INSTITUTE REPORT NO. 222

AD-A175 296

INTERACTION OF PHENYLDICHLOROARSINE WITH BIOLOGICAL MOLECULES

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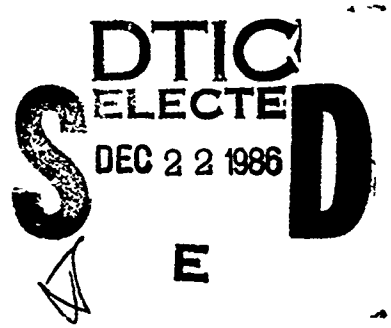

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O'Connor, McGown, and Dill

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER INSTITUTE REPORT NO. 222	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Interaction of Phenyldichloroarsine with Biological Molecules		5. TYPE OF REPORT & PERIOD COVERED Final - 1 May '85-1 May '86 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Richard J. O'Connor, MS; Evelyn L. McGown, PhD; and Kilian Dill, PhD		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Division of Comparative Medicine & Toxicology Letterman Army Institute of Research Presidio of San Francisco, CA 94129-6800		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Project #3M162734A875 Task BD Work Unit #306
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research & Development Command Fort Detrick Frederick, MD 21701-5010		12. REPORT DATE August 1986 13. NUMBER OF PAGES 19
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Lewisite analogue; arsenical; phenyldichloroarsine; British anti-Lewisite; meso-2,3-dimercapto succinic acid; glutathione; ^{13}C-nuclear magnetic resonance.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

~~^{14}C~~ Carbon 14 ... Carbon 13 ... ~~^{13}C~~ ... ~~^{13}C~~-NMR

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ABSTRACT

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PREFACE

Kilian Dill was a participant in the U.S. Army Summer Faculty Research and Engineering Program during summer of 1985. His current address is:

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ACKNOWLEDGMENTS

The authors would like to express their appreciation to CPT Mike McCreery for his help with the project.

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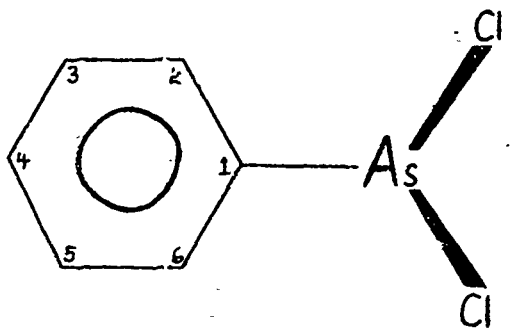
INTRODUCTION

Phenyldichloroarsine (PDA) is a vesicant arsenical whose molecular toxicology is little understood (1). It is an analog of Lewisite (transchlorovinyldichloroarsine) and has a similar vesicant potency (2,3). In order to study the efficacy of various antidotes to these compounds, it is of paramount importance to study the molecular binding, transport, and the eventual cellular damage that PDA can produce.



Mono-substituted trivalent arsenicals such as PDA react with thiol groups to produce multiple monothiol species and with vicinal dithiol compounds to produce thermodynamically stable cyclic compounds (1). Because of the specific reactivity of PDA with thiol groups, it is likely that the biological damage caused by PDA results from the reaction with thiol-containing molecules present in biological systems.

In order to gain insight into the biological actions of PDA, with the goal of finding improved arsenic antidotes, we investigated the interaction of PDA with the red blood cell and its cytoplasmic thiol (SH)-containing molecules. Within the red cell, hemoglobin contributes the bulk of exposed free

SH groups, 1.8-3.6 umol/10^9 cells, depending upon the species. The protein contains an additional 3.6-7.2 umol SH/10^9 cells which are inaccessible unless the protein is denatured. Glutathione (GSH) contributes 0.15-0.30 umol SH/10^9 cells (4). Red cell membranes and ergothioneine account for < 5% of the total free SH content (5).

In the following report we present our results with ^{14}C-PDA and mouse red blood cells and human hemoglobin. We also present nuclear magnetic resonance (NMR) spectroscopic analyses of PDA adducts with several small molecular weight SH compounds.

METHODS

PDA

Phenyldichloroarsine (PDA) was purchased from Research Organic/Inorganic Chemical Corp., Sun Valley, CA. It was purified by repeated distillation at 128°C under 15 mm Hg of nitrogen and the purity (> 99%) was verified by infrared spectrophotometry. Stock solutions of PDA were prepared at a concentration of 50 mM in anhydrous ethanol. ^{14}C-PDA (5.6 mCi/mmole) was purchased from Wizard Laboratories, Davis, CA.

The aqueous form of PDA is easily oxidized to phenylarsonic acid. Our ^{14}C-PDA preparation was synthesized by a method which utilizes the oxidation of iodide ion to iodine to reduce the arsenical to its desired oxidation state (III). If trace amounts of iodine are left in the product, either free or as an adduct with PDA, it will promote oxidation of PDA when it is exposed to water. We found this was a significant problem with our commercial ^{14}C-PDA. To minimize the problem, we first distilled the ^{14}C-PDA under high vacuum, leaving behind the phenylarsonic acid (and any other non-volatile impurities). The PDA was then stored under vacuum to deprive it of oxygen and to maintain strictly anhydrous conditions. Stored in this manner, the isotope was stable for weeks.

PDA and Intact Red Blood Cells

In order to ascertain whether the red blood cell quantitatively takes up PDA and, if so, whether PDA

binds to the membrane or some cytoplasmic component, we undertook the radio-labelling of red blood cells with ^{14}C-PDA. Whole blood was obtained from six white mice (ICR strain; approximately 30 g) via heart puncture and centrifuged at 300 x g for 30 minutes. The supernatant and buffy coat were removed, the cells resuspended and washed twice in saline. Packed cells were then resuspended with an equal volume of saline followed by the addition of the ^{14}C-PDA (approximately 0.25 umol/10^9 cells). The reaction was allowed to proceed for two hours. The cells were then spun down, the supernatant saved, and the cells were lysed with distilled water. The membranes were pelleted by centrifugation and washed several times by resuspending the membranes in water, repelleting and removing the supernatant. The pellets and washings were also saved for counting.

PDA and Human Hemoglobin

To study PDA binding to human hemoglobin (Hb), stroma-free hemoglobin was obtained from the Blood Research Division, mixed with ^{14}C-PDA in varying proportions, put into dialysis bags and dialyzed against 40 ml saline. ^{14}C was determined in both the internal contents and external fluid at timed intervals.

Preparation of PDA Adducts

PDA-glutathione was prepared by the following method: PDA was added to a beaker containing 20 ml of anhydrous ethanol. To this a twofold molar excess of reduced glutathione was added in four equal portions over a period of approximately 15 minutes. Glutathione gradually dissolved in the ethanol as it reacted with PDA. The solution was continuously stirred for another hour and then evaporated to dryness using gaseous nitrogen. All other adducts of PDA were made by simply placing the appropriate molar amounts of PDA and DMSA or BAL into the desired solvent. Adducts were formed immediately.

NMR Studies

Proton-decoupled, natural abundance ^{13}C-NMR spectra were obtained on a Varian XL-300 FT-NMR spectrometer operating at 75.429 megahertz (7.05 tesla). A

25 KHz window and an 18 usec (90°) pulse width were used throughout these studies. The spectra of the PDA-glutathione adduct were obtained in a water/ deuterated water (3:1) mixture; PDA in D-4 methanol and D-6 acetone; the PDA-DMSA adduct in D-4 methanol and D-6 acetone; the PDA-BAL adduct in D-4 methanol and D-6 acetone.

RESULTS AND DISCUSSION

PDA Interaction with Intact Red Blood Cells

When we used freshly distilled ^{14}C-PDA, the intact red cells took up approximately 98% of the ^{14}C. When older ^{14}C preparations were used, the amount that remained extracellular increased, presumably due to the breakdown product, phenylarsonic acid. The latter compound was not taken up by red blood cells (R.J. O'Connor and E.L. McGown, unpublished results). When the cells were lysed, > 90% of the ^{14}C was in the cytoplasm and < 10% was membrane-associated.

PDA and Hemoglobin

The results above indicated that PDA penetrated the red cell membrane and was bound to some component in the cell cytoplasm. To investigate the possibility that hemoglobin was the cytoplasmic component to which PDA was bound, human hemoglobin-PDA equilibrium dialysis experiments were performed. It was found that 96% of the radiolabel had dialyzed out. We attempted to determine the number of "tight" binding sites per Hb molecule by using the Scatchard plot (Figure 1). There appeared to be only 0.05 sites per Hb molecule. These results indicated that PDA interacts weakly with Hb (0.05 sites) or that it does not bind to Hb (PDA may be binding to an impurity). Overall, we concluded that when PDA enters the red blood cell it binds to some component other than Hb, possibly glutathione.

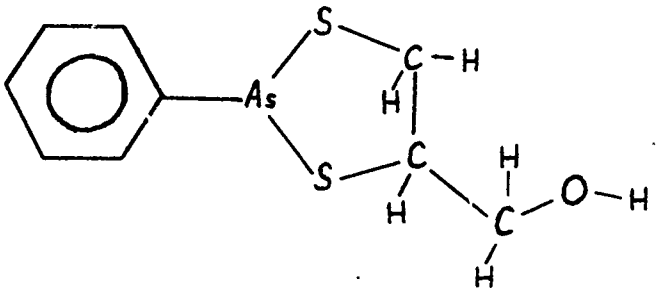
^{13}C-NMR Studies of PDA and its Adducts

In the following discussion, chemical shifts are given relative to external tetramethylsilane (TMS); the respective solvents were used as internal references.

PDA. ^{13}C spectra of PDA were recorded in both D-6 acetone and D-4 methanol. In methanol four resonances were readily discernible for PDA (Figure 2); these all occurred in the aromatic region of the spectrum. The peak at 147.87 ppm arose from the non-protonated aromatic carbon of PDA. This was readily rationalized by its line width (6) and extremely long spin-lattice relaxation time of $\geq$ 60 seconds (s) (vide infra). The other three resonances occurred at 132.35, 130.97, and 129.74 ppm. From intensity considerations and resonance patterns, we reasoned that the peak furthest downfield must be assigned to the C-4 of PDA, whereas the other two resonances must arise from the two equivalent C-2,4 and C-3,5 atoms, respectively.

In acetone the same resonances occurred at 147.18, 132.65, 131.00, and 129.78 ppm. Some of these were substantially broadened, which may result from solvent-solute interactions. Further studies are needed to delineate this.

PDA and BAL. PDA readily reacts with BAL to form a 5-membered ring adduct as shown below (1).



We attempted to undertake two studies of this complex: one study in methanol to monitor the adduct being formed and another in acetone to monitor the T1's of various carbon atoms of BAL before and after the adduct was formed.

The reaction of PDA with BAL was initially undertaken in D-4 methanol (Figure 3). The formation of the adduct had a pronounced effect on the chemical shifts of the PDA resonances. New resonances appeared at 144.4 (1), 131.64 (1), 130.06 (2), and 129.40 (2) ppm, respectively. The number in parentheses reflects the carbon count inferred from peak intensities. This

indicated that one of the carbons shifted as much as 3.4 ppm due to adduct formation. BAL in D-4 methanol exhibited three resonances at 65.77, 45.54, and 30.00 ppm. Formation of the PDA-BAL adduct had a more pronounced effect on the BAL ^{13}C-resonances than on the ^{13}C-resonances of the phenyl carbons of PDA. The new resonances appeared at 64.68, 59.26, and 43.45 ppm. Clearly, adduct formation had resulted in a shift of as much as 13.4 ppm for two of the carbon atoms.

The second study of the PDA-BAL complex was performed in D-6 acetone. The PDA resonances for the complex occurred at 147.06 (1), 132.22 (1), 130.77 (2), and 129.52 (2) ppm, respectively. It appeared that adduct formation in the D-6 acetone solvent had little effect on ^{13}C chemical shifts of PDA resonances. This is a point that should invite further investigation. BAL in D-6 acetone exhibited two observable resonances at 65.46 (triplet) and 45.56 (doublet) ppm when monitored (proton coupled). Unfortunately, the third resonance lay under one of the solvent's (D-6 acetone) resonances. When the PDA-BAL adduct was monitored (proton coupled), the BAL resonances occurred at 64.25 (triplet), 59.36 (doublet), and 40.65 (triplet) ppm. The resonance that shifted from 45.56 to 59.36 ppm was clearly the C-2 carbon of BAL, and the resonance that hardly shifted (65.46 to 64.25 ppm) was the C-1 carbon. The remaining resonance was attributed to the C-3 of BAL. This interpretation was directly supported by the data in the previous study in which D-4 methanol was the solvent.

In order to ascertain the relative mobility (via the rotational correlation time, τ_R) of certain carbon atoms of BAL in the "free" form and in the adduct, T1 values for BAL and the BAL-PDA adduct were obtained. In the free form, the T1's of C-1 and C-2 of BAL in acetone (336 mM) were 5.6s and 8.1s, respectively. The acetone resonance obscured the ^{13}C resonance of BAL C-3. In the BAL-PDA complex in D-6 acetone (336 mM), T1 values of 4.4, 8.1, and 5.0 s, respectively, were observed for BAL C-1, C-2, and C-3. Thus, in acetone, the T1's of BAL appear to be unaffected by adduct formation.

The T1's of the PDA carbon atoms of the PDA-BAL complex in D-6 acetone were 53 s (129.4 ppm) 2.7 s (132.4 ppm), 6.1 s (130.8 ppm) and 5.6 s (129.5 ppm). These were not compared with the ^{13}C-T1 values of free PDA. It is interesting to note that the T1 of C-1 PDA was approximately 1 minute.

PDA and Glutathione

Glutathione is a tripeptide (γ-L-glutatamyl-L-cysteinyl-glycine) that is found at a concentration of 2 mM in the human red blood cell. It contains a cysteine residue that has a sulfhydryl group which can readily react with PDA. Our results given below, along with the peak integration data of the various spectra (not presented here) indicate that glutathione forms a 2:1 adduct with PDA in ethanol. This result is similar to the recently published data concerning a glutathione-vanadium complex (7).

The resonances for glutathione [333 mM in a water/deuterated water (3:1) mixture] at pH 7.2 are listed in Table 1. The resonance assignments are based on literature data for glutathione (7) and related dipeptides composed of Glu (8).

Table 1. ^{13}C Chemical Shift Data for Glutathione

Shift in ppm		Assignment
177.1		
175.8	Carbonyl	
174.8	Carbon Atoms	Cys
172.5		
56.4		Cys C^{α}
54.9		Glu C^{α}
44.1	Aliphatic	Gly C^{α}
32.1	Carbon Atoms	Glu C^{γ}
26.9		Glu C^{β}
26.5		Cys C^{β}

Table 2 lists chemical shifts which were obtained after glutathione formed a 2/1 adduct with PDA (165 mM) sample, pH 7.4, in a water/deuterated water mixture. External TMS in a capillary was used as a reference. Figure 4 shows the ^{13}C-NMR spectra of glutathione and glutathione reacted with PDA.

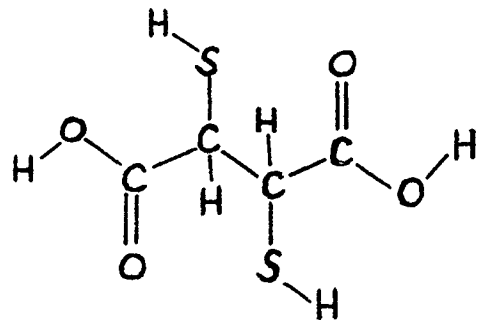
Table 2. ^{13}C Chemical Shift Data for PDA-Glutathione Adduct

Shift in ppm		Assignment
176.8 175.4 174.6 172.Ø	Carbonyl Carbon Atoms	Cys
139.4 132.4 13Ø.1	PDA Carbon Atoms	
55.5		Cys C^{α}
54.8	Aliphatic	Glu C^{α}
44.2	Carbon Atoms	Gly C^{α}
33.5		Cys C^{β}
32.2		Glu C^{γ}
26.9		Glu C^{β}

It can readily be seen that the carbon atoms whose chemical shifts were perturbed upon formation of the adduct are those of cysteine. These results indicate that the sulfhydryl groups protrude, unhindered, into the aqueous media and that the adduct formation does little to disturb the structure of the tripeptide. We also noted in our studies that when DMSA was added to the solution, the PDA-glutathione complex was readily abolished.

PDA-DMSA

DMSA is a compound that contains vicinal sulfhydryl groups.



These groups readily react with PDA to form a thermodynamically stable 5-membered ring. DMSA is only sparingly soluble in D-6 acetone. However, it reacted with PDA in acetone to from the 1:1 DMSA-PDA adduct. A signal for the methine carbons of DMSA in the complex was observed at 59.3 ppm. Another signal also appeared at 57.5 ppm, and it appeared to increase substantially with time. It probably was an oxidation product of DMSA.

CONCLUSIONS

We have shown that when PDA is added to red blood cells, it will penetrate the RBC and bind (react) with some component in the cell interior. Our NMR results indicate that the intracellular component is probably glutathione.

RECOMMENDATIONS

A number of NMR experiments need to be performed to characterize further the PDA-adducts (BAL, DMSA). Examples are:

(1) Effect of solvent on the chemical shifts of these complexes. How important is a protic or aprotic solvent?

(2) T1's should be determined for the PDA-BAL complex in solvents other than acetone. Again, a protic solvent may have a different effect.

(3) ^{75}As-NMR of these species.

(4) Undertake 2D-NMR studies of these various complexes.

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APPENDIX

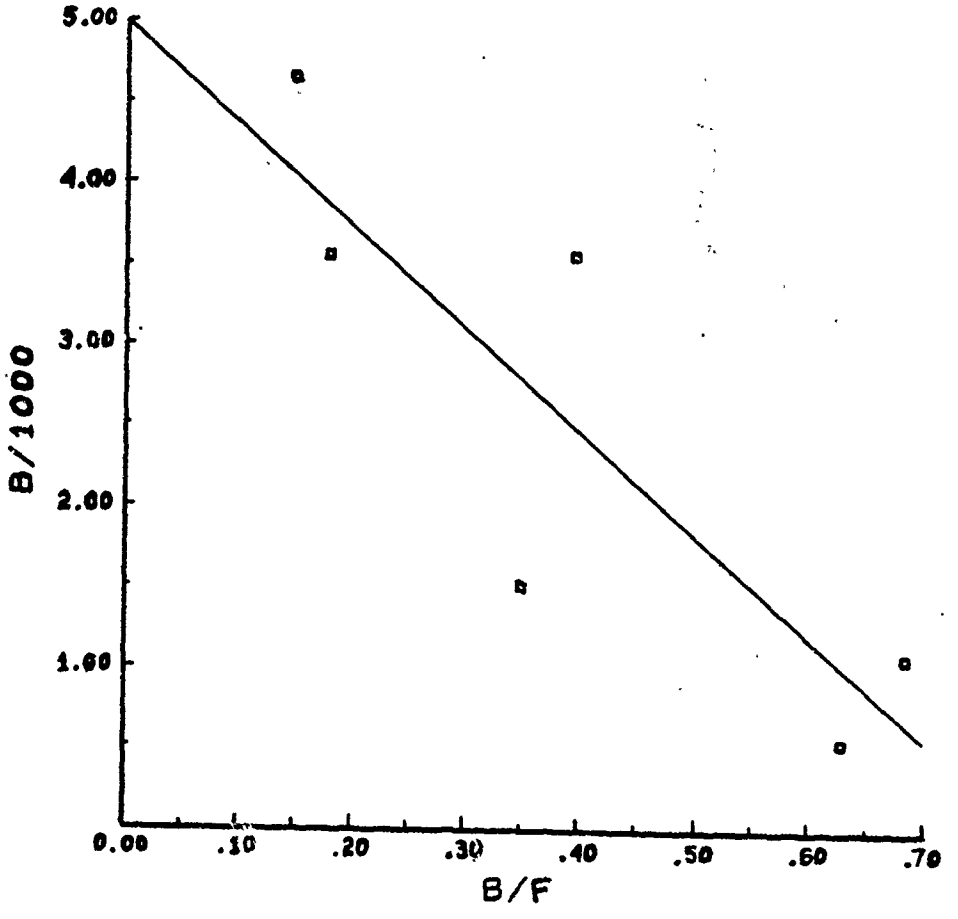

Figure 1. Scatchard Plot of PDA Binding to Hemoglobin

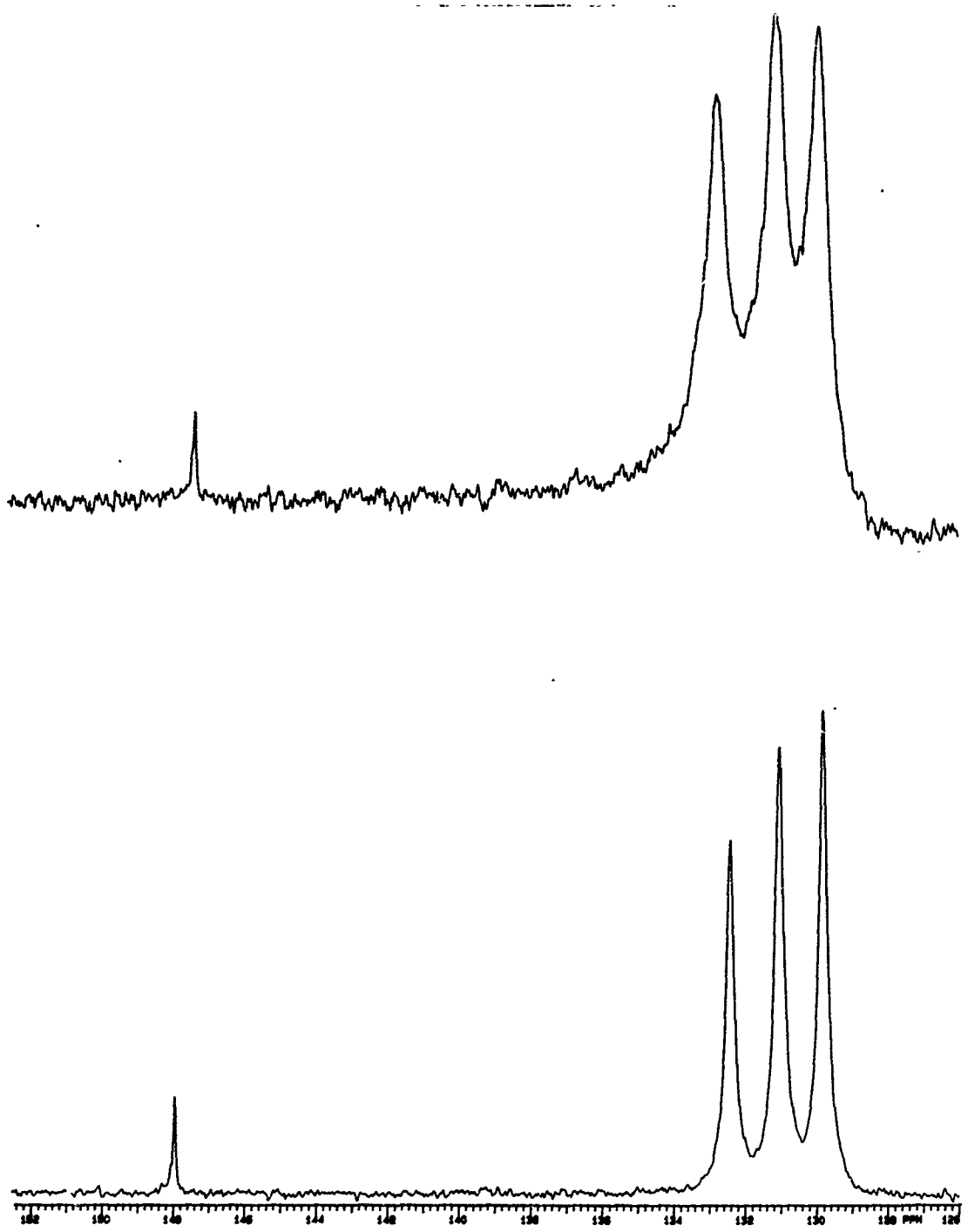

Figure 2. Proton-Decoupled ^{13}C-NMR Spectrum of the Aromatic Region of PDA in D-6 Acetone (upper spectrum) and in D-4 Methanol (lower spectrum).

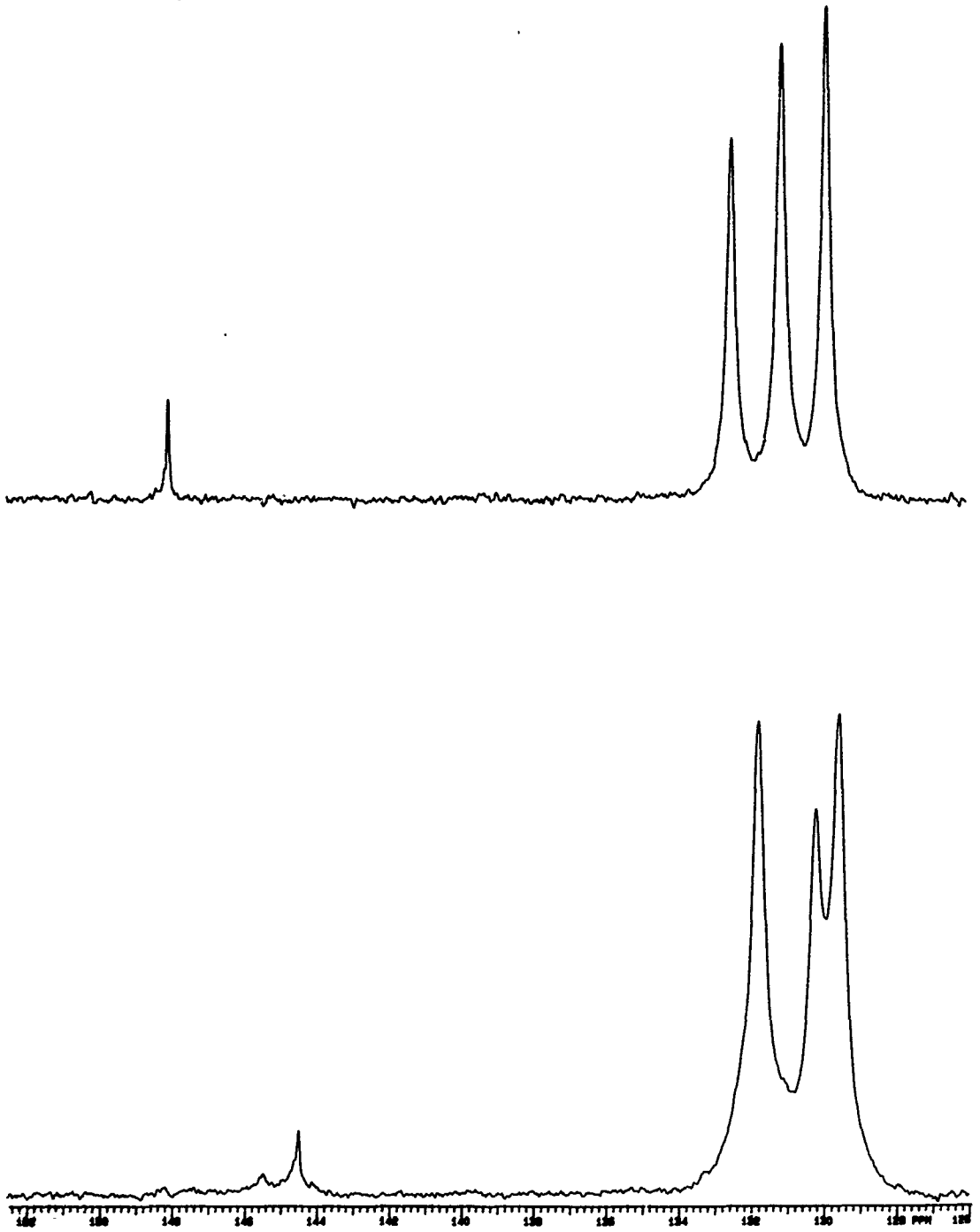

Figure 3. Proton-Decoupled ^{13}C-NMR Spectrum of the Aromatic Region of Free PDA (upper spectrum) and PDA reacted with BAL (lower spectrum)

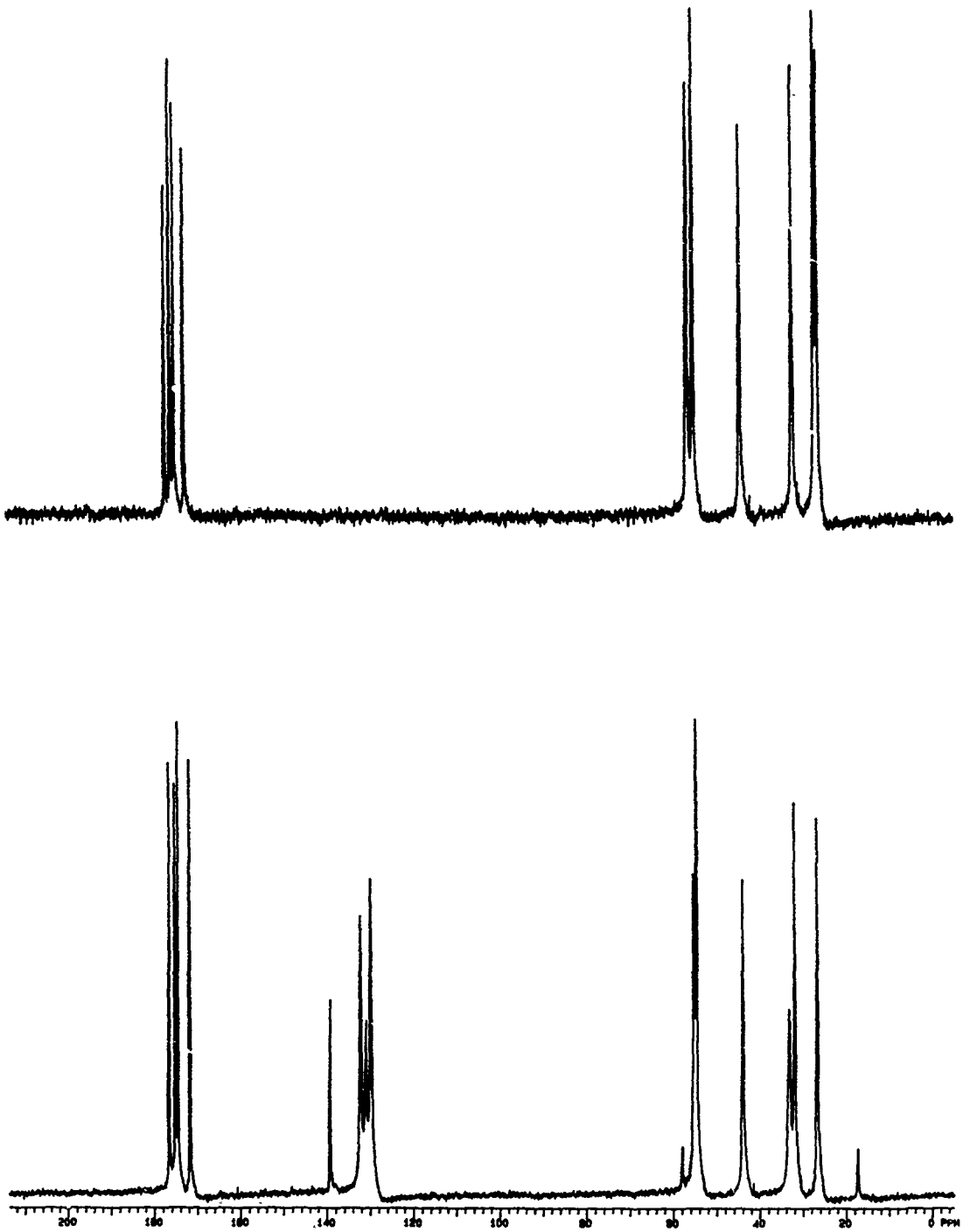

Figure 4. Proton-Decoupled ^{13}C-NMR Spectrum of Native Glutathione (upper spectrum) and Glutathione Reacted with PDA (lower spectrum)

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