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Paraquat- and Diquat-Induced Oxygen Radical Generation and Lipid Peroxidation in Rat Brain Microsomes

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NADPH-menadione reductase activity by rat brain microsomes (Ms) was decreased 40–50% by 10 μM dicumarol, a potent inhibitor of DT-diaphorase, whereas no change in NADPH-paraquat (PQ) and -diquat (DQ) reductase activity was observed. NADPH-DQ reductase activity in brain Ms was 2.5-fold higher than NADPH-PQ reductase activity. The formation of PQ and DQ radicals was verified optically and observed directly by ESR spectroscopy in the NADPH-PQ and -DQ reductase reactions by brain Ms under anaerobic conditions. PQ- and DQ-induced superoxide formation was confirmed by the detection of DMPO-OOH ESR signals and followed by chemiluminescence (CL) of a *Cypridina luciferin* analogue (CLA). The kinetics and intensity of the CL were consistent with the observations that the reduction in DQ is faster than that in PQ. Thiobarbituric acid reactive substances (TBARS) and phospholipid hydroperoxides in brain Ms increased in the presence of NADPH and Fe^{3+}. The generation of both lipid peroxidation products derived from brain Ms decreased with increasing concentrations of PQ and DQ. The inhibitory effect of DQ is more pronounced than that of PQ. The formation of PQ- and DQ-induced reactive oxygen species was not associated with lipid peroxidation in rat brain Ms.

Key words: brain injury, lipid peroxidation, oxygen radicals, paraquat, parkinson's disease.

Paraquat (PQ; 1,1-dimethyl-4,4-bipyridilium) and diquat (DQ; 1,1-ethylene-2,2-dipyridilium) are widely used in agriculture as herbicides. It has been documented that PQ is highly toxic to mammalian species and that the cytotoxicity involves oxygen radical generation through redox cycling (*1–3*). One-electron reduction of PQ or DQ results in the formation of the corresponding radicals, which donates an electron to oxygen (*4*). The resulting superoxide undergoes dismutation to H_2O_2. The reaction of H_2O_2 with PQ and DQ radicals forms hydroxyl radicals, which elicit lipid peroxidation and cause oxidative damage to biomolecules in many tissues. Therefore, the cytotoxicity of PQ and DQ is explained in terms of the formation of reactive oxygen species (ROS) in cells.

Exposure to high levels of these compounds causes lung, liver, kidney, and brain injury. These injuries depend upon the accumulation of these compounds in cells and the relative activities of the enzymes that catalyze the one-electron reduction of PQ and DQ to those enzymes that scavenge ROS (*5–11*). Several lines of evidence have shown that PQ penetrates the rat brain (*13*), and that neurotoxicity occurs after PQ ingestion (*14–18*). The one-electron reduction of quinones, PQ and DQ, with subsequent superoxide formation is assumed to be catalyzed by liver, lung and brain microsomal NADPH-cytochrome P-450 reductase and xanthine oxidase (*19–23*).

A marked organ specificity is found in PQ-induced lipid peroxidation by mouse liver, lung and brain Ms (*10*). In this document, lipid peroxidation in mouse brain Ms in the presence of NADPH and iron is reported to increase with increasing concentrations of PQ, whereas lipid peroxidation in liver Ms decreases with increasing concentrations of PQ. PQ-induced lung damage after PQ uptake has been studied in detail and NADPH-dependent lipid peroxidation in lung Ms is strongly inhibited in the presence of PQ (*9*). The mechanism for PQ- and DQ-dependent lung injury remains uncertain. Low level chemiluminescence (CL) due to ROS in rat liver Ms is induced by the addition of NADPH and PQ, whereas the lipid peroxidation in rat liver Ms is concomitantly abolished (*11*). The question is raised whether PQ and DQ cause or inhibit lipid peroxidation by rat brain Ms. We investigated the PQ and DQ reduction mechanism by rat brain Ms. The chemical species of phospholipid hydroperoxides derived from brain Ms in the presence of NADPH and iron have been determined by CL-HPLC methods. The effects of PQ and DQ on NADPH-dependent lipid peroxidation in rat brain Ms have also been reported

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Abbreviation CLA, *Cypridina* luciferin analog; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide, DQ, diquat, DTPA, diethylenetriamine pentaacetic acid, MK, menadione, PC, phosphatidylcholine, PE, phosphatidylethanolamine, PQ, paraquat, ROS, reactive oxygen species, TBARS, thiobarbituric acid reactive substance.

MATERIALS AND METHODS

Preparation of Rat Brain Microsomes—Male 8-week-old SD rats were delivered from Japan SLC (Hamamatsu). They were fed a normal laboratory diet (as pellets) and water *ad libitum*, and maintained for one week in the laboratory. Rats were anesthetized with 50 mg/kg of pentabarbital, and blood in the brain was cleared by perfusing ice-cold saline into the carotid artery. The brain tissues were quickly removed and frozen in liquid nitrogen, and stored at –80°C until use. The microsomal fraction was obtained from fifty brain tissue specimens per preparation by the method reported previously (*24*). The rat brains were homogenized in 4 volumes of ice-cold 100 mM Tris-HCl buffer, pH 7.4, using a glass homogenizer with a Teflon pestle (three strokes at 1,000 rev/min). The homogenates were centrifuged at 1,000 ×*g* for 15 min, and then the supernatant was centrifuged at 12,000 ×*g* for 15 min. The microsomal fraction was precipitated by ultracentrifugation of the postmitochondrial supernatant at 105,000 ×*g* for 60 min. The microsomes were washed by resuspending in 100 mM Tris-HCl buffer, pH 7.4, and subsequently ultracentrifuged. Protein concentrations were determined by the method of Lowry *et al.* (*25*) with bovine serum albumin as a standard.

Biochemical Assays—NAD(P)H-PQ and -DQ reductase reactions were measured at 340–400 nm with a Shimadzu UV-300 dual-wavelength spectrophotometer. Under anaerobic conditions, PQ and DQ radicals were detected using a Varian E-109B ESR spectrometer, and were observed optically at 606 and 443 nm, respectively (*1*). Oxygen radical formation was demonstrated using the ESR spin-trap 5,5-dimethy-1-pyrroline *N*-oxide (DMPO), and by chemiluminescence of a *Cypridina* luciferin analog (CLA, 2-methyl-6-phenyl-3,7-dihydro-imidazo[1,2-*a*]pyrazin-3-one), with an Aloka luminescence reader BLR-301. Lipid peroxides were measured as the formation of thiobarbituric acid reactive substances (TBARS) (*26*), and the amounts of phospholipid hydroperoxides (PCOOH; phosphatidylcholine hydroperoxide, PEOOH; phosphatidylethanolamine hydroperoxide) were determined by CL-HPLC (*27*). All reactions were carried out in 100 mM Tris-HCl buffer, pH 7.4, at 25°C unless otherwise noted.

RESULTS

The NADPH-oxidase reaction catalyzed by rat brain Ms was enhanced by the addition of PQ and DQ, and increased in the order: DQ > PQ (Fig. 1A). No change in the enhanced NADPH-oxidase reaction was observed when the reactions were carried out in the presence of 10 μM dicumarol, an inhibitor of DT-diaphorase (Fig. 1A). NADPH-menadione (MK) reductase activity was clearly inhibited in the presence of the same concentration of inhibitor (Fig. 1Ac). No increase in the NADH-oxidase reaction in the presence of DQ or PQ was found, nor was an effect of dicumarol upon the NADH-oxidase reaction by rat brain Ms observed (Fig. 1B). These results indicate that the reduction of DQ or PQ is mainly catalyzed by NADPH-cytochrome P-450 reductase and not by DT-diaphorase. PQ or DQ radicals produced by rat brain Ms were observed optically at their λ max, 443 and 606 nm, respectively, after a lag phase, under anaerobic conditions (Fig. 2A). It appears that the length of the lag period depends upon the residual oxygen in the reaction mixtures. No PQ or DQ radicals were seen under aerobic conditions, because the rate constants for the reaction of PQ and DQ radicals with oxygen are very fast ($6–8 \times 10^8$ M^{-1} s^{-1}) (*4, 6*). The kinetics of PQ and DQ radical formation indicate that about 5 μM PQ radicals and 10 μM DQ radicals accumulated over a reaction time of 15 min (Fig. 2A).

Figure 2B shows the ESR spectra of PQ and DQ radicals formed in the NADPH-PQ and -DQ reductase reaction by rat brain Ms under anaerobic conditions, which are identical to those radicals produced by hepatocytes (*23*).

The one-electron reduction of PQ and DQ causes the for-

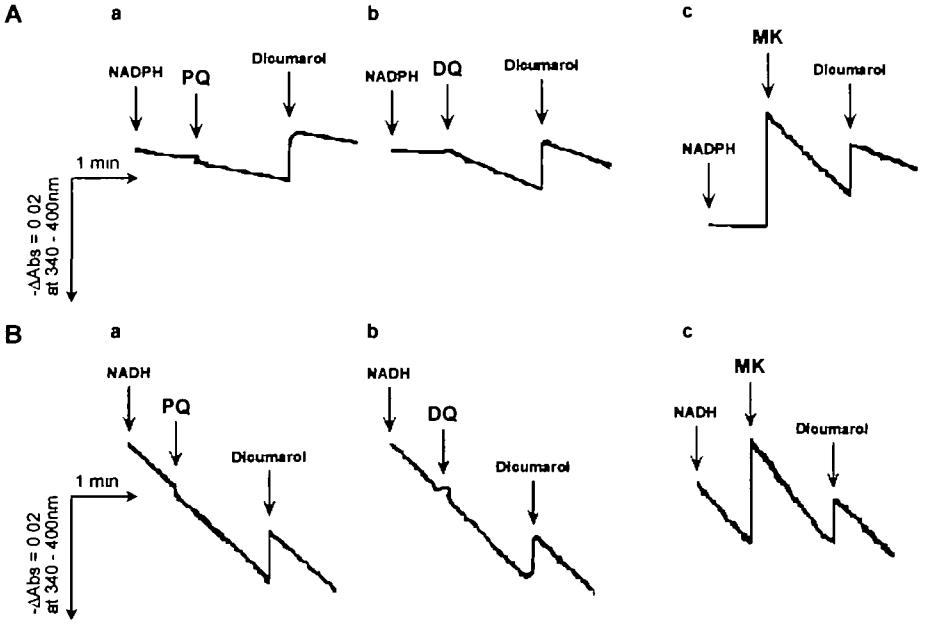

Fig 1 **NAD(P)H-PQ, -DQ, and -MK reductase reactions catalyzed by rat brain microsomes.** Reaction mixtures contained 0.5 mg protein/ml rat brain microsomes, 50 μM PQ (a), DQ (b) or MK (c), and 80 μM NADPH (A) or NADH (B). 10 μM dicumarol was added at the indicated times.

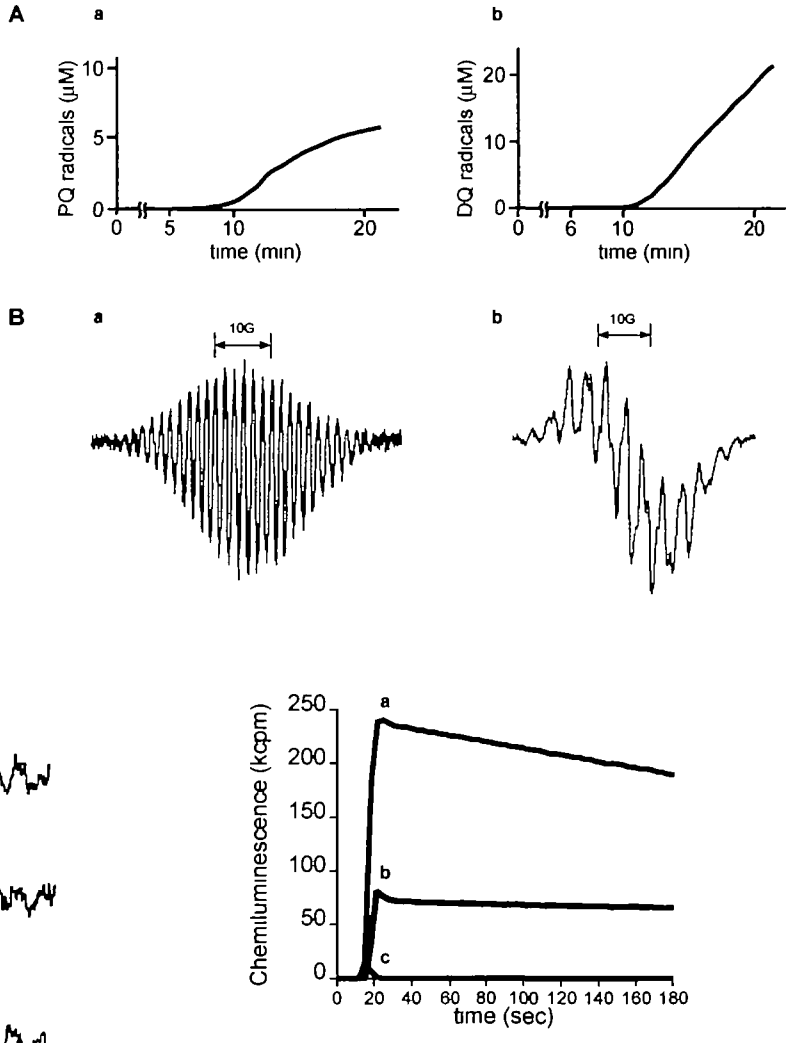

Fig 2. **PQ and DQ radicals produced by rat brain microsomes in the presence of NADPH under anaerobic conditions.** Reaction mixtures contained 0 5 mg protein/ml rat brain microsomes, 1 0 mM PQ (a), or DQ (b), 1 0 mM KCN, and 0 5 mM NADPH A· The reactions were followed at 606 nm for PQ radicals (a) and 443 nm for DQ radicals (b) B ESR spectra of PQ (a) and DQ (b) radicals. The instrumental conditions were center of magnetic field, 3,389 G, modulation amplitude, 1 0 G, receiver gain, 2×10^4, time constant, 0 25 s, power, 20 mW, and scan speed, 6.25 G/min

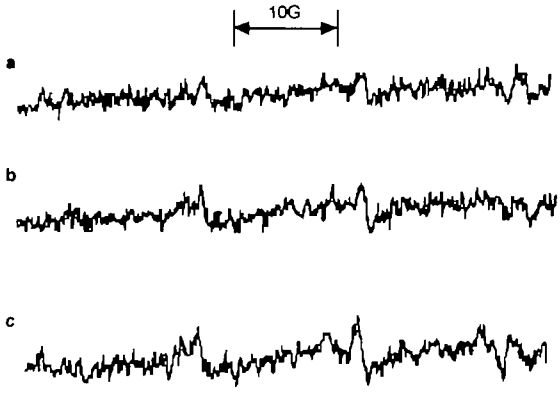

Fig 3 **Oxygen radical formation in NADPH-PQ (a), -DQ (b), and -MK (c) reductase reactions catalyzed by rat brain microsomes.** Reaction mixtures contained 0 5 mg protein/ml rat brain microsomes, 50 μM PQ (a), DQ (b), or MK (c), 1 0 mM NADPH, 0 5 mM DTPA, and 100 mM DMPO The instrumental conditions were center of magnetic field, 3,389 G, modulation amplitude, 1 0 G, receiver gain, 2×10^4, time constant, 0 125 s, power, 20 mW, and scan speed, 25 G/min

Fig 4 **Chemiluminescence of CLA induced in NADPH-PQ (b) and -DQ (a) reductase reactions catalyzed by rat brain microsomes.** Reaction mixtures contained 0 5 mg protein/ml rat brain microsomes, 50 μM PQ (b) or DQ (a), 1 0 mM NADPH and 10 μM CLA in the absence or presence of 0 1 μM SOD (c)

mation of reactive oxygen species (ROS). The formation of oxygen radicals in the NADPH-PQ and -DQ reductase reaction by rat brain Ms was observed under aerobic conditions by ESR spin-trapping. The ESR signals were weak but assigned to a combination of one characteristic of DMPO-OOH and DMPO-OH (Fig. 3) The DMPO-OH signal was increased when the reactions were carried out in the presence of 0.1 μM SOD (data not shown). The results indicate that the hydroxyl radicals were generated through the reaction of H_2O_2 with PQ or DQ radicals. We, therefore, made use of CLA as a CL probe for detecting superoxide. The CL of CLA is also sensitive for detecting superoxide and singlet oxygen (*28*). Kinetic traces for PQ- and DQ-induced CL by rat brain Ms are shown in Fig. 4. It should be noted that no CL of CLA was observed in the absence of PQ or DQ. The DQ-induced CL (Fig. 4a) was three times higher than the PQ-induced CL (Fig. 4b). The CL was abolished when the reactions were carried out in the presence of SOD (Fig. 4c). The difference in the intensity of the steady-state CL induced by PQ and DQ is consistent with the relative activity of NADPH-PQ to -DQ reductase reaction in rat brain Ms (Fig. 1). The effect of SOD indicates that the CL of CLA is mainly caused by the reaction of CLA with superoxide.

It has been suggested that most of the toxic effects of superoxide are due to the generation of hydroxyl radicals. The formation of hydroxyl radicals through the reaction of H_2O_2 with PQ and DQ radicals has been reported (*5, 6*). When rat brain Ms were incubated at 37°C for 50 min in the presence of 1 mM NADPH, the concentration of lipid peroxides determined as TBARS increased from 0.5 to 2.0 nmol/mg protein. However, in the presence of both PQ and NADPH, the formation of lipid peroxides was not enhanced but decreased. We have further observed the effects of PQ

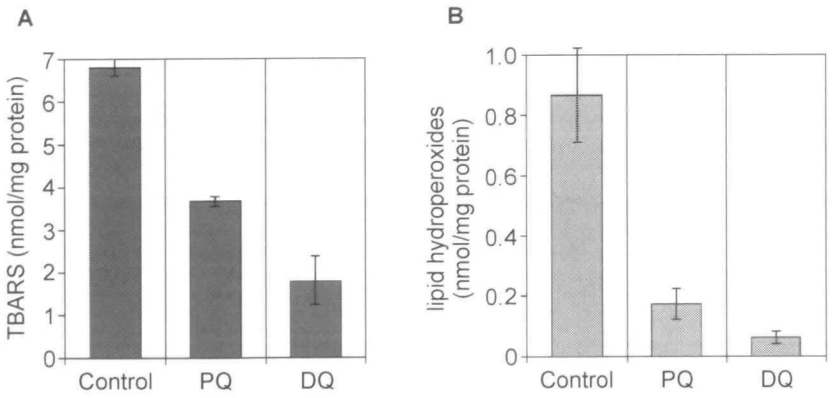

Fig 5 **Effects of PQ and DQ on NADPH-dependent lipid peroxidation in rat brain microsomes.** Reaction mixtures contained 2.0 mg protein/ml rat brain microsomes, 50 μM PQ or DQ, 1.0 mM NADPH, and 10 μM $FeCl_3$ Reaction mixtures were incubated at 37°C for 50 min The concentration of lipid peroxides was determined as TBARS (A) (*26*) and by CL-HPLC (B) (*27*). Each value represents the mean of three experiments. Control indicates the concentration of lipid peroxides formed in the absence of PQ or DQ

and DQ upon iron-catalyzed lipid peroxidation of brain Ms in the presence of NADPH (Fig. 5). Under the same conditions, the formation of TBARS and phospholipid hydroperoxides (PCOOH+PEOOH) determined by CL-HPLC also decreased in the presence of PQ and DQ Furthermore, the decrease in both TBARS and phospholipid hydroperoxides was dependent on the concentration of either PQ or DQ (Fig. 6). The inhibitory effects of DQ on lipid peroxidation were higher than those of PQ, and the concentration causing 50% inhibition was about 10–15 μM for PQ and 0 5–1.0 μM for DQ In Fig. 5, the amount of phospholipid hydroperoxides was about 15% of that estimated as TBARS. This seems reasonable, because CL-HPLC detects only phospholipid hydroperoxides (PCOOH+PEOOH), while TBARS represent aldehydes in addition to phospholipid hydroperoxides.

In the presence of PQ and DQ, the rate of NADPH oxidation and oxygen radical generation was increased In contrast, neither the formation of TBARS nor that of hydroperoxides (PCOOH+PEOOH) was increased. The inhibitory effects of PQ and DQ on NADPH-dependent lipid peroxidation in rat brain Ms were similar to those in rat liver Ms (*11*).

DISCUSSION

The redox mechanism of PQ by rat liver Ms has been reported (*1, 2, 11, 23*). NADPH-P450 reductase, present in rat liver, kidney, lung, and brain Ms, mainly catalyzes the one-electron reduction of PQ and DQ in the presence of NADPH, leading to the formation of PQ and DQ radicals The redox properties of PQ and DQ radicals have been documented in detail (*4–6*). PQ and DQ radicals rapidly donate an electron to oxygen, because the redox potential of the PQ/PQ radical couple is low enough to produce superoxide (*5, 6*). Figure 1 shows that NADPH-PQ, -DQ, and -MK reductase activities were observed in brain Ms. The NADPH-P450 reductase activity in rat brain Ms as estimated from the NADPH-$K_3Fe(CN)_6$ reductase and cytochrome *c* redutase reactions by rat brain Ms is 10–12% of that in liver Ms (data not shown). MK serves as a good electron accepter for DT-diaphorase, which, because it catalyzes two-electron reductions of quinones, is classified as an antioxidant enzyme. NADPH-MK reductase activity in rat brain Ms was clearly inhibited in the presence of dicumarol, whereas no change in the NADPH-PQ reductase activity was observed in the presence of inhibitor (Fig. 1). The effects of dicumarol indicate that the NADPH-PQ and -DQ reductase reactions are mainly catalyzed by NADPH-P450 reductase and not by DT-diaphorase in rat brain Ms.

PQ-induced lipid peroxidation in brain Ms in the presence of NADPH is a matter of debate. It has been reported that PQ enhances lipid peroxidation in mouse brain Ms in the presence of both NADPH and iron (*10*). The biochemical mechanism by which this combination causes lipid peroxidation and leads to cell death has been suggested to be similar to the syndrome of Parkinson's disease (*17, 18*). Parkinson's disease involves progressive cell death in the substantia nigra, which contains relatively high concentrations of iron. PQ has been shown to mobilize bound iron in ferritin through its reductive reaction with PQ radicals (*29, 30*). Released ferrous iron initiates a Fenton-type Haber-Weiss reaction. On the contrary, the present results show that PQ and DQ strongly inhibit the formation of TBARS and phospholipid hydroperoxides. TBARS formation in liver Ms in the presence of NADPH depends upon the experimental conditions, because hemoproteins and transition metals contaminating the Ms preparation catalyze the oxidation of membrane lipids. We used perfused rat brains to remove contaminating iron. We have shown that oxygen radical formation is stimulated by the addition of PQ and DQ to a reaction mixture containing rat brain Ms and NADPH The reaction of PQ radicals with H_2O_2, which leads to the formation of hydroxyl radicals, has been reported (*5, 6*). The resulting hydroxyl radicals react with membrane lipids to initiate lipid peroxidation. We have shown that the formation of TBARS in the presence of NADPH and iron is decreased by increasing concentrations of PQ, DQ (Figs. 5 and 6) and MK (data not shown). Quinones such as MK behave as radical quenchers. Figure 5 shows that PCOOH and PEOOH derived from rat brain Ms are generated during the incubation of brain Ms with NADPH and iron. It has been shown that ferrous iron should be formed through the reaction of ferric iron with reduced P450, superoxide and PQ radicals, which initiates a Fenton-type Haber-Weiss reaction (*29–31*). A marked difference was observed in the NADPH-PQ reductase activity between rat brain and rat liver Ms, which is ascribed to the relative activity of NADPH-P450 reductase present in Ms. The levels of NADPH-P450 reductase and P450 in rat brain Ms are one-tenth those in rat liver Ms (*18*). The present results are consistent with the observation that PQ-induced oxygen radical formation is not related to the lipid peroxidation of rat liver Ms (*11*). However, the mecha-

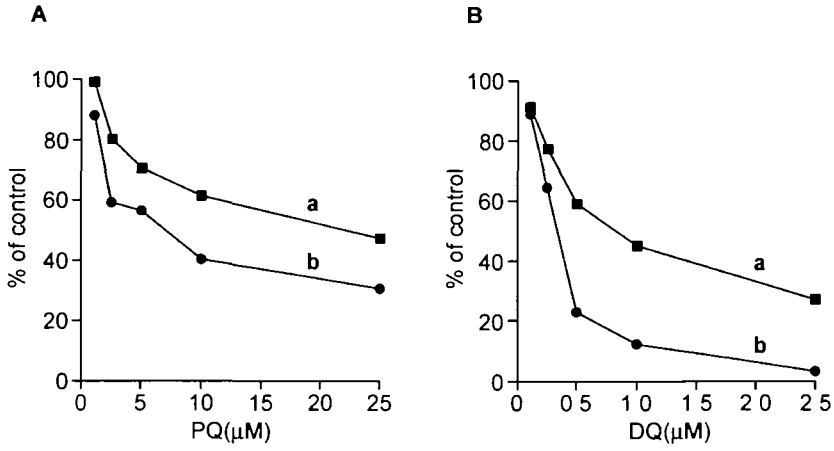

Fig 6 **Effects of PQ and DQ concentration on NADPH-dependent lipid peroxidation in rat brain microsomes.** Reaction mixtures contained 2 0 mg protein/ml rat brain microsomes, various concentrations of PQ (A) or DQ (B), 1 0 mM NADPH, and 10 μM $FeCl_3$ Reaction mixtures were incubated for 50 min. The concentration of lipid peroxides was determined as TBARS (a) (*26*) and by CL-HPLC (b) (*27*) 100% denotes the concentation of lipid peroxides formed in the absence of PQ or DQ

nism by which PQ and DQ inhibit the iron-induced lipid peroxidation of rat liver and brain Ms remains to be determined. We can rule out the possibility that PQ- and DQ-induced lipid peroxidation of the membrane is the major mechanism for the rat brain toxicity of PQ and DQ.

PQ has been used as a herbicide, and there is a strong correlation between the incidence of Parkinson's disease and the level of PQ used (*15, 16*). Because of the similarity in the structure of PQ and 1-methyl-4-phenyl pyridinium (MPP^+), PQ, and MPP^+ probably undergo the same biological activation by NADPH-dependent enzymes (*32–34*). Although PQ is reduced by NADPH-P450 reductase, NO synthase and xanthine oxidase, no reduction of MPP^+ by these enzymes has been reported (*33*). It has been confirmed that the redox potential of the MPP^+/MPP couple is below –500 mV so that the enzymatic reduction of MPP^+ is implausible. It has been shown that MPP^+ inhibits mitochondrial electron flow and causes a decrease in membrane potential (*34, 35*) The uptake of PQ by hepatocytes causes a decrease in the GSH level due to the formation of H_2O_2 and superoxides (*7, 8*). These results together with the present results indicate that the mechanism of PQ toxicity to neurons is different from that of MPP^+ toxicity.

Several reports have suggested that the steady state level of active oxygen regulates the cellular activity (*36–38*). When cells are exposed to low levels of superoxides, their growth is stimulated. Low concentrations of H_2O_2 also stimulate cell growth, whereas a high concentration of H_2O_2 depresses growth and elicits apoptosis. It has been confirmed that the uptake of PQ causes lung, liver, kidney and brain injury. Several investigators have provided evidence that PQ causes the uncoupling of the neuronal NO synthase reaction, which results in the formation of superoxides through its diaphorase activity (*39, 40*). The activation of PQ by brain Ms includes the redox cycling of PQ under aerobic conditions. The resulting superoxides are rapidly dismuted into H_2O_2 by SOD. H_2O_2 is scavenged by GSH-peroxidase at the expense of GSH. Therefore, active oxygen intermediates (H_2O_2 and superoxide) may indirectly regulate the activity of cells through the reaction of GSSG with proteins that regulate the cellular redox state. Alternatively, active oxygen itself may regulate cells through its direct reaction with regulatory proteins (*38*).

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