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# Paraquat Uptake into Freshly Isolated Rabbit Lung Epithelial Cells and Its Reduction to the Paraquat Radical under Anaerobic Conditions

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## SUMMARY

Uptake of paraquat (PQ; 10 μM) into lung cell fractions enriched in alveolar type II cells or Clara cells was linear with time, and after 60 min the intracellular concentration was approximately 10-fold higher than that in the medium. In contrast, alveolar macrophages were not able to accumulate PQ from the extracellular medium. PQ uptake in preparations of type II and Clara cells, but not alveolar macrophages, was inhibited by an equimolar concentration of putrescine or spermidine and by a combination of the metabolic inhibitors, potassium cyanide and iodoacetate (1 mM each). The reduction of PQ (1 mM) under anaerobic conditions was investigated in lung cells by ESR spectroscopy. The amplitude of the ESR signal of the PQ radical increased with time with intact or sonicated type II and Clara cell preparations, but with macrophages it increased only when the cells were sonicated. The signal in sonicated cells but not whole cells was decreased by addition of antibodies to NADPH-cytochrome P-450 reductase, suggesting that the PQ radical is generated intracellularly under these conditions.

PQ (1,1-dimethyl-4,4′-bipyridilium; methyl viologen; CAS: 1910-42-5) is a nonselective contact herbicide. The most characteristic feature of PQ toxicity is lung damage and, following administration to rats, rabbits, and mice, a very high concentration of PQ has been shown to accumulate in lung tissues relative to other organs of the body (1–3). Toxicity in rats given PQ is manifested by extensive alveolar pulmonary edema and hemorrhage, fibrosis, and disruption of the epithelial cells (4, 5). Although several studies have indicated that the rabbit is less susceptible or even resistant to PQ-related pulmonary damage (6, 7), other workers have demonstrated lesions in rabbit lungs (8, 9).

Morphological studies have indicated that the most extensively damaged cells in the lung are the type I and type II alveolar epithelial cells (8, 10, 11), and PQ is directly toxic to rat type II cells *in vitro* (12). There is also indirect evidence that PQ is accumulated in these cell types (13). The PQ dication is transported into rat lung slices by an energy-dependent system responsible for the uptake of endogenous diamines and polyamines (14, 15). One of the objectives of this study was to directly investigate the PQ uptake system in freshly isolated rabbit alveolar type II cells and to compare the results with those obtained using Clara cells and alveolar macrophages. A previous study using isolated rat lung cells demonstrated an active uptake process for the PQ dication in type II cells but not in alveolar macrophages (16). There have been no such studies with Clara cells, although there is some evidence for PQ uptake by the bronchiolar epithelium in rats (17) and damage to bronchiolar cells (Clara and ciliated) after PQ administration to mice (18).

The PQ dication is known to undergo cyclic oxidation-reduction. Enzymatic one-electron reduction of the PQ dication results in formation of the PQ cation free radical (19). In the presence of oxygen, the PQ cation radical is immediately reoxidized, and the superoxide radical is generated. This can lead to production of more toxic oxygen species, e.g., the hydroxyl radical and singlet oxygen, which are implicated in the initiation of membrane-damaging lipid peroxidation and the development of pulmonary toxicity (19), although possibly only after cellular reductive capacity has been exhausted. The PQ cation radical is stable under anaerobic conditions and can be recognized by its characteristic ESR spectrum. A second aim of this study was to demonstrate the generation of the PQ radical inside intact lung cells and to quantitate its rate of formation in the different cell types.

## Materials and Methods

**Chemicals.** Protease (type I), DNase I, trypsin inhibitor (type II-S), and nitroblue tetrazolium used in the lung cell isolation and iden-

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**ABBREVIATIONS:** Ab, antibodies; HEPES, *N*-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HpBS, buffered balanced salt solution, pH 7.4, containing 25 mM HEPES; IA, iodoacetate; KCN, potassium cyanide; PEG, polyethylene glycol; PQ, paraquat.

tification procedures were purchased from Sigma Chemical Company (St. Louis, MO). F12K growth medium was obtained from Grand Island Biological Company (Grand Island, NY) and Percoll from Pharmacia Fine Chemicals (Piscataway, NJ).

[*methyl*-$^{14}$C]PQ chloride (111 mCi/nmol, 98% radiochemical purity) and tritiated water (2.5 mCi/ml) were obtained from Amersham Corporation (Arlington Heights, IL). PEG, [1,2-$^{3}$H] (1.2 mCi/g), and PEG, [1,2-$^{14}$C] (0.57 mCi/g), were purchased from New England Nuclear (Boston, MA).

Unlabeled PQ (methyl viologen hydrate) was obtained from Aldrich Chemical Company (Milwaukee, WI) and unlabeled PEG 4000 (Carbowax) was from Fisher Scientific Company (Fair Lawn, NJ). Putrescine dihydrochloride, spermidine trihydrochloride, and IA (sodium salt) were purchased from Sigma. KCN was from Fisher Scientific and Aquasol liquid scintillation cocktail from New England Nuclear. Minimal essential medium F-11 was supplied by Grand Island Biological Company and fetal bovine serum was supplied by Sigma.

**Animals.** Male New Zealand White rabbits (2–2.5 kg) were obtained from Dutchland Farms (Denver, PA). They were allowed free access to water and food.

**Pulmonary cell isolation.** Alveolar type II cells, Clara cells, and alveolar macrophages were isolated and separated by slight modification of the procedure of Devereux and Fouts (20–22). The rabbits were anesthetized by intravenous injection of 3 ml of sodium pentobarbital (50 mg/ml) containing 50 mg of sodium heparin. The lungs were cleared of blood by perfusion *in situ* with Krebs-Ringer bicarbonate solution, pH 7.4, containing 4.5% bovine serum albumin and 5 mM glucose (fortified K-RB), and the macrophages were obtained by lung lavage as described previously (22). The lungs were digested by filling with 0.2% protease I in HpBS, pH 7.4, containing 25 mM HEPES (20) and incubating for 2 min at 37° and then 8 min at room temperature. After chopping the lungs, the pieces were suspended in ice-cold cell isolation buffer (HpBS/F12K/fortified K-RB, 3:1:1) containing 0.05% trypsin inhibitor (200 ml/lung). The suspension was degassed for 30 sec, filtered, centrifuged at 800 × *g* for 10 min, and resuspended in approximately 10 ml of cell isolation buffer containing 0.05% DNase I.

Initial separation of the cells into four fractions was carried out by centrifugal elutriation in a JE-6B elutriator rotor (Beckman Instruments, Palo Alto, CA). The first fraction, collected at 2200 rpm and 9 ml/min, consisted mostly of cell debris and was discarded. Fraction 2 (2200 rpm, 14 ml/min) was further purified by Percoll density-gradient centrifugation. The cell suspension was layered on 25 ml of 28% Percoll in HpBS. After centrifugation for 20 min at 800 × *g*, the cells enriched at the interface between Percoll and HpBS were, on the average, 79% type II cells, identified by a modified Papanicolaou stain without acid alcohol (20) with less than 5% contamination by Clara cells. The third fraction (2200 rpm, 17 ml/min) contained a mixture of cells and was also discarded. Fraction 4 was collected at 1200 rpm and 20 ml/min and was further purified by a discontinuous Percoll gradient. The cell suspension was layered on gradients consisting of 25 ml of 28% Percoll in HpBS on top of 5 ml of 60% Percoll. The cells collected from the interface between the two Percoll solutions after centrifugation at 800 × *g* for 20 min contained up to 38% Clara cells, with less than 1% contaminating type II cells, as identified by nitroblue tetrazolium staining (20).

The isolated lung cells were suspended in MEM, pH 7.4, containing 10% fetal bovine serum. Cell counts were by an Electozone/Celloscope particle size analyzer (Particle Data Inc., Elmhurst, IL).

**Uptake of PQ into lung cells.** To measure uptake, 3-ml aliquots of cells (4–5 × 10$^{6}$/ml) were incubated with 10 μM unlabeled PQ and 0.1 μCi [$^{14}$C]PQ at 37° in a shaking water bath under an atmosphere of 95% $O_2$/5% $CO_2$. PEG (0.1%, w/v) containing [$^{3}$H]PEG (1 μCi/3 ml) was added as an extracellular marker (4). Samples (0.5 ml) were removed at 0, 20, 40, and 60 min and centrifugated for 5 min at 600 × *g*. The supernatant was discarded and the cell pellet was lysed by addition of 0.2 ml of 10% perchloric acid. After vortexing, the suspension was centrifuged for 1 min in a model 5412 Eppendorf centrifuge. Aliquots (3 × 50 μl) were counted using Aquasol liquid scintillation cocktail. The volume of extracellular medium trapped with the cell pellet was determined from the [$^{3}$H]PEG present. This value was used to correct for the [$^{14}$C]PQ present in the extracellular medium and to accurately calculate the intracellular PQ concentration.

The intracellular volume of the three cell types was calculated by a similar technique using [$^{3}$H]$H_2O$ and [$^{14}$C]PEG (23).

In studies of the effect of putrescine (10 μM and 100 μM), spermidine (10 μM), and a combination of KCN/IA (1 mM each) on PQ uptake, these compounds were added to the cell suspension as ×100 solutions in HpBS, just prior to the addition of PQ. Viability of the cells at the beginning and end of experiments was determined by trypan blue dye exclusion. Neither PQ nor any of the inhibitors of uptake caused a decrease in cell viability when compared with untreated cells.

**Detection of the PQ free radical by ESR.** The isolated cells were suspended in medium at a cell density of approximately 20 × 10$^{6}$/ml for type II cells, 10 × 10$^{6}$/ml for Clara cells, and 100 × 10$^{6}$/ml for macrophages. A sample of cells (0.5 ml) was bubbled with nitrogen in a test tube for 2 min prior to addition of PQ (1 mM). The cells were transferred to an ESR flat cell and bubbled with nitrogen for a further 1.5 min. ESR scans were then recorded every 4 min until approximately 40 min after addition of PQ. The increase in amplitude with time of the ESR signal of the PQ radical was measured and expressed in arbitrary units per 10$^{6}$ cells at a fixed gain of 4 × 10$^{4}$. All ESR spectra were obtained with a Varian E-104 X-band spectrometer (9.5 GHz) with a magnetic field modulation frequency of 100 kHz, a nominal microwave power of 20 mW, and a magnetic field modulation amplitude of 2 × .33 G.

In studies of the effect of Abs to rabbit liver NADPH-cytochrome P-450 reductase upon the appearance of the ESR signal, the Ab were added to the cell suspension before bubbling with nitrogen and the addition of PQ. The Ab were prepared in the goat as previously described (24) and were kindly supplied by Dr. R. M. Philpot, National Institute of Environmental Health Sciences.

Statistical analysis was performed using a *t* test for two means (25). The level of statistical significance was chosen as $p < 0.05$.

## Results

Uptake of PQ into cell fractions enriched in type II and Clara cells was linear with time (Fig. 1), and after 60 min the intracellular concentration was approximately 10-fold higher than that in the medium. Alveolar macrophages did not accumulate PQ. Over the 60-min incubation period, the internal and external PQ concentrations in macrophages equilibrated, resulting in a [PQ] in/[PQ] out ratio of 0.87.

In the uptake studies, the intracellular PQ concentrations reported were corrected for the association of PQ that occurred with the various lung cell preparations at zero time. These zero time values were 15 pmol of PQ/10$^{6}$ cells in the type II preparations, 12.5 pmol of PQ/10$^{6}$ cells in the Clara cell preparations, and 13.3 pmol of PQ/10$^{6}$ cells in the macrophage preparations. These zero time values almost certainly do not represent intracellular PQ, for very similar numbers were obtained in cells killed by exposure to high concentrations of KCN/IA, and there were no increases with incubation time in the amount of PQ associated with the dead cells. The zero time values most likely represent PQ bound externally to the plasma membrane and released upon deproteination with perchloric acid.

The accumulation of PQ by preparations of intact type II and Clara cells was reduced by a combination of KCN (1 mM) and IA (1 mM), but there was no change in the uptake of PQ by macrophages (Table 1). A lower concentration of KCN/IA (0.5 mM) had a lesser but still significant effect in the type II cells. PQ uptake by type II and Clara cell fractions was also

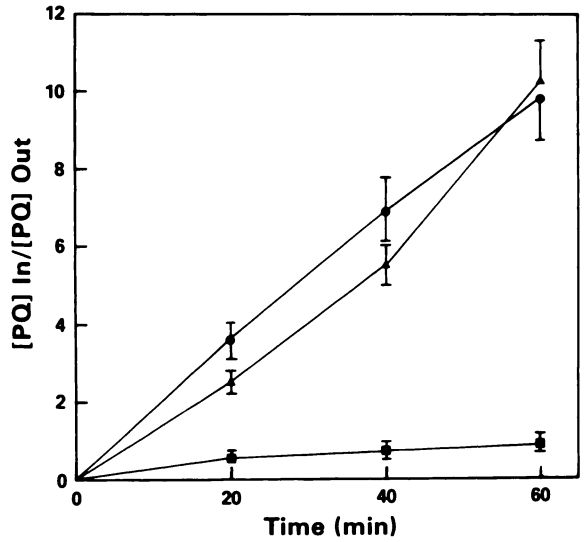


**Fig. 1.** The uptake of PQ (10 μM) by type II cells, Clara cells, and alveolar macrophages. Cells were incubated with [$^{14}$C]PQ as described in Materials and Methods. The ratio of intracellular to extracellular PQ concentration ([PQ] in/[PQ] out) was calculated for type II cells, $n$ = 12 (▲); Clara cells, $n$ = 11 (●); and macrophages ($n$ = 9) (■). *Error bars* represent standard error. Intracellular PQ concentrations used are corrected values after subtraction of the zero time association of PQ with the lung cell preparations.

decreased by an equimolar concentration of the diamine putrescine (Table 1). The polyamine spermidine similarly inhibited PQ uptake (results not shown). Neither putrescine nor spermidine had any effect on the uptake of PQ by alveolar macrophages. Addition of a 10-fold higher concentration of putrescine (100 μM) resulted in a more marked inhibition of PQ uptake in the type II cells and Clara cells and also a slight decrease in the macrophages. Under these conditions, however, it is likely that putrescine is having a small effect in addition to inhibition of PQ uptake, because the zero time value for PQ associated with the lung cells was 18.5% lower in macrophages and 20% lower in the type II cells than in control macrophages or type II cells, or cells exposed to 10 μM putrescine.

When PQ (1 mM) was incubated under anaerobic conditions with type II cells or Clara cells, the PQ cation radical was detected by ESR spectroscopy (Fig. 2). However, no ESR signal was detected in incubations containing intact macrophages. The amplitude of the ESR signal of the PQ cation radical increased with time (over 40 min) in whole type II and Clara cells (Fig. 3). When type II and Clara cells were sonicated prior to incubation, an ESR signal was observed which was slightly lower in intensity than in the whole cells, but which also increased with time (results not shown). The PQ cation radical was detected when PQ was incubated with sonicated macrophages. The signal was of lower intensity than that observed in sonicated type II and Clara cells and did not increase with time over the 40-min incubation period; it reached a plateau after approximately 14 min (Fig. 3).

Addition of 50 μl of Ab to NADPH-cytochrome P-450 reductase to incubations containing intact type II or Clara cells had no effect on the amplitude of the ESR signal (data not shown). In contrast, there was an antibody concentration-dependent decrease in ESR signal amplitude in incubation mixtures containing sonicated type II cells and 20 or 50 μl of Ab preparation (Fig. 4). Formation of the ESR signal of the PQ cation radical was completely inhibited in sonicated Clara cells by 50 μl of Ab.

## Discussion

PQ dication accumulation has been demonstrated previously in rat lung slices (14) and in rat alveolar type II cells in primary cultures but was found not to occur in macrophages (16). In the present study, similar results were obtained using freshly prepared type II cells and macrophages isolated from rabbit lung. Accumulation of PQ was also observed in enriched fractions of rabbit Clara cells. There have been no previous studies of PQ uptake by isolated Clara cells, but bronchiolar damage has been observed *in vivo* following administration of this herbicide to rats and mice (17, 18). On the average, only 32% of the enriched Clara cell population could be positively identified as Clara cells, and it is possible that another cell type in this fraction (type I cells?) may be responsible for the observed uptake. However, the major contaminants of this fraction are macrophages, which are not able to accumulate PQ from the extracellular medium.

Uptake of PQ into macrophages was unchanged in the presence of a combination of the metabolic inhibitors KCN and IA. This result, taken with the inabilty of macrophages to accumulate PQ, suggests that uptake in this cell type is by passive diffusion through the cell membrane, resulting in equilibration of internal and external PQ concentrations. In contrast, the accumulation of PQ in type II and Clara cell preparations was

TABLE 1

**The effect of KCN/IA and putrescine on the uptake of [$^{14}$C]PQ (10 μM) by type II cells, Clara cells, and alveolar macrophages**

Cells were incubated with [$^{14}$C]PQ as described in Materials and Methods.

| | Intracellular PQ concentration (pmol/10$^6$ cells) | | | | | |
|---|---|---|---|---|---|---|
| | Type II | | Clara | | Macrophage | |
| | 20 min | 60 min | 20 min | 60 min | 20 min | 60 min |
| Control | 20 ± 1 (12)[a] | 83 ± 2 (12) | 24 ± 1 (11) | 64 ± 2 (11) | 5 ± 1 (9) | 6 ± 1 (9) |
| + KCN/IA 1 mM | 6 ± 2 (3)[b] | 14 ± 3 (3)[b] | 6 ± 1 (3)[b] | 16 ± 1 (3)[b] | 2 (1)[c] | 8 (1) |
| + KCN/IA 0.5 mM | 10 ± 2 (3)[b] | 24 ± 4 (3)[b] | –[d] | – | – | – |
| + Putrescine 10 μM | 6 ± 3 (3)[b] | 18 ± 3 (3)[b] | 7 ± 1 (3)[b] | 14 ± 3 (3)[b] | 5 ± 2 (3) | 10 ± 2 (3) |

[a] Mean ± SE; numbers in parentheses, $N$.
[b] $p < 0.05$ compared with uptake in control cells.
[c] One determination only.
[d] –, not determined.

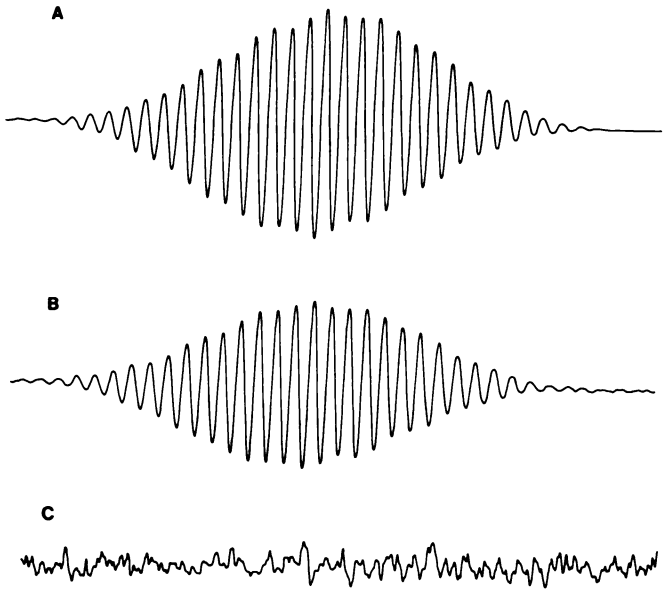


**Fig. 2.** The ESR spectra obtained after a 39-min (A and B) or an 18-min (C) anaerobic incubation of PQ (1 mM) with (A) type II cells, $10.2 \times 10^6$/0.5 ml; (B) Clara cells, $5.4 \times 10^6$/0.5 ml; and (C) macrophages, $4.5 \times 10^7$/0.5 ml. The scan was recorded over 4 min at a receive gain of $1 \times 10^4$ (A), $5 \times 10^3$ (B), and $5 \times 10^4$ (C).

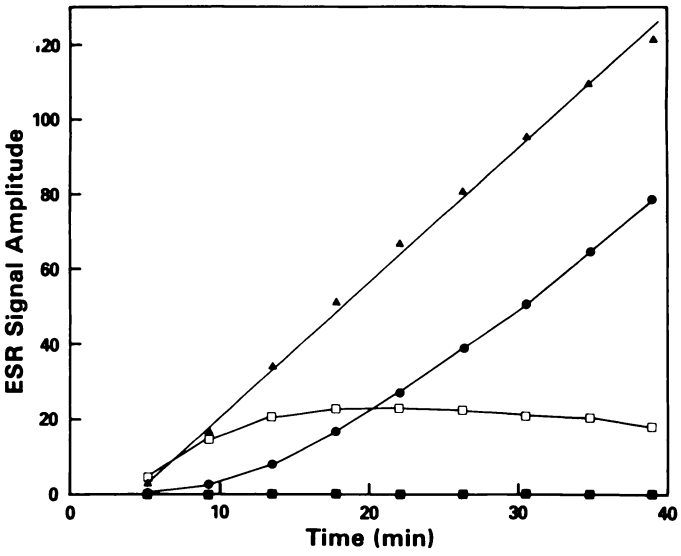


**Fig. 3.** Amplitude of ESR signal of PQ cation radical (expressed in arbitrary units for $10^6$ cells at a fixed gain of $4 \times 10^4$) obtained during anaerobic incubation of PQ (1 mM) with lung cell suspensions. Where indicated, cells were sonicated for 20 sec. ▲, type II cells; ●, Clara cells; ■, macrophages; □, sonicated macrophages. Values are from a representative experiment in which all cell types were isolated from the same animals, and experiments were run on the same day.

inhibited by KCN and IA, implicating an energy-dependent uptake process in these cell types. Uptake of PQ in rat type II cells in primary culture was similarly susceptible to the effect of metabolic inhibitors (16).

Previous experiments with rat lung slices led to the proposal that PQ is a substrate for the system normally responsible for the accumulation of endogenous diamines and polyamines in the lung (15). In the present study, the uptake of PQ into type II and Clara cell fractions, but not macrophages, was significantly inhibited by equimolar concentrations of the diamine putrescine and the polyamine spermidine. Lung slice studies

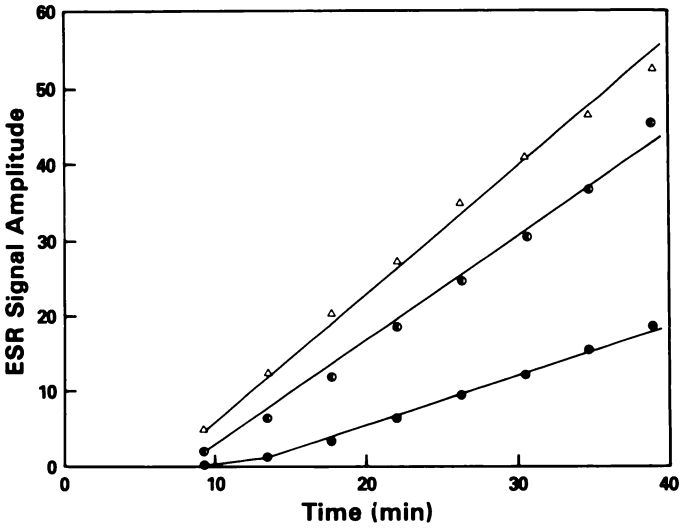


**Fig. 4.** Amplitude of ESR signal of PQ cation radical (expressed in arbitrary units for $10^6$ cells at a fixed gain of $4 \times 10^4$) obtained during anaerobic incubation of PQ (1 mM) with sonicated type II cells. Where indicated, 20 or 50 μl of Ab to NADPH-cytochrome P-450 reductase (33 mg/ml) were added to 500-μl aliquots prior to bubbling with nitrogen and addition of PQ. ▲, sonicated type II cells; ◐, same + 20 μl of Ab; ●, same + 50 μl of Ab. Values are from a representative experiment in which all spectra were obtained on the same day.

have provided indirect evidence that the alveolar type I and type II cells are the site of the polyamine uptake system (15), but here, presented for the first time, is a direct demonstration of this uptake system in two epithelial cell types of the lung.

PQ is known to be reduced when incubated anaerobically with lung microsomes and either NADPH or an NADPH-generating system (19, 26), and a strong ESR signal of the PQ free radical is observed (26). The ESR signal characteristic of the PQ radical was obtained in the present study during anaerobic incubation of PQ with intact type II or Clara cells. Rabbit Clara cells have a higher content of NADPH-cytochrome P-450 reductase activity than do type II cells (21), yet the increase in ESR signal amplitude with time was greater for the type II cells, probably reflecting the higher purity of the type II cell fraction. A slightly lower signal amplitude was observed where sonicated Clara or type II cells were used. Under these conditions, PQ access to NADPH-cytochrome P-450 reductase is unrestricted, but the cellular NADPH will be at a lower concentration in the total volume of the incubation medium.

The PQ radical could be detected in macrophage incubations only after the cells were sonicated. The amplitude of the ESR signal was lower than that obtained with sonicated type II or Clara cells, consistent with the lower metabolic capacity of the alveolar macrophages. It is probable that the low permeability of macrophages to PQ is the reason for the inability to detect an ESR signal in whole cells, because macrophages have appreciable NADPH-cytochrome P-450 (cytochrome *c*) reductase activity (27).

For the ESR studies, the isolated lung cells were incubated for up to 40 min under anaerobic conditions. In parallel anaerobic incubations there was a 20–30% decrease in viability in the type II and Clara cells and less than a 5% decrease in viability of the macrophages during this time. There have been previous reports of the generation of free radicals in whole cells and of their detection by ESR (28–30). These studies utilized

intact protozoans, however, and not cells isolated from a whole mammalian tissue.

An important aspect of our study was to determine whether or not the PQ cation radical was generated intracellularly in preparations of intact Clara and alveolar type II cells. We answered this question by adding Ab to NADPH-cytochrome P-450 reductase to both intact and sonicated preparations. There was a decrease in the rate of PQ radical generation by sonicated type II and Clara cells upon addition of the Ab. A comparable result was obtained previously upon addition of a similar Ab preparation to mouse liver microsomes (19). In contrast, Ab to rabbit cytochrome P-450 reductase did not produce a significant change in the amplitude of the PQ cation radical ESR signal in incubations of whole type II or Clara cells. Large Ab molecules are unable to cross intact cell membranes and, consequently, to inhibit intracellular reductase. Therefore, our results are consistent with generation of the PQ radical intracellularly in intact cell preparations and not by fragments of cells present in the incubation medium.

In conclusion, our results clarify some aspects of PQ-mediated lung toxicity. We have demonstrated the presence of an energy-dependent uptake system for PQ in alveolar type II cells and Clara cells of rabbit lung and have shown that endogenous diamines and polyamines inhibit the uptake of PQ in these cells. We have also demonstrated the intracellular conversion of PQ to its cation radical in type II and Clara cells, the stem cells of the alveolar and bronchiolar epithelium, respectively. Differences in the susceptibility of various lung cell types *in vivo* may be due to a balance between the amount of PQ accumulated within the cell and then reduced to its cation radical, and the ability of the cell to detoxify the reactive species (e.g., superoxide radical, hydroxyl radical) that are produced by redox cycling of PQ in an aerobic environment.

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