Spin-echo NMR Investigation of the Interaction of Phenyldichloroarsine with Glutathione in Intact Erythrocytes

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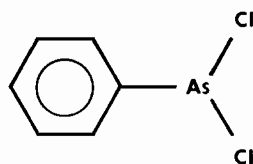
The potent cytotoxicity of trivalent organic arsenicals such as phenyldichloroarsine (PDA) (Scheme 1) is attributed to their reactions with sulfhydryl-containing molecules [1–5]. Arsenicals are transported systemically in the blood cells [6, 7].



Scheme 1.

PDA is readily absorbed by human erythrocytes and appears primarily to form a 1:2 adduct with intracellular glutathione [6, 7]. The bis glutathione phenylarsine adduct [ϕ-As(GS)$_2$] can readily be synthesized and is quite stable [6]. In the red blood cell, however, there are other sulfhydryl-containing compounds which may also react with PDA, such as hemoglobin and ergothioneine. In this study we utilized ^{1}H spin-echo NMR spectroscopic techniques to investigate the fate of PDA in the intact red blood cell and in a solution containing constituents found normally in the red blood cell. In the present study we utilized guinea pig red blood cells because they contain more glutathione (GSH) than other species [6, 7] and their hemoglobin does not appear to have any available sulfhydryl groups that seem to react with PDA [7].

Our findings show that PDA reacts with glutathione in guinea pig red blood cells to give ϕ-As(GS)$_2$ (Fig. 1), which in turn appears to be involved in a secondary association with guinea pig hemoglobin.

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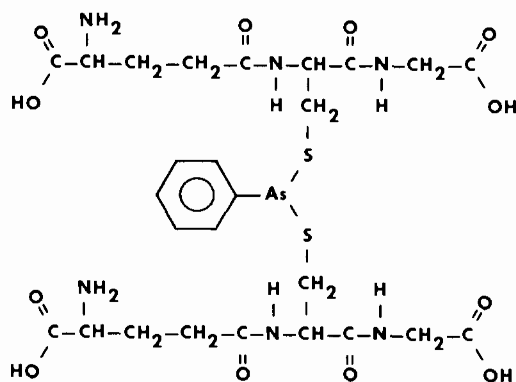

Fig. 1. Primary structure of ϕ-As(GS)$_2$.

Experimental

Materials and Methods

PDA was commercially available and purified as previously described [6]. Hartley strain guinea pigs (*Cavia porcellus*) were purchased from Simonsen Labs, Gilroy, Calif., housed individually, maintained on a 12-h light/dark cycle, and fed Purina Lab Chow *ad libitum*. They were sacrificed with carbon dioxide gas and exsanguinated by cardiac puncture. Ethylenediaminetetraacetic acid (EDTA) was used as the anticoagulant. The plasma was removed and the erythrocytes were deuterated by repeated washings in deuterated phosphate-buffered saline solution [PBS] and centrifugation. We were able to replace approx. 97% of the exchangeable hydrogens. ^{1}H NMR spectra were recorded on a Varian XL-300 spectrometer.

Results and Discussion

Figure 2 shows that when [U-^{14}C]PDA was mixed with a suspension of guinea pig red blood cells in a

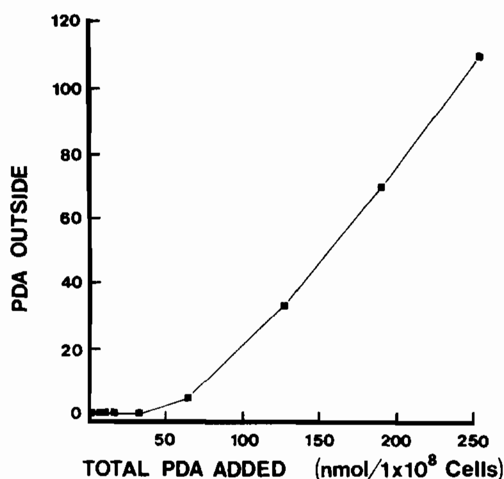

Fig. 2. Uptake of [U-^{14}C]PDA by guinea pig erythrocytes. ^{14}C counts were taken of the external solution.

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PBS, the ^{14}C label was readily taken up by the cells with a saturation point (where the line begins to curve) of approx. 25 nmol PDA/10^8 cells. Further additions of PDA resulted in increased extracellular PDA; though not all the PDA was found extra cellular and this probably resulted from weak secondary binding sites (such as on hemoglobin) within the red blood cell. The saturation point appeared to be reached when internal GSH to PDA ratio became approx. 2:1.

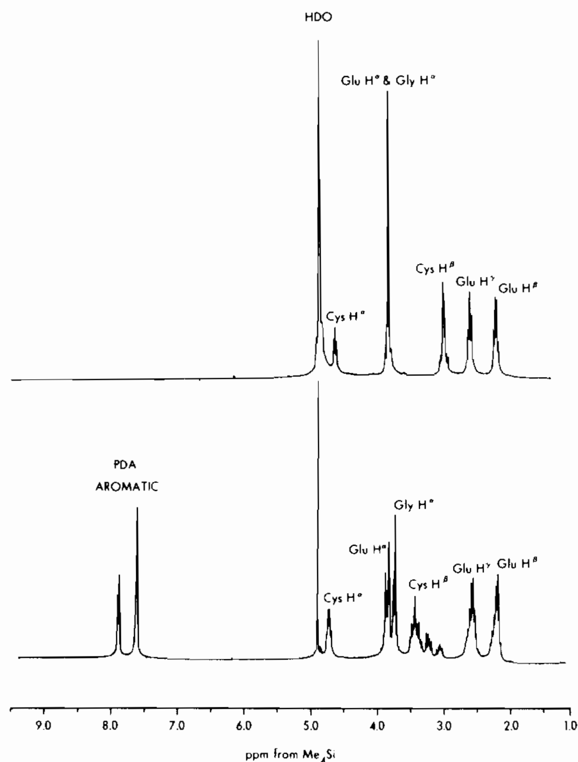

Fig. 3. ^{1}H NMR spectra of glutathione and ϕ-As(GS)$_2$. A spectral window of 4 000 Hz was used and time-domain data were collected in 16 000 addresses with a recycle time of 11 s. Upper trace is from 118 mM GSH in D_2O, pH* 6.6 after 32 accumulations. Lower trace is from 69 mM ϕ-As(GS)$_2$ in D_2O, pH* 6.8 after 80 accumulations. pH* indicates uncorrected pH meter readings.

Figure 3 shows the ^{1}H NMR spectra of glutathione and ϕ-As(GS)$_2$ in D_2O; resonance assignments were obtained from published work [6, 8, 9]. The ^{1}H NMR of deuterated erythrocytes is provided in Fig. 4. Clearly, the broad ^{1}H resonances present for the lipid and cytoplasmic large protein components, such as hemoglobin, completely obscure the minor sharp resonances that would be observed for GSH.

Using the Carr–Purcell–Meiboom–Gill spin-echo pulse technique [10], we were able to eliminate the

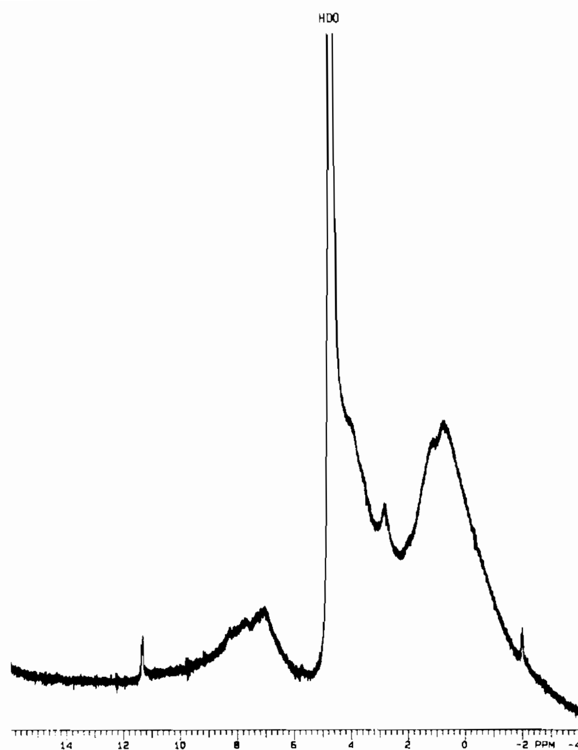

Fig. 4. ^{1}H NMR spectrum of deuterated intact guinea pig erythrocytes. Spectral conditions are identical to those given in Fig. 3. The intense resonance at approx. 4.3 ppm is the residual HDO.

resonances corresponding to the large molecules having small T_2 values and preferentially refocus the spins pertaining to small molecules such as GSH and ergothioneine that would have significantly larger T_2 values. A portion of the ^{1}H spectrum (1–4 ppm downfield from Me_4Si) of deuterated intact erythrocytes obtained with the spin-echo technique is given in Fig. 5 (bottom) in which only sharp resonances are seen. Not all of the glutathione resonances were observable; the H^{α} of Cys resides very close to the residual HDO resonance. The intense resonance at 3.5 ppm is assigned to ergothioneine [8, 9].

Using our measured value of 40 nmol GSH/10^8 guinea pig erythrocytes, we estimated the total GSH content of the guinea pig erythrocyte sample in Fig. 5 to be 12 μmol. As graded additions of PDA were made to this sample, two things were observed: (1) the resonances of GSH broadened and (2) the equivalence point was reached when the PDA to GSH ratio was approx. 1:2. Similar results were also obtained when PDA was added to lysed guinea pig erythrocytes or to solutions containing dialyzed guinea pig hemolysate to which an appropriate amount of GSH had been added.

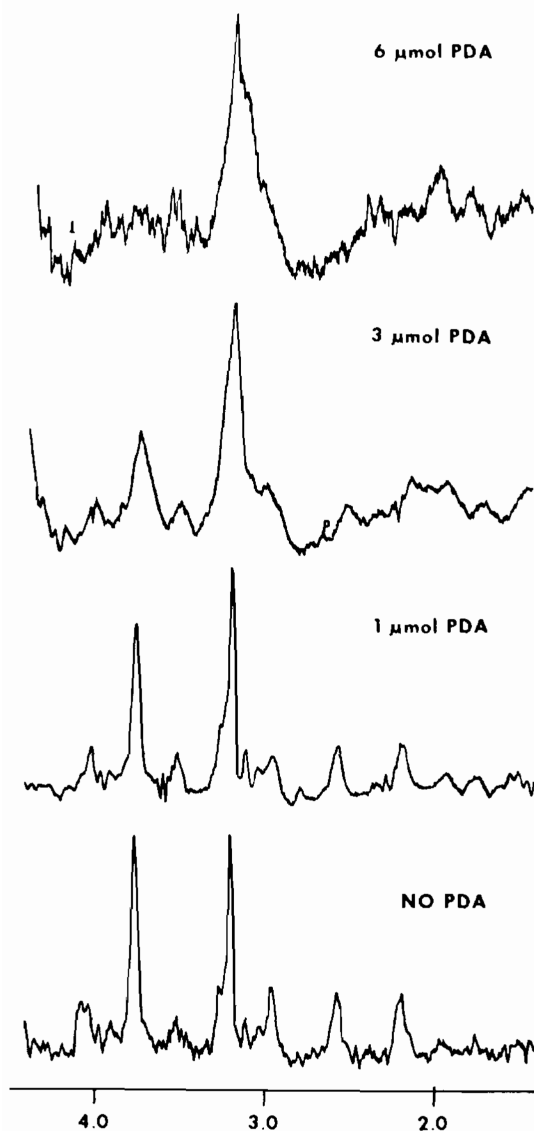

Fig. 5. ^{1}H spin-echo NMR spectra of intact guinea pig erythrocytes upon graded additions of PDA. Typically, 100–500 accumulations were required per spectrum.

The fact that ϕ-As(GS)$_2$ by itself exhibited sharp resonances indicates that a simple formation of this adduct does not drastically change the correlation time. The broadening of the resonances in the cell preparations suggests that a macromolecule such as hemoglobin or membrane surface is interacting with the adduct. PDA does not react with hemoglobin of most species except the rat whose hemoglobin has additional sulfhydryl groups. The present study with guinea pig erythrocytes indicates that PDA enters the erythrocyte and forms the ϕ-As(GS)$_2$ adduct. Thus, the loss of resonances in our current spin echo experiment upon addition of PDA must indicate that this change in correlation time may result from the interaction of ϕ-As(GS)$_2$ with some portion of the guinea pig hemoglobin.

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