0090-9556/83/1106-0526$02.00/0
DRUG METABOLISM AND DISPOSITION
Vol. 11, No. 6

Printed in U.S.A.

THE RELATIONSHIP BETWEEN PARAQUAT ACCUMULATION AND COVALENT BINDING IN RAT LUNG SLICES

TIMOTHY M. SULLIVAN AND MARK R. MONTGOMERY

Research Service, Veterans Administration Hospital and Department of Pharmacology and Therapeutics, University of South Florida Medical Center

(Received May 19, 1983; accepted June 20, 1983)

ABSTRACT:

The accumulation and covalent binding of paraquat in rat lung slices were both linear for 6 hr in room air incubations. Binding continued to increase in slices transferred to paraquat-free buffer after 3 hr of incubation in paraquat although accumulated paraquat decreased. Binding in 100% O_2 was decreased slightly. Active accumulation in 100% N_2 did not occur, but binding proceeded at one-third the rate observed in room air. Ascorbate decreased accumulation in room air, although binding was unaffected. Reductants had no effect on binding in 100% nitrogen. Paraquat binding in slices of various organs was in the order of lung > liver > heart > kidney cortex. Mitochondrial proteins were found to have the highest concentration of bound paraquat in lung slices followed in order by microsomal protein > nuclear protein = cytosolic protein. The binding of paraquat is postulated to involve a reduced species, presumably the monovalent radical.

Paraquat (methyl viologen, 1,1'-dimethyl-4,4'-bipyridylium dichloride) is a useful herbicide, but is also a potent pulmonary-specific toxin (1–4). Lung slices *in vitro* (5) and pulmonary tissue *in vivo* (6– 8) have been shown to concentrate PQ[1] by an energy-dependent process. The toxic actions of paraquat apparently are due to its redox characteristics, which facilitate the transfer of electrons from enzymatic sources to molecular oxygen, producing activated oxygen toxicants (1, 4).

Covalent binding has been implicated in the toxic mechanism of many xenobiotics (9), but not in the case of PQ. Ilett *et al.* (8) found no evidence of protein binding in the tissues of PQ-poisoned animals. In contrast, covalent binding of PQ to lung protein *in vitro* has been demonstrated (10, 11), but several aspects of the reported binding data are inconsistent with previous observations of the accumulation and biochemistry of PQ. Binding of PQ *in vitro* is reported (11) not to increase with incubation time beyond 30 min, although accumulation has been shown to be linear with time for at least 4 hr (5, 12). Also, hypoxic incubation is reported (11) to increase binding although accumulation is severely inhibited (5, 12). The present study was designed to clarify the relationship between *in vitro* PQ accumulation and binding.

Materials and Methods

Methyl-^{14}C-labeled PQ was purchased from Amersham (Arlington Heights, IL). Unlabeled PQ, ascorbic acid sodium salt, and sodium dithionite were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade obtained from commercial sources. Male Long-Evans rats (Blue Spruce Farms, NY), 180–220 g, were used throughout the study. Lungs were removed under ether anesthesia and cut into 0.5-mm thick slices (12). All incubations were conducted at 37°C in a shaking water bath with 120 oscillations/min under ambient air, 100% O_2 or 100% N_2 as indicated.

Accumulation. Accumulation studies were performed by incubating 40–60 mg of lung slices in 7 ml of Krebs-Ringer phosphate buffer (KRP), pH 7.4 (NaCl, 130 mM, KCl, 5.2 mM, $CaCl_2$, 1.9 mM, $MgSO_4$, 1.3 mM, Na_2HPO_4, 10 mM, and glucose 11 mM). A final PQ concentration of 10^{-5} M with 0.1 μCi ^{14}C-PQ/ml was determined to be optimal for incubations up to 6 hr. One study was included to measure the effect of buffer volume on accumulation. Accumulation was stopped by removing the tissue and rinsing with PQ-free KRP buffer. The tissues were then prepared for lsc. Counting efficiencies were determined by the external standard method. Accumulation data are expressed as nmol of PQ accumulated/g of lung slices (pre-incubation wet weight).

Binding. Binding studies were conducted by incubating 150 mg of lung slices in 21 ml of KRP buffer, maintaining the proportion used in the accumulation incubations. One study was performed to examine the effect of buffer volume on binding.

The reactions were stopped by adding 2.1 ml of 70% perchloric acid and the slices were transferred to 5 ml of 5% TCA and homogenized. The precipitate was resuspended in another 5 ml of 5% TCA and the extraction procedures of Hollinger and Giri (10) were followed. The precipitates were dried overnight, then a weighed amount (about 10 mg) was assayed by lsc. The protein content was determined (13), and the binding data are expressed as nmol of PQ bound/g of acid-insoluble protein (10, 11). PQ is not metabolized by mammals (8, 14).

Two categories of controls were processed through the extraction procedure with each group of samples. Nonincubated controls for background counting rates were prepared by homogenizing 150 mg of lung slices and extracting as described. Additional samples were prepared to measure nonspecific PQ binding residual after acidification. These samples were incubated in ^{14}C-PQ-containing buffer to which the perchloric acid had been added prior to addition of the lung slices.

The binding of PQ in slices of heart, liver, and kidney cortex was estimated in the same manner as described for the lung. The kidneys were bisected and the medulla removed before preparing the cortical slices.

Subcellular Distribution of Bound ^{14}C Activity. Six g of lung slices were obtained from eight rats and incubated for 3 hr in 840 ml of KRP, 10^{-5} M PQ with 0.1 μCi ^{14}C-PQ/ml. The slices were homogenized in 100 ml of

[1] Abbreviations used are: PQ, paraquat; TCA, trichloroacetic acid; lsc, liquid scintillation counting.

Supported by a grant from the Veterans Administration Medical Research and National Institutes of Health Grant ES02846. Presented in part at the 22nd Annual Meeting of the Society of Toxicology, Las Vegas, Nevada, March 7–11, 1983 (*Toxicologist* 3, 50, 1983).

Send reprint requests to: Dr. Tim Sullivan, VA Hospital (151), 13000 North 30th Street, Tampa, FL, 33612.

PQ-free KRP and filtered through cheesecloth. The filtrate was centrifuged at 500*g* for 5 min and the pellet (nuclear fraction) was resuspended in buffer as 5-ml aliquots. The supernatant was centrifuged at 10,000*g* for 15 min, and the pellet (mitochondrial fraction) was resuspended in 5-ml aliquots of buffer. The supernatant was centrifuged at 100,000*g* for 60 min and the pellet (microsomal fraction) resuspended in 5-ml aliquots of buffer. The remaining supernatant (cytosol fraction) was distributed in 5-ml aliquots. All of the 5-ml samples were deproteinized simultaneously with 0.5 ml of 40% TCA and the precipitates were extracted as described.

Statistical Analysis. Statistical analysis was based on the Student's *t* test; $p < 0.05$ was chosen to indicate significance. Linear regressions are based on the least squares calculation.

TABLE 1

The effect of buffer volume on the accumulation and binding of paraquat in rat lung slices

Data are expressed as $\bar{X} \pm$ SE, $N = 3$. Lung slices (250–300 mg) were incubated in room air for 3 hr in 10^{-4} M paraquat. KRP buffer as described in text was used.

ml KRP Buffer/Incubation	Paraquat Accumulated/g Tissue	Paraquat Bound/g Protein
	nmol	*nmol*
3	577 ± 34	22.8 ± 1.3
35	840 ± 58[a]	28.7 ± 1.5[a]
70	985 ± 98[a]	41.9 ± 0.3[a]

[a] $p < 0.05$ compared to the results from the 3.0-ml incubations.

Results

Efficiency of the Covalent Binding Extraction Procedure. Aliquots of supernatants from the extraction procedure were assayed to document exhaustive extraction of the ^{14}C label. In addition, the extraction efficiency was examined by repeating the entire procedure a second time on a homogenate of lung slices. One and one-half g of lung slices were incubated with 10^{-5} M PQ for 2 hr and extracted as before. Then, half of the precipitate was extracted again following the entire procedure. Five lsc samples were prepared from each precipitate. The precipitate which was extracted twice contained only 9% less ^{14}C specific activity than the precipitate which was extracted once ($p < 0.05$).

Effect of Buffer Volume on Accumulation and Binding. Volumes of 3.0, 35, and 70 ml were used to incubate 250–300 mg of slices in 10^{-4} M PQ (table 1). Accumulation and binding both increased significantly in 35 ml of buffer (46 and 26%, respectively) and in 70 ml of buffer (71 and 84%, respectively), compared to 3 ml. Very large buffer volumes are not practical for multiple samples due to cost requirements, so all subsequent incubations were prepared as described in *Materials and Methods*. The PQ concentration was maintained at 10^{-5} M in all studies other than those of table 1.

Relationship of Accumulation to Binding. Identical experimental designs were used to evaluate the influence of oxygenation on accumulation and binding of paraquat in the lung slice preparations (fig. 1, *A* and *B*). Lung slices were incubated in room air for

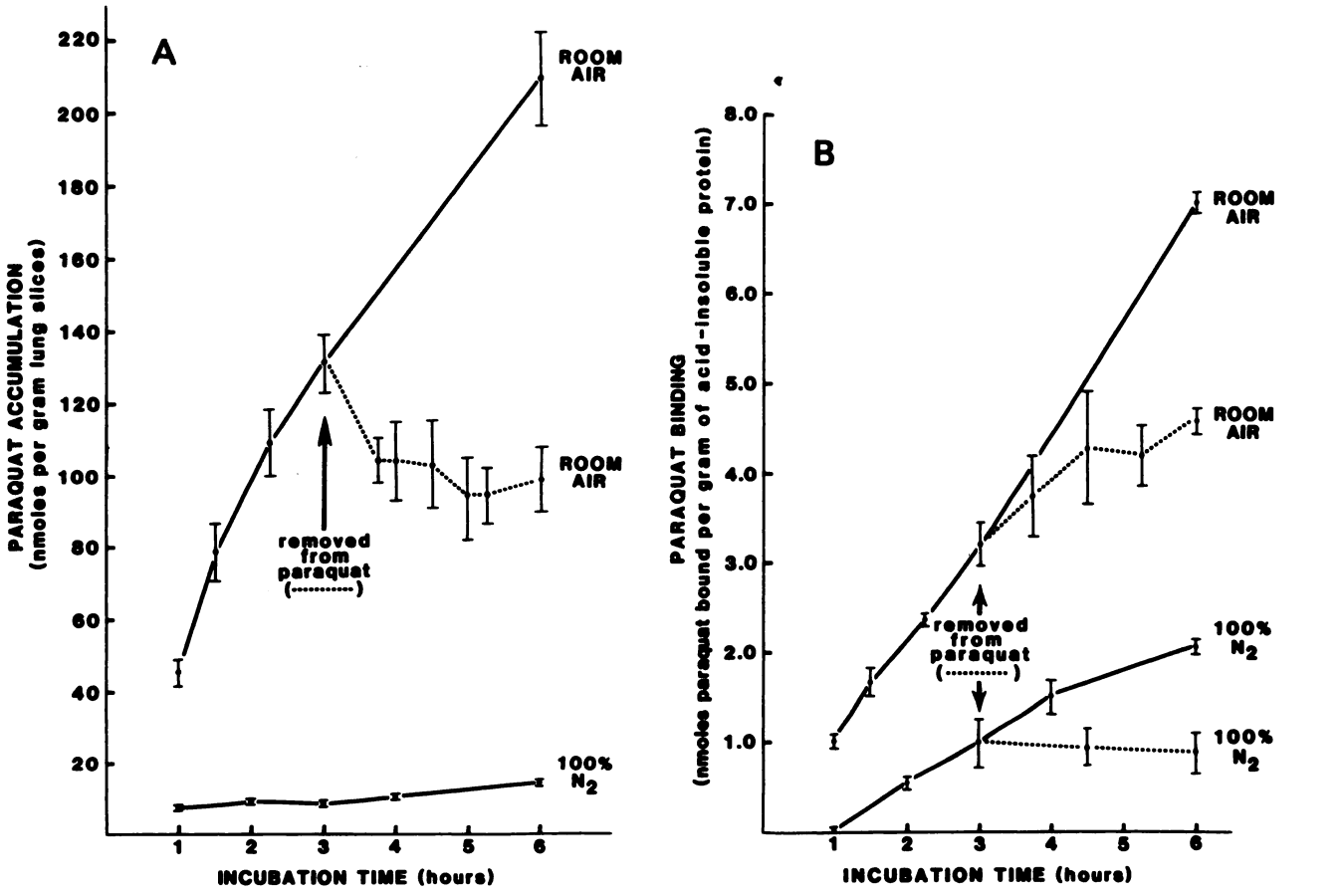

FIG. 1. *The time course of accumulation (A) and binding (B) of paraquat in rat lung slices.*

Lung slices were incubated in room air as described in *Materials and Methods* with 10^{-5} M paraquat. Samples removed from paraquat after 3 hr were transferred to identical incubation buffer without paraquat for additional incubation up to 3 hr. Indicated samples were maintained in 100% nitrogen during incubation. Incubations were at 37°C. Data are $\bar{X} \pm$ SE, with 3–6 samples/mean.

6 hr in the presence of 10^{-5} M PQ. Accumulation deviates little from linearity over the 6 hr of incubation, and can be approximated by a linear model with a slope of 31.5 nmol/hr and $r^2 = 0.86$ (fig. 1*A*). When slices were transferred to PQ-free buffer after an initial 3 hr of incubation with PQ, a decrease of about 20% ($p < 0.05$) in the amount of PQ accumulated was observed within 45 min. Continued incubation without PQ did not result in additional decreased accumulation. Incubation of lung slices under 100% N_2 prevented active PQ accumulation (fig. 1*A*) as has been previously described (12).

As seen with accumulation, binding in room air was also linear throughout the 6-hr incubation period (fig. 1*B*). However, in contrast to accumulation, the lung slices continued to bind PQ after removal from the PQ-containing buffer. Apparently, the PQ which remains concentrated within the slices represents a sufficient substrate pool for the binding reaction(s). Total binding of PQ increased 41% ($p < 0.05$) during the final 3 hr of incubation after removal from PQ. Also in contrast to accumulation, binding during incubation under 100% N_2 increased in a time-dependent manner, but at a rate less than incubations conducted in room air. The slopes of the least squares regressions are 1.24 ± 0.04 nmol PQ bound/g hr^{-1} for room air incubations ($r^2 = 0.99$) and 0.42 ± 0.04 nmol/g hr^{-1} under nitrogen ($r^2 = 0.85$). Binding under nitrogen did not continue to increase when the lung slices were removed from PQ, in contrast to the results observed with aerobic incubation.

Effect of Incubation in 100% Oxygen. No significant effect of 100% oxygen on PQ accumulation was observed (fig. 2*A*, $p > 0.15$ at 6 hr), consistent with previous reports (12). There was, however, a significant decrease ($p < 0.05$) in the binding of PQ at 4 and 6 hr of incubation under 100% oxygen (fig. 2*B*). Binding in 100% oxygen was 79% of the binding in room air after 4 hr of incubation and was 82% after 6 hr.

Effect of Chemical Reductants. Ascorbate has been reported to reduce PQ accumulation *in vitro* (15). Fig. 3 compares the data of Montgomery *et al.* (15) with the present observations on the *in vitro* effect of 10 mM sodium ascorbate on PQ binding in room air incubations. The previous study reported that ascorbate decreased PQ accumulation only at incubation times greater than 1 hr. Covalent binding, in contrast, was significantly reduced only within the first hour of incubation and was not inhibited at longer incubation times.

The effect of sodium ascorbate on PQ binding under hypoxia was also investigated. Lung slices were incubated under 100% N_2 for 3 hr with 0, 0.1, 1.0, or 10 mM concentrations of ascorbic acid. The means observed in this study ranged from 0.69 to 1.10 nmol PQ bound/g of protein with no significant differences between any of the groups.

In an additional experiment, lung slices were incubated for 3 hr in room air in buffer containing PQ but without ascorbic acid to allow PQ to be actively accumulated. The tissues were then transferred to PQ-free buffer containing 0, 0.1, 1.0, or 10 mM ascorbate and incubated under 100% N_2 for an additional 2 hr. The same procedure was followed using sodium dithionite instead of ascorbate. No concentration of ascorbate or dithionite had a significant effect ($p > 0.05$; data not shown).

Paraquat Binding in Slices from Selected Organs. To evaluate the organ specificity of the binding process, slices of liver, kidney cortex, heart, and lung were incubated for 4 hr (table 2). Binding was in the order lung > liver > heart > kidney cortex. Binding in liver slices was 70% of that in the lung slices, while binding in the heart and kidney cortex was 54 and 34%, respectively.

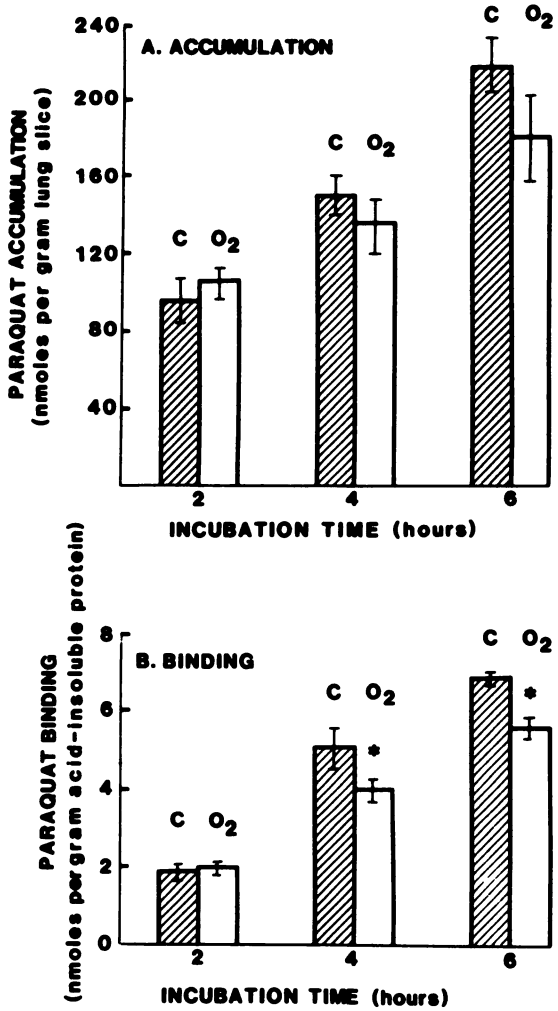

FIG. 2. *The effect of 100% oxygen on the accumulation (A) and binding (B) of paraquat in rat lung slices.*

C, controls incubated in room air. O_2, incubations in 100% oxygen. Data are $\bar{X}$ ± SE for 3–6 samples/time point. *Asterisks* indicate $p < 0.05$ compared to appropriate control. Paraquat was 10^{-5} M.

Subcellular Localization of Bound ^{14}C Activity. The specific activities of the acid-insoluble protein precipitates obtained from differential centrifugation of treated lung slices are shown in table 3. The mitochondrial fraction contained a 40% higher concentration of bound paraquat than the microsomal fraction and 75% higher than either the nuclear or cytosolic fractions.

Discussion

The accumulation and binding of PQ *in vitro* are strongly dependent on buffer volume. Previous investigations of PQ binding with small buffer volumes (11) showed no increase in binding after 30 min of incubation. The present study indicates that, under appropriate conditions, binding is a linear function of time for at least 6 hr of incubation.

The rate of active PQ accumulation in room air is 31.5 nmol PQ/g tissue/hr, and 1.24 nmol PQ/g protein become bound per hour. We have determined that acid-insoluble proteins comprise 15% of the nonperfused lung wet weight. Therefore, approximately 0.6% of the accumulated PQ becomes bound in room air incubations.

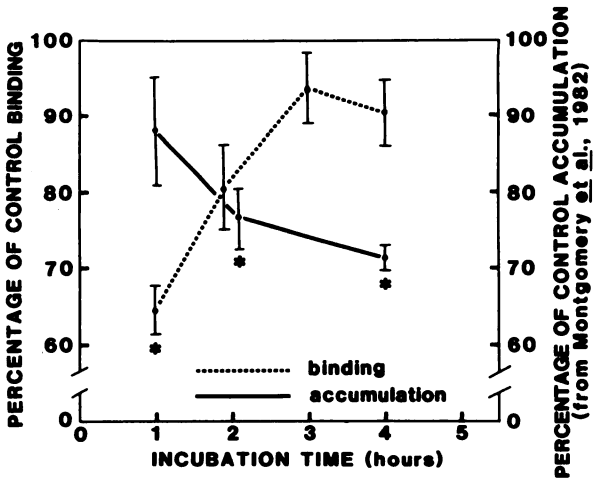

FIG. 3. *The effect of 10 mM sodium ascorbate on the accumulation and binding of paraquat in rat lung slices.*

Data are $\bar{X} \pm$ SE of the percentage of control (no added ascorbate) samples at each time point; $N = 4$ for the accumulation data and $N = 3$ for binding. The accumulation data are from Montgomery *et al.* (15). Paraquat was 10^{-5} M, and incubations were conducted in room air. *Asterisks* indicate $p < 0.05$ compared to appropriate control.

TABLE 2

The in vitro binding of paraquat to slices of selected organs from the rat

Data are $\bar{X} \pm$ SE, $N = 6$. One hundred and fifty mg of 0.5-mm slices of the organs were incubated for 4 hr in room air in 10^{-5} M paraquat.

Organ	Paraquat Bound/g Protein
	nmol
Kidney cortex	1.63 ± 0.15
Heart	2.58 ± 0.11
Liver	3.35 ± 0.33
Lung	4.76 ± 0.35

TABLE 3

Subcellular localization of bound ^{14}C activity following incubation of rat lung slices with ^{14}C-paraquat

Six g of lung slices were incubated for 3 hr in 10^{-5} M ^{14}C-paraquat. Data are $\bar{X} \pm$ SE for 3–6 samples/fraction. Cellular fractionation is described in *Materials and Methods.*

Cellular Fraction	^{14}C/protein	Paraquat/Protein
	nCi/g	*nmol/g*
Nuclear	31.0 ± 2.2	4.07 ± 0.29
Mitochondrial	54.6 ± 2.7	7.17 ± 0.35
Microsomal	39.6 ± 0.3	5.20 ± 0.03
Cystosolic	30.7 ± 0.2	4.05 ± 0.03

PQ accumulates within hypoxic lung slices to the concentration present in the buffer (10 mM, or 10 nmol/g), and supports hypoxic binding at one-third the rate of normoxic binding. Giri and Lunsman (11) reported that hypoxic binding was 77% greater than normoxic binding. The earlier study used twice the lung slice mass in one-seventh the buffer volume compared to the present study, which may contribute to the contradiction in results.

The fact that PQ does bind in hypoxic incubations, presumably without enzymatic reduction, suggests that either the PQ^{2+} species is being bound or that the divalent cation is being reduced by other processes prior to binding. Neither sodium ascorbate nor sodium dithionite increased hypoxic binding, however. This observation does not preclude formation of the PQ radical (PQ^{+}). Endogenous chemical reducing equivalents may be sufficient to maintain PQ as the radical under 100% N_2. Ledwith (16) has reported thermodynamic data which indicate that the radical is the favored form of PQ. Hypoxic binding was one-third of normoxic binding even though PQ accumulation was as much as 20-fold higher in normoxia. This implies that the efficiency of the binding reaction(s) (as a fraction of the total PQ available) is greater in hypoxic tissues than in aerobic. Indeed, the availability of O_2 to act as oxidant for PQ^{+} could decrease the fraction of PQ which is in the reactive PQ^{+} state, even in the presence of maximal rates of enzymatic PQ reduction.

Further support for the premise that a reduced PQ species is responsible for binding is provided by the results of incubation in 100% oxygen. There was no significant effect of 100% O_2 on accumulation, although there was a tendency for decreased accumulation which was not seen in earlier work (12). However, binding was significantly decreased by 100% oxygen. The reduction of PQ^{2+} may be less affected by high O_2 tension than is the reoxidation of PQ^{+}, since reducing equivalents are generated without direct involvement of oxygen whereas reoxidation of PQ^{+} by O_2 should obey mass action kinetics. An increased rate of reoxidation of PQ^{+} would reduce the amount of PQ^{+} available for binding.

The contrasting effects of ascorbate on accumulation and binding in room air are important. Binding was not affected at incubation times greater than 1 hr despite decreased accumulation, implying that the binding process occurs more efficiently with added ascorbate. Ascorbate (10 mM) may enhance the stability of the PQ^{+} radical, increasing its predominance in the total population of all PQ species. The synergistic depletion of oxygen by the ascorbate-PQ couple may also increase PQ^{+} concentration by lowering its rate of oxidation (15), while simultaneously inhibiting accumulation.

These studies suggest that a reduced species of PQ may be the reactive intermediate in the binding process. The hypothesized PQ^{+} radical has the highly reactive, electrophilic characteristics of the mixed function oxidative intermediates presumed to be responsible for covalent binding of chloroform, bromobenzene, and other xenobiotics. The proposal that PQ^{+} is the species responsible for binding is consistent with current theories on the redox cycling of PQ and consistent with the noted lack of mixed function oxidation of PQ. The possibility remains, however, that PQ^{2+} and/or other species are responsible for the high affinity binding observed in our studies. This binding was greater in lung than in other organs. In lung slices, mitochondrial and microsomal proteins bound greater concentrations of PQ than nuclear or cytosolic proteins. The contribution of covalent protein binding to PQ toxicity remains to be elucidated.

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