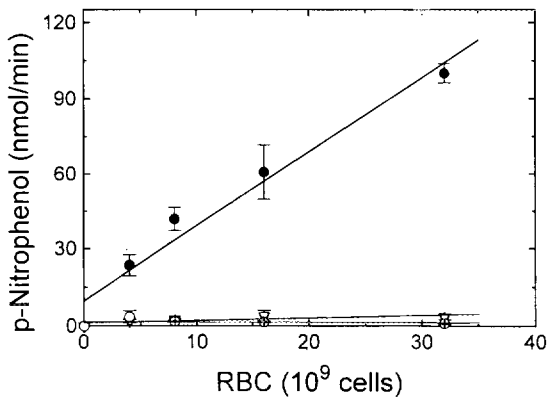

FIG. 6. Paraoxon hydrolysis by carrier erythrocytes as a function of RBC volume: carrier RBC loaded with phosphotriesterase (●), hypotonically dialyzed cells with no enzyme (○), and isotonically rather than hypotonically washed RBC (▽).

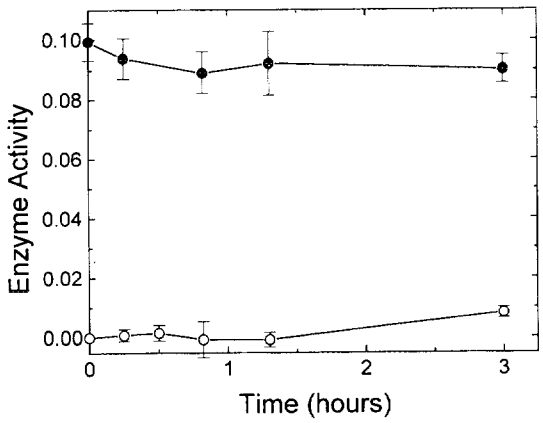

FIG. 7. Leakage of phosphotriesterase from carrier erythrocytes *in vitro*: (●) cell fraction and (○) buffer fraction.

and therapeutic antagonism of chemical intoxication (Leung *et al.*, 1986, 1991). Although the initial approach is to use erythrocytic carrier cells, ultimately other carriers such as liposomes and artificial cells will be employed. Earlier studies (Leung *et al.*, 1991) reported that the use of rhodanese in CRBCs enhanced the protection against cyanide poisoning. These studies are being expanded to the encapsulation of phosphotriesterase within resealed erythrocytes and the ability of these carrier cells to hydrolyze paraoxon *in vitro*. Hypotonic dialysis presents an osmotic shock to the RBC and creates transient pores on the cell membrane. These transient openings formed on the erythrocyte membrane during hypotonic dialysis (Seeman, 1967) provide the basis for the encapsulation of drugs and enzymes within RBCs. Enzymes and drugs are able to diffuse through these membrane pores into the cell and subsequent resealing and annealing entrap the enzyme molecules inside the cell. Zolla *et al.* (1990) found that the efficiency of protein encapsulation into human erythrocytes was size dependent. Molecules of similar sizes diffuse through the transient openings of the membrane in a similar manner. Larger molecules do not penetrate through membranes as readily. Phosphotriesterase has a molecular weight of approximately 36,000, and a 30% encapsulation efficiency of this enzyme is consistent with reports from another laboratory (DeLoach, 1983) and with other enzymes, i.e., rhodanese (Leung *et al.*, 1986, 1991).

Evidence is presented that phosphotriesterase can be successfully encapsulated into resealed murine erythrocytes by hypotonic dialysis. Once entrapped within resealed erythrocytes, these resealed cells are capable of rapidly hydrolyzing paraoxon (Figs. 4, 5, and 6). The addition of paraoxon to the reaction mixture containing erythrocytes entrapped with phosphotriesterase resulted in rapid paraoxon hydrolysis which was indicated by rapid increase in *p*-nitrophenol concentration as a function of cell volume and time. The enzyme was apparently encapsulated within the cell with minimal incorporation into the membrane. This was established by cell lysis and repeated washing of the cell membrane. No phosphotriesterase activity was observed in the membrane residue. Thus, paraoxon was readily available to the enzyme entrapped inside of the CRBC. The high turnover number of the enzyme (K_{cat} = 2100/sec) assured rapid hydrolysis of paraoxon within the cell. Although movement of paraoxon across membrane is governed by equilibrium constants, under the conditions of these experiments, this whole process denotes to almost unidirectional movement of paraoxon to inside of the cell. Conditions were now optimal for rapid hydrolysis of the organophosphorus substrate, as the high lipid solubility of paraoxon contributed to its rapid diffusion through the membrane. Because of the broad substrate spectrum, the enzyme within CRBC also may be capable of hydrolyzing many other organophosphorus compounds.

Enzymes that hydrolyze paraoxon and other organophosphorus compounds have been reported in human and other mammalian species (Zech and Zürcher, 1974), insects (Watanabe *et al.*, 1991), and bacteria (Dumas *et al.*, 1989). Free exogenous enzyme preparations have been used to antagonize various organophosphorus compound poisoning (Cohen and Warringa, 1957; Broomfield, 1992; Ashani *et al.*, 1991). However, the unfavorable physiological disposition and uptake by the reticular endothelial system greatly limits the free enzymes as potential prophylactic and therapeutic agents. Studies by Leung *et al.* (1986, 1991) using rhodanese in cyanide antagonism indicate that such unfavorable disposition factors can be overcome by the carrier erythrocyte system, as the stability and *in vivo* persistence of the enzyme were significantly prolonged. This seems also true with phosphotriesterase, as animals challenged with paraoxon appear to be protected. In very preliminary studies all animals receiving CRBC phosphotriesterase survived, whereas none of the animals receiving free enzymes survived. The persistence of the enzyme entrapped within erythrocytes can be greatly extended to the lifespan of the erythrocytes. Erythrocyte encapsulation of various proteins has been reported to be successful because the proteolytic activity within an erythrocyte is minimal (Ihler, 1983). This provides the basis for using erythrocytes as a biodegradable carrier system (Leung *et al.*, 1986).

Concerns about use of the resealed erythrocytes as carriers for enzymes often include leakage of the enzyme from the RBC, life expectancy of the RBC, and physiological and immunological reactions associated with the administration of the carrier RBC. Leakage constitutes a very small percentage of the injected dose of erythrocyte-encapsulated drug (DeLoach and Barton, 1982). Erythrocytes used as carriers are stable and remain in circulation from 10 to 36 days depending on animal species (DeLoach, 1983; DeLoach *et al.*, 1980, 1981). Life expectancy of these carrier

erythrocytes does not significantly differ from that of the normal erythrocytes. Since only a small volume of the carrier erythrocytes is administered to an animal, it does not have significant adverse effects on blood viscosity. Immunological reactions from the carrier erythrocytes are minimal since the donor and the receiver of the erythrocytes are the same species. The encapsulation of the phosphotriesterase into erythrocytes and rapid hydrolysis of paraoxon by these CRBC appear to show promise as a new approach to antagonize the toxic effect of organophosphorus compounds.

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