The Journal of Neuroscience, June 1992, 12(6): 2362–2369

Interaction of the Putative Essential Nutrient Pyrroloquinoline Quinone with the *N*-Methyl-D-Aspartate Receptor Redox Modulatory Site

Elias Aizenman,[1] Karen A. Hartnett,[1] Can Zhong,[2] Paul M. Gallop,[3] and Paul A. Rosenberg[2]

[1]Department of Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, [2]Department of Neurology, Children's Hospital and Harvard Medical School, and [3]Laboratory of Human Biochemistry, Children's Hospital, Department of Biological Chemistry, Harvard Medical School, and Department of Oral Biology, Harvard School of Dental Medicine, Boston, Massachusetts 02115

The putative essential nutrient pyrroloquinoline quinone (PQQ) can efficiently mediate reduction and oxidation reactions in a variety of systems. Therefore, we investigated whether this compound could alter the function of the NMDA receptor via a recently described redox modulatory site. In rat cortical neurons *in vitro*, 50 μM PQQ could reverse the enhancement of 30 μM NMDA-induced whole-cell ionic currents produced by the reducing agent dithiothreitol (DTT; 2–4 mM). PQQ also depressed native responses in a DTT-reversible fashion. In addition, 50–200 μM PQQ produced a significant degree of neuroprotection in an acute model of NMDA-mediated neurotoxicity in astrocyte-rich cultures of rat cerebral cortex. Under certain conditions, PQQ can lead to the formation of oxygen-derived free radicals, and we have previously observed that these reactive species can oxidize the NMDA receptor. Nevertheless, the enzymatic free radical scavengers superoxide dismutase and catalase (10 μg/ml each) did not abolish the actions of PQQ. This observation held true even in astrocyte-poor cortical cultures, where neuronal processes are directly exposed to the extracellular milieu. Therefore, under *in vitro* conditions in which PQQ is presented without an exogenous electron donor, it appears as if the entire neuroprotective effect of PQQ is attributable to a direct oxidation of the NMDA receptor redox site. These results suggest the possibility of a novel role for PQQ, PQQ-like substances, and quinone-containing proteins in the brain, and may represent a novel therapeutic approach for the amelioration of NMDA receptor–mediated neurotoxic injury.

Pyrroloquinoline quinone (PQQ, methoxatin), 4,5-dihydro-4,5-dioxo-1-*H*-pyrrolo[2,3-f]quinone-2,7,9-tricarboxylic acid (Salisbury et al., 1979), is an anionic, water-soluble substance that can efficiently transfer electrons between a variety of reductants and oxidants (Gallop et al., 1989; Paz et al., in press). In mammals, PQQ has been suggested to serve as a soluble redox cofactor that can mediate oxidation and reduction of a variety of compounds, and lead to the formation as well as removal of superoxide (Gallop et al., 1989, 1990; Paz et al., in press). PQQ appears to be a normal constituent in animal tissues and fluids, including those in humans (Flueckiger et al., in press; Paz et al., in press). Recently, PQQ was shown to be an essential nutrient in rodents since mice fed a diet deficient in this substance had friable skin, grew poorly, became osteolathyritic, and failed to reproduce (Killgore et al., 1989; Smidt et al., 1991). These signs of PQQ deprivation could be reversed upon supplementing the diet with PQQ. In addition, other investigators have found that administration of PQQ may prevent oxidative stress injury involved in cataract formation (Nishigori et al., 1989), liver injury (Watanabe et al., 1988), and lipid peroxidation (Hamagishi et al., 1990).

We have previously shown that oxygen free radicals can effectively oxidize putative sulfhydryl residues on the NMDA-specific glutamatergic receptor (Aizenman et al., 1990). These sulfhydryl residues likely constitute a functional modulatory site on this receptor that is sensitive to redox reagents, hence termed the redox modulatory site (Aizenman et al., 1989). Thus, sulfhydryl-reducing agents such as dithiothreitol (DTT) have been observed to enhance NMDA-mediated responses dramatically in a variety of preparations, while oxidizing agents can diminish these responses. In this investigation, we have evaluated the oxidizing properties of PQQ on the function of NMDA receptors in rat cortical neurons in tissue culture.

Some of these results have been published in abstract form (Aizenman et al., 1991; Rosenberg et al., 1991).

Materials and Methods

Tissue culture. Cortices were obtained from embryonic day 16 C-D rats and were dissociated according to previously described methods to produce either astrocyte-rich (Dichter, 1978; Rosenberg, 1988) or astrocyte-poor (Rosenberg, 1991) neuronal cultures. Briefly, cortices were incubated in Earle's salt solution containing 0.03% trypsin for 2 hr at 37°C and then in calcium- and magnesium-free Earle's salt solution for an additional 20 min at 37°C; cell viability was assessed by trypan blue exclusion. The plating suspension was adjusted to 300,000–500,000 cells/ml with growth medium [v/v mixture of 80% Dulbecco's modified Eagle's minimum essential medium (DMEM), 10% Ham's F-12 nutrient mixture, 10% heat-inactivated iron-supplemented calf serum, 25 mM HEPES, 24 U/ml penicillin, 24 μg/ml streptomycin, 2 mM glutamine]. The dissociated cells were plated onto 35 mm plastic dishes containing five 12 mm cover glasses each. The cover glasses had been previously

Received Oct. 25, 1991; revised Jan. 10, 1992; accepted Jan. 17, 1992.

We thank Dr. Ian J. Reynolds for critically reviewing the manuscript. This work was supported by NIH grants to E.A. (NS29365 and DA04975), P.M.G. (DE08649), and P.A.R. (NS26830); a grant from the Joseph P. Kennedy Jr. Foundation to P.A.R.; and a Mental Retardation Core Grant to the Children's Hospital, Boston.

Correspondence should be addressed to E. Aizenman, Department of Physiology, E1456-BST, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

coated with either poly-L-lysine (astrocyte poor) or collagen/poly-L-lysine (astrocyte rich). Astrocyte-poor cultures were produced by exposure to 5 μM cytosine arabinoside for 48 hr, beginning at 4 d *in vitro*. After inhibition, medium was replaced with DMEM/minimum essential medium (MEM)/F12 (4:5:1), with the N2 supplements of Bottenstein and Sato (Bottenstein, 1983), catalase at 1 μg/ml (Walicke et al., 1986), plus 1% serum. Glutamine was added to a final concentration (exclusive of serum) of 2 mM. Medium was not subsequently replaced. These cultures typically survived 4–6 weeks *in vitro*. To help prevent evaporation, the 35 mm dishes were placed on pads of water-soaked filter paper in 60 mm Petri dishes. Astrocyte-poor cultures were utilized for toxicity experiments after 3–5 weeks in culture. The number of neurons in these cultures was approximately 70% of the total number of cells (Rosenberg, 1991). To produce astrocyte-rich cultures, non-neuronal cell proliferation was inhibited with 2 μM cytosine arabinoside 2 weeks after dissociation, and the growth medium was replaced three times per week. Neurons comprised 5–10% of the total number of cells in these cultures (Rosenberg, 1991). Astrocyte-rich cultures were used for electrophysiological recordings and toxicity experiments 1 week after inhibition. The ultrastructure of astrocyte-rich and astrocyte-poor cultures has been examined (Harris et al., 1989). Neuronal processes and synapses in astrocyte-rich cultures are almost entirely buried beneath a layer of astrocytes and astrocytic processes. Only the neuronal somata appear to be in direct contact with the extracellular medium. In astrocyte-poor cultures, neuronal processes, synapses, and soma are in direct contact with the medium.

Recording conditions. Electrophysiological measurements were performed at room temperature (25°C) using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). The resistances of the patch electrodes when filled with internal solution and placed in the recording bath were measured to be 3–5 MΩ. Current signals were amplified using an Axopatch 1-C patch-clamp amplifier (Axon Instruments), filtered with an 80 dB/decade low-pass Bessel filter (0.5–1 kHz), and digitized (1–2.5 kHz) with a computer interface system (TL-1-125 DMA Labmaster, Scientific Solutions). Data were collected and analyzed using commercially available software (pCLAMP, Axon Instruments). The reference electrode was a Ag–AgCl wire connected to the extracellular solution by a 2 M KCl, 1% agarose bridge.

The extracellular solution for the physiological measurements was based on Hanks' salts and contained (in mM) NaCl, 137; $NaHCO_3$, 1; $NaHCO_4$, 0.34; KCl, 5.36; KH_2PO_4, 0.44; $CaCl_2$, 2.5; HEPES, 5; dextrose, 22.2; glucose, 10; and phenol red, 0.011 gm/liter; pH adjusted to 7.2 with 0.3 *N* NaOH. Half micromolar tetrodotoxin and 10 μM (−)-bicuculline methiodide were added to reduce spontaneous synaptic activity. Glycine (1 μM) was added to mostly saturate (>90%; Kleckner and Dingledine, 1988) the strychnine-insensitive glycine recognition site on the NMDA receptor (Johnson and Ascher, 1987). During the recording period, cells were continuously superfused at a rate of 0.5 ml/min with the extracellular solution. NMDA was applied to the cells under study by pressure ejection (PicoPump, World Precision Instruments) from micropipettes placed in close proximity (20–30 μm) to the cell. The micropipette was made by carefully breaking the tip of conventional intracellular microelectrodes to a size of approximately 10 μm. The pulse of air pressure (10–20 psi) applied to the pipette was controlled via the computer. This microperfusion system ensured relatively fast local application and withdrawal of drugs. Dithiothreitol (DTT; Calbiochem), 5,5′-dithio-bis-(2-nitro-benzoic acid) (DTNB; Calbiochem), superoxide dismutase (SOD; Fluka), catalase (Sigma), and pyrroloquinoline quinone (PQQ; Fluka) were applied via the superfusate. *N*-methyl-D-aspartate (NMDA) was obtained from Sigma, (−)-bicuculline methiodide from Research Biochemicals, and tetrodotoxin from Calbiochem.

Toxicity assays. Assessment of cell survival was performed by counting surviving neurons utilizing trypan blue exclusion (Pixley and Cotman, 1985; Rosenberg and Aizenman, 1989). Experiments were usually designed according to the following scheme. We utilized cortical cultures plated on the 12 mm coverslips (five coverslips per dish). On the day of the experiments, each coverslip was transferred to a chamber of a 24 well tissue culture plate that contained MEM (no phenol red). Thus, up to five different conditions could be tested on neurons from a single culture dish. A typical experiment utilized coverslips from three separate dishes in order to perform an experiment in triplicate with up to five different treatments. Removal of a drug or other treatment was achieved by gentle serial dilution (200:1, equivalent to three complete washes) to minimize trauma to the cells. The media used for rinsing in astrocyte-poor cultures contained 250 μM 2-amino-5-phosphonovalerate (APV) and 0.01% BSA since these cultures are destroyed by multiple media changes, and can be protected from this effect by APV (P. A. Rosenberg, unpublished observations). If a particular treatment involved more than one exposure (i.e., drug A, followed by drug B), then all groups received the same number of media changes. Toxicity was assessed 18–24 hr following exposure. For the trypan blue method, the cells were rinsed once with physiological buffer containing 0.01% BSA and incubated with 0.2% (w/v) trypan blue in buffer for 2 min. Cells were then rinsed three times and fixed with 2.5% (v/v) glutaraldehyde in buffer. Cell counts were performed under phase-contrast optics with an inverted microscope at 200× magnification across the diameter of each coverslip with the use of a grid-containing eyepiece (approximately 10% of the total surface of the coverslip). Cell counts were performed by a person blinded to the arrangement of treatment groups.

Detection of free radical generation. The production of free radicals by DTT and PQQ was measured using a nitroblue tetrazolium (NBT) assay (Gallop et al., 1989; Aizenman et al., 1990; Paz et al., 1991). To a solution of NBT (1 mg/5 ml Earle's salt solution without phenol red), increasing quantities of DTT and PQQ (from a stock solution of 2 mM DTT and 50 μM PQQ) were added. The reduction of NBT to form formazan was detected spectrophotometrically at 492 nm.

Results

Electrophysiological effects of PQQ

Whole-cell recordings were obtained from a total of 17 cells. We initially measured the effects of PQQ on NMDA-mediated whole-cell currents in cortical neurons with the use of patch electrodes. A 2 min incubation of the cell under study with 50 μM PQQ could significantly decrease the native NMDA (30 μM) response in a DTT (2–4 mM)-reversible fashion (Fig. 1). In addition, 50 μM PQQ (2 min) could completely reverse any potentiating actions that 2–4 mM DTT (2 min) had on NMDA-mediated responses (see inset in Fig. 1). In all these experiments ($n = 7$), NMDA-elicited responses were obtained after DTT or PQQ had been rinsed off the cells. PQQ (50 μM) on its own did not produce any changes in membrane current noise, nor did it affect NMDA responses already diminished by a 2 min incubation with a 500 μM solution of the sulfhydryl-oxidizing agent DTNB (not shown; Aizenman et al., 1989; Reynolds et al., 1990). Furthermore, as PQQ-diminished responses were also observed to be unaffected by 500 μM DTNB, both these oxidizing substances are likely acting at the same site with equal efficacy. Finally, the PQQ-modified response remained sensitive to NMDA antagonists such as CGS-19755 (100 μM), confirming our earlier observations that the overall pharmacological properties of the NMDA receptor remain unaltered following reduction or oxidation (Aizenman et al., 1989).

PQQ-mediated oxidation may occur via the generation of oxygen free radicals (Gallop et al., 1989, 1990; Paz et al., in press). Since we have observed that these reactive oxygen species are effective at oxidizing the NMDA receptor redox site and decrease its function (Aizenman et al., 1990), we performed some recordings in the presence of the enzymatic free radical scavengers SOD and catalase ($n = 6$). Inclusion of these enzymes in the bathing solution throughout the experiment at a concentration of 20 μg/ml each did not prevent the oxidizing actions of PQQ (Fig. 2). Similar results were observed even when the cultures were pretreated with SOD and catalase up to 3 hr in order to allow for possible differences in site access by the enzymes versus PQQ. Thus, 30 μM NMDA responses after 50 μM PQQ treatment in the absence of SOD and catalase were 41.9 ± 10.4% ($n = 8$) of the 2–4 mM DTT-potentiated responses. Similarly, responses obtained after PQQ treatment in the presence of the free radical scavengers were 45.3 ± 14.4% ($n = 6$)

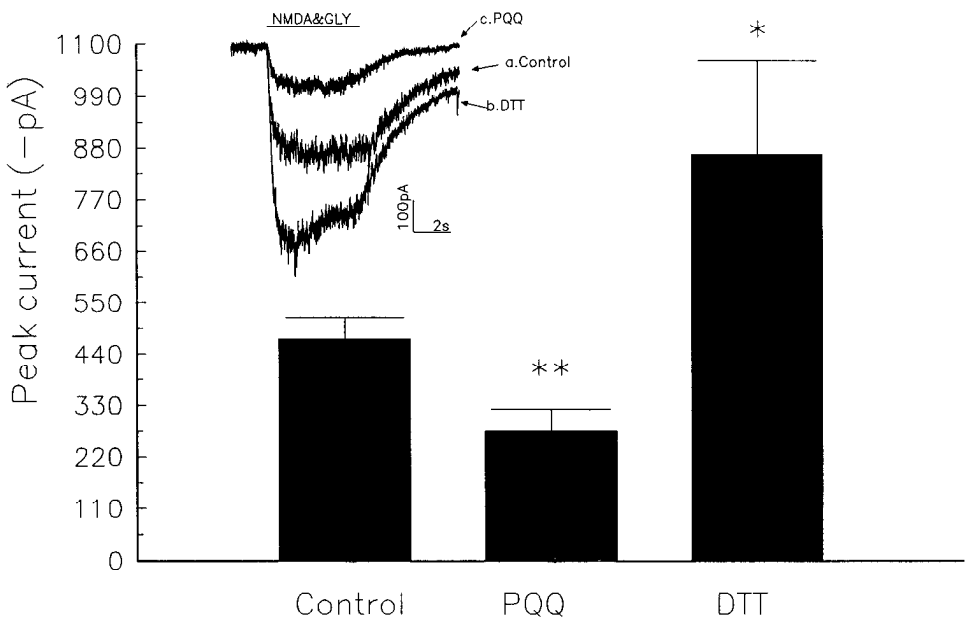

Figure 1. Effects of PQQ and DTT on NMDA+glycine-evoked whole-cell currents in cortical neurons: mean peak response (±SEM; $n = 7$) to 30 μM NMDA plus 1 μM glycine obtained by whole-cell voltage-clamp (−60 mV) recordings of neurons either under control conditions or after a 2 min incubation with either PQQ (50 μM) or DTT (2–4 mM). Responses after either DTT or PQQ treatments were obtained after these drugs were washed out. For some cells, the DTT treatment preceded the PQQ treatment (*inset, a–c,* represents the temporal order of the responses), while for other cells the treatments were reversed; however, no differences were found between the two protocols so that these data were pooled. A one-way ANOVA revealed significant differences between treatment groups ($F = 6.4$; $p = 0.008$). Post hoc tests between the groups with a Bonferroni correction for multiple comparisons revealed significant differences between the control and the PQQ groups (**, $p < 0.001$) and between the PQQ and DTT groups (*, $p < 0.01$).

of the DTT-potentiated responses. These two values did not differ significantly (Student's *t* test). These results suggest that the action of PQQ on the NMDA-induced currents was not mediated via the formation of oxygen free radical species.

Neuroprotective properties of PQQ in astrocyte-rich cultures

Stimulation of NMDA receptors in central neurons can lead to delayed cell death in *in vitro* model systems (Rothman et al., 1987; Choi et al., 1988). In addition, NMDA receptor–mediated neurotoxicity has been linked to a variety of CNS disorders (Meldrum and Garthwaite, 1990). Therefore, we were interested in testing whether oxidation of the NMDA receptor by PQQ could afford protection against NMDA neurotoxicity in our culture system. Since PQQ, as mentioned above, can lead to formation of oxygen-derived free radicals that are highly toxic to neurons (Walicke et al., 1986; Rosenberg, 1988), it was also important to establish whether prolonged exposures to PQQ-containing solutions could also injure cells.

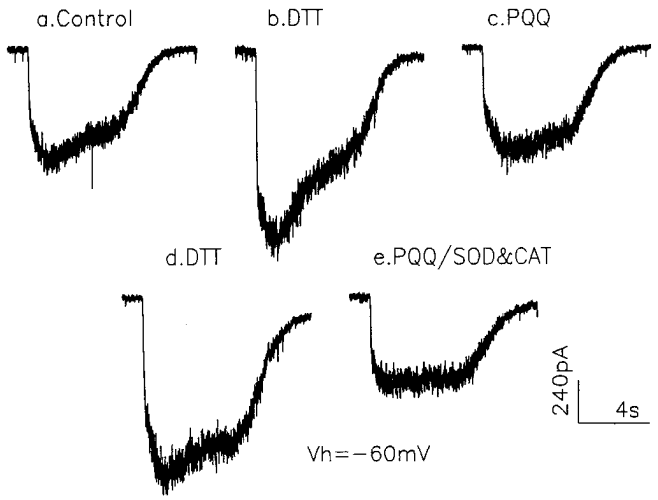

Figure 2. Effects of enzymatic free radical scavengers on the oxidizing actions of PQQ at the NMDA receptor: sequential whole-cell responses to 30 μM NMDA plus 1 μM glycine in a cortical neuron in culture under control conditions (*a*), or after incubation with 2 mM DTT (*b*), 50 μM PQQ (*c*), 2 mM DTT (*d*), and 50 μM PQQ in combination with 20 μg/ml SOD and 20 μg/ml catalase (*CAT*) (*e*). The cell was voltage clamped at −60 mV. NMDA responses were obtained after treatment washout. Note that presence of free radical scavengers does not prevent PQQ from reversing the DTT effect. Similar results were obtained in an additional five cells.

As we have previously reported (Aizenman et al., 1990), DTT can significantly enhance the toxicity produced by NMDA in astrocyte-rich cultures of rat cerebral cortex. In the present investigation, we observed that 50 μM PQQ was effective in reversing the potentiating effects that 500 μM DTT had on the delayed toxicity produced by a short (5 min) exposure to 50 μM NMDA (Fig. 3*A*; $n = 6$). PQQ added prior to DTT had no neuroprotective actions. It is noteworthy that extensive washing was performed between the PQQ and DTT treatments to prevent interaction between these two substances. In five out of six additional experiments, 200 μM PQQ was sufficient to produce a significant blockade of the lethal effects that were produced by a 30 min exposure to 200 μM NMDA alone (Fig. 3*B*). Neither a 30 min nor an overnight exposure to PQQ produced any neurotoxicity, and in fact there was a tendency for an increased number of viable neurons in the overnight-treated cultures when compared to controls (Fig. 3*B*, fifth bar).

In a separate set of experiments, we tested whether SOD alone (10 μg/ml; $n = 3$) or SOD and catalase together (10 μg/ml each; $n = 4$) could prevent PQQ from reversing the DTT-induced enhancement of NMDA toxicity. Once again, these experiments were aimed at establishing whether free radical production could account for the actions of PQQ. We observed that treatment with the free radical scavengers could not abolish the saving effects of PQQ (data not shown). This is in direct contrast with our previous observation that the oxidizing effect of the free radical–generating system of xanthine and xanthine oxidase on

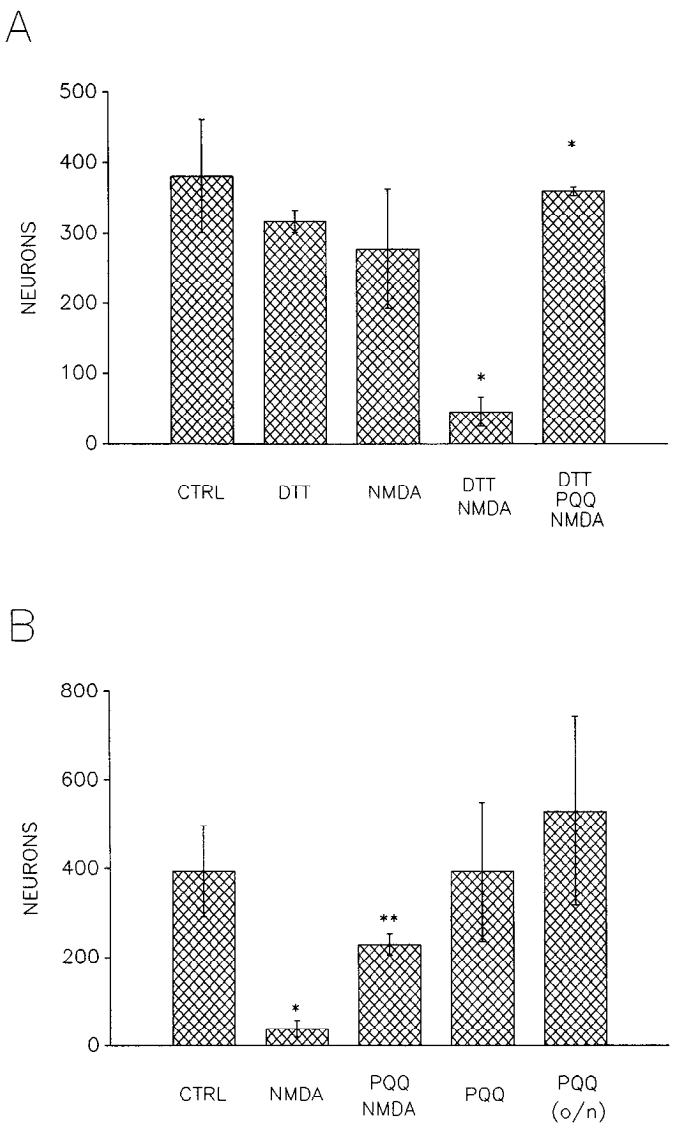

Figure 3. Neuroprotective actions of PQQ in astrocyte-rich cultures of cerebral cortex. *A,* Assessment of neuronal survival in sister coverslip cultures following a variety of treatments. *CTRL,* vehicle alone; *DTT,* 5 min exposure to 500 μM DTT; *NMDA,* 5 min exposure to 50 μM NMDA; *DTT/NMDA,* 5 min exposure to 500 μM DTT, followed by a 5 min exposure to 50 μM NMDA; *DTT/PQQ/NMDA,* 5 min exposure to 500 μM DTT, followed by a 5 min exposure to 50 μM PQQ, and finally followed by a 5 min treatment to 50 μM PQQ and 50 μM NMDA. Values shown are means ± SD for a single experiment performed in triplicate. A one-way ANOVA revealed significant differences between treatment groups ($F = 18.9$; $p = 0.0001$). Post hoc tests among selected groups with a Bonferroni correction for multiple comparisons revealed a statistical difference between the *CTRL* group and the *DTT/NMDA* group (*, $p < 0.01$), and between the *DTT/NMDA* and the *DTT/PQQ/NMDA* group (*, $p < 0.01$). Similar results were obtained for a total of six experiments. *B,* Assessment of neuronal survival in sister coverslip cultures after the following treatments: *CTRL,* vehicle alone; *NMDA,* a 30 min exposure to 200 μM NMDA; *PQQ/NMDA,* a 30 min exposure to 200 μM PQQ followed by a 30 min exposure to 200 μM PQQ and 200 μM NMDA; *PQQ,* a 30 min exposure to 200 μM PQQ; *PQQ(o/n),* an overnight exposure to 200 μM PQQ. Values are means ± SD for a single experiment performed in triplicate. A one-way ANOVA revealed significant differences between treatment groups ($F = 6.2$; $p = 0.01$). Post hoc tests among selected groups with a Bonferroni correction for multiple comparisons revealed a significant difference between the *CTRL* group and the *NMDA* group (*, $p < 0.01$), and between the *NMDA* and the *NMDA/PQQ* groups (**, $p < 0.001$). Similar results were obtained in five out of six experiments, with one experiment failing to show a significant saving by PQQ.

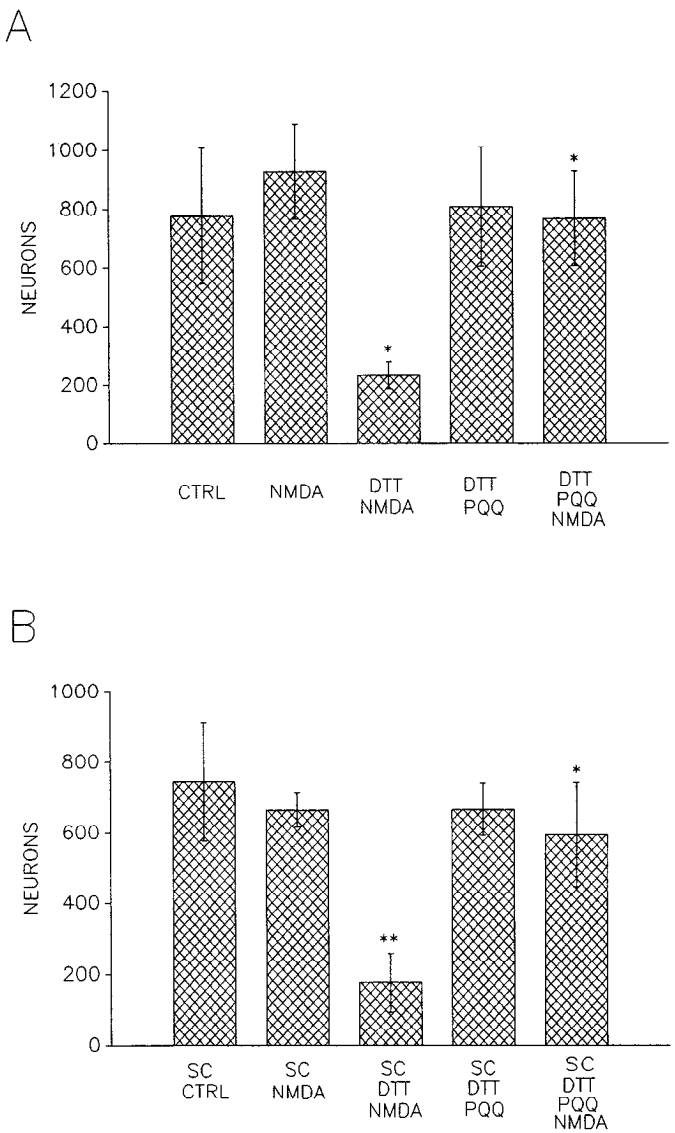

Figure 4. Neuroprotective actions of PQQ in astrocyte-poor cultures of cerebral cortex. *A,* Assessment of neuronal survival in sister coverslip cultures following a variety of treatments. Astrocyte-poor cultures were placed in wells and exposed to medium with or without 2 mM DTT for 10 min. Medium was then completely replaced with medium containing 250 μM APV, and then exposed to medium with or without 50 μM PQQ for an additional 10 min. Medium was then replaced with medium containing 0.01% BSA with or without 75 μM NMDA (30 min). Medium was then replaced with APV-containing medium, and toxicity was assessed 22 hr later. Values shown are means ± SD for a single experiment performed in triplicate. A one-way ANOVA revealed significant differences between treatment groups ($F = 7.4$; $p = 0.005$). Post hoc tests among selected groups with a Bonferroni correction for multiple comparisons revealed a statistical difference between the *CTRL* group and the *DTT/NMDA* group (*, $p < 0.05$), and between the *DTT/NMDA* and the *DTT/PQQ/NMDA* groups (*, $p < 0.05$). Similar results were obtained for a total of four experiments. *B,* Assessment of neuronal survival in sister coverslip cultures following similar treatments as described above except that SOD and catalase (*SC*; 10 μg/ml) were included in all the groups. Values shown are means ± SD for a single experiment performed in triplicate. A one-way ANOVA revealed significant differences between treatment groups ($F = 11.9$; $p = 0.001$). Post hoc tests with a Bonferroni correction for multiple comparisons revealed a statistical difference between the *CTRL* group and the *DTT/NMDA* group (**, $p < 0.01$), and between the *DTT/NMDA* and the *DTT/PQQ/NMDA* group (*, $p < 0.05$). Similar results were obtained for a total of four experiments.

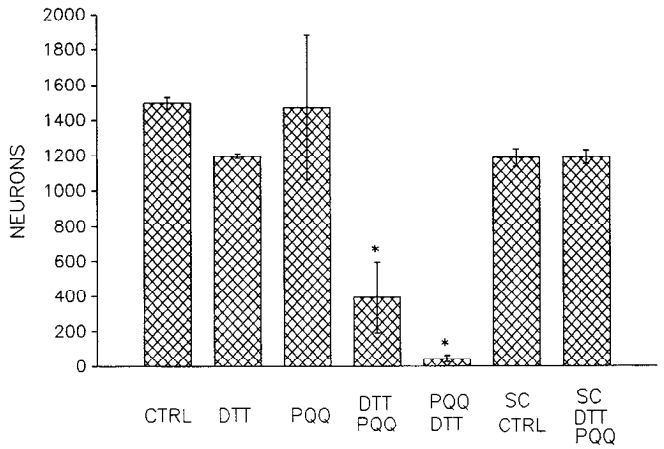

Figure 5. Incomplete rinsing reveals a toxic component to PQQ/DTT treatments: assessment of neuronal survival in sister coverslips following various treatments. Astrocyte-poor cultures were placed in medium with or without 2 mM DTT or 50 μM PQQ for 10 min. Media was then removed and replaced with either control medium or medium containing either PQQ or DTT (10 min). This incomplete rinse left approximately 3% of the original concentration of either PQQ or DTT. One set of coverslips were exposed to PQQ after the DTT treatment in the presence of SOD and catalase (*SC*; 10 μg/ml each). Toxicity was assessed 25 hr later. Values shown are means ± SD for a single experiment performed in triplicate. A one-way ANOVA revealed significant differences between treatment groups ($F = 22.1$; $p < 0.0001$). Post hoc tests among selected groups with a Bonferroni correction for multiple comparisons revealed a statistical difference between the *DTT/PQQ, PQQ/DTT* groups and the *SC/DTT/PQQ* group (*, $p < 0.05$). Similar results were obtained for a total of three experiments.

the NMDA receptor redox site was significantly inhibited by SOD and catalase (Aizenman et al., 1989). As we have observed before (Aizenman et al., 1990; see also Fig. 4*B*), SOD and catalase by themselves could not reverse the actions of DTT.

Neuroprotective properties of PQQ in astrocyte-poor cultures

To exclude the possibility that the lack of effect of SOD and catalase was an access problem due to astrocytes "shielding" neuronal processes and synapses from the enzymes (Rosenberg and Aizenman, 1989; Harris et al., 1989), a series of neurotoxicity experiments were performed on astrocyte-poor cultures of rat cerebral cortex (Rosenberg, 1991). As in the astrocyte-rich cultures, DTT (2 mM) significantly enhanced toxicity produced by 75 μM NMDA, and this enhancement could be totally prevented by 50 μM PQQ either in the absence (Fig. 4*A*) or presence (Fig. 4*B*) of SOD (10 μg/ml) and catalase (10 μg/ml). As mentioned earlier, SOD and catalase did not reverse the effects of DTT on NMDA toxicity (Fig. 4*B*, third bar). Very little toxicity of 75 μM NMDA alone was noted in these experiments, as the rinse media contained 250 μM APV, which was necessary to protect neurons in these cultures from the lethal effects of media changes. However, this amount of APV was not sufficient to overcome the enhanced NMDA toxicity produced by DTT. In contrast, other studies (Aizenman and Hartnett, in press) have revealed that 100 μM CGS-19755, another competitive NMDA antagonist (Murphy et al., 1988), can prevent these actions of DTT.

Toxicity of DTT plus PQQ

In all of the aforementioned experiments, we performed a complete rinse between the DTT and PQQ additions to avoid interaction between these two compounds. We noted, however, that when washes were not interposed between the PQQ and the DTT treatments (an incomplete rinse leaving approximately 3% of the original concentration of either PQQ or DTT), most neurons in the astrocyte-poor cultures were lost after subsequent incubation for 24 hr (Figs. 5, 6). Neither 2 mM DTT nor 50 μM PQQ by themselves were toxic to astrocyte-poor cultures (Fig. 5). SOD and catalase (10 μg/ml each) protected against this toxicity of DTT plus PQQ (Figs. 5, 6). This toxicity was not observed when thorough washes were interposed between treatments (Fig. 6*E*). Interestingly, the lethal effects of the combination of PQQ and DTT were not present in astrocyte-rich cultures.

The toxicity of sequential addition of DTT and PQQ, or PQQ and DTT, seemed likely to be due to small amounts of the two compounds being present together due to the incomplete rinsing. We thus performed a series of experiments where neurons were exposed to serial dilutions of 2 mM DTT and 5 μM PQQ added together (Fig. 7). Considerable toxicity was observed at a 1:30 dilution of this mixture (final concentrations: 67 μM DTT, 167 nM PQQ); all neurons were dead at a 1:10 dilution (final concentrations: 200 μM DTT, 500 nM PQQ). These results suggested that PQQ, in the presence of DTT, was capable of redox cycling, leading to the amplified production of free radicals (Gallop et al., 1989). This possibility was addressed by measuring the production of free radicals by DTT and PQQ with an NBT assay (Gallop et al., 1989; Aizenman et al., 1990; Paz et al., 1991). Neither PQQ alone nor DTT up to 200 μM significantly reduced NBT during a 30 min exposure. In contrast, DTT plus PQQ rapidly reduced NBT. As it can be readily seen in Figure 8, free radicals were in fact generated by increasing concentrations of the DTT/PQQ mixture, indicating that DTT serves as an electron donor to PQQ, which then subsequently passes electrons to either oxygen or NBT in a redox cycling process. This amplified production of free radicals is the most likely basis for the toxicity of DTT plus PQQ demonstrated in Figure 7, which was shown to be specifically mediated by oxygen-derived free radicals due to the protection afforded by SOD and catalase.

Discussion

Our results show that PQQ can interact with the redox modulatory site on the NMDA receptor in cortical neurons. PQQ can oxidize this site and decrease NMDA-induced whole-cell currents. The consequences of this interaction are reversible by DTT and are sufficient to afford neuroprotection in an *in vitro* model of NMDA toxicity. In addition, PQQ appears to oxidize the NMDA receptor redox site by a process not mediated directly by oxygen-derived free radicals, given our results that show that SOD and catalase do not prevent the effects of PQQ. This observation held true even in astrocyte-poor cultures where the neuronal processes are directly exposed to the culture medium and access to the receptor by the enzymatic free radical scavengers is presumably not impaired (Harris et al., 1989). Although PQQ can participate in reactions generating oxygen-derived free radicals (Gallop et al., 1989), formation of these species does not appear necessary for NMDA receptor oxidation. We were able to detect, however, the formation of free radicals when DTT and PQQ were present together. Moreover, oxygen-derived free radicals formed by the DTT/PQQ reaction were toxic to neurons in astrocyte-poor but not in astrocyte-rich cultures. Thus, the neurons in astrocyte-rich cultures may be protected by astrocytic shielding, or by endogenous free rad-

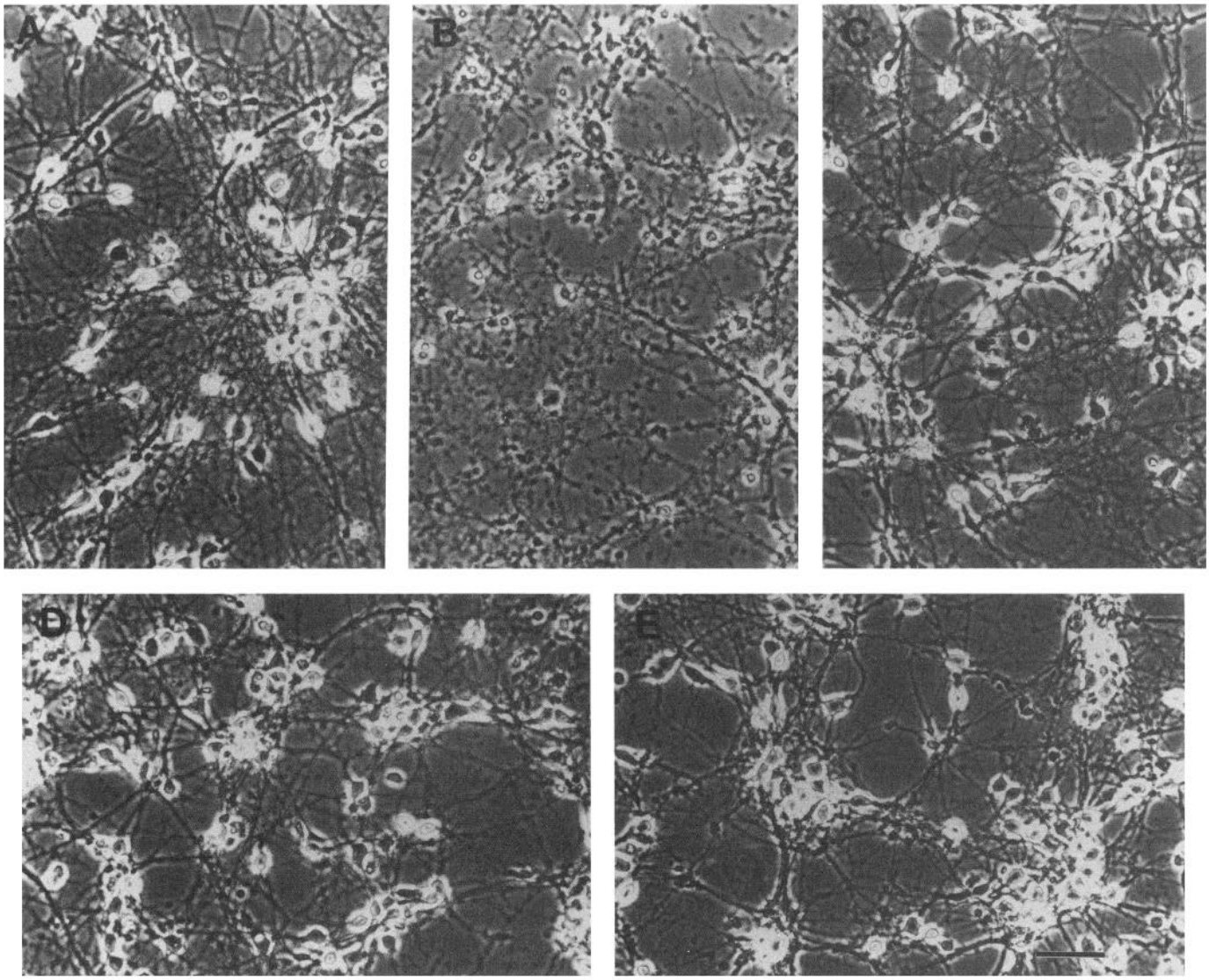

Figure 6. Delayed neurotoxicity after sequential additions of DTT and PQQ: phase-contrast micrographs of sister coverslips containing astrocyte-poor cultures exposed to similar treatments as described in Figure 5. *A*, control culture; *B*, culture exposed to 2 mM DTT (10 min) followed by incomplete rinse and 50 μM PQQ (10 min), after 22 hr; *C*, culture exposed to 2 mM DTT (10 min) followed by incomplete rinse and 50 μM PQQ (10 min), after 30 additional minutes; *D*, culture exposed to 2 mM DTT (10 min) followed by incomplete rinse and 50 μM PQQ in the presence of SOD and catalase (10 μg/ml; 10 min), after 22 hr; *E*, culture exposed to 2 mM DTT (10 min) followed by three complete washes and 50 μM PQQ (10 min), after 22 hr.

ical scavengers present in this culture system, or both. The fact that free radicals may be generated by PQQ in the presence of an adequate electron donor suggests that in some instances a second mechanism might be involved in PQQ-mediated neu-

→

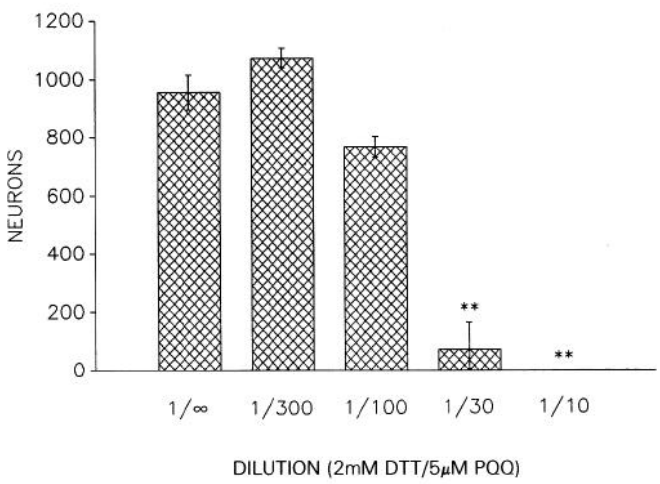

Figure 7. Toxicity of PQQ and DTT added together. Assessment of neuronal viability in sister coverslips of astrocyte-poor cultures after various treatments. Astrocyte-poor cultures were exposed to medium containing 250 μM APV plus 0.01% BSA, with decreasing dilutions of 2 mM DTT plus 5 μM PQQ. Toxicity was assessed 25 hr later. Values shown are the mean ± SEM for a single experiment performed in triplicate. A one-way ANOVA revealed significant differences between treatment groups ($F = 170.1$; $p < 0.0001$). Post hoc tests among selected groups with a Bonferroni correction for multiple comparisons revealed significant killing at 1:30 and 1:10 dilutions (**, $p < 0.01$). Similar results were obtained for a total of two experiments.

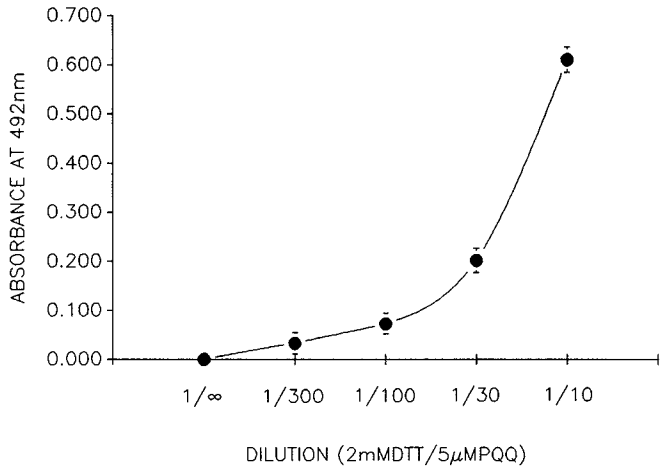

Figure 8. Formation of formazan by DTT/PQQ. To a solution of NBT (1 mg/5 ml), increasing quantities of DTT and PQQ were added from a stock solution of 2 mM DTT plus 5 μM PQQ. Solutions were incubated in the dark for 30 min at room temperature. Formation of formazan was detected by measuring the absorbance of the solutions at 492 nm. Values are the mean ± SD of four measurements.

roprotection; we have previously observed that nonlethal concentrations of oxygen-derived free radicals can be neuroprotective by oxidizing the NMDA receptor redox site (Aizenman et al., 1990). This mechanism might be of particular importance in the brain, where various electron donors are known to be present in the extracellular fluid, for example, glutathione and ascorbate. Nevertheless, under the *in vitro* conditions used for the present experiments in which PQQ is used without an exogenous electron donor, it appears as if the entire neuroprotective effect of PQQ is attributable to a direct oxidation of the NMDA redox site.

PQQ has been reported to be widely distributed in animals, plants, and microorganisms (Ameyama et al., 1985; Smidt et al., 1991). Since there are indications that PQQ is not synthesized in mammalian tissue but has an important nutritional role as an essential nutrient (Killgore et al., 1989; Smidt et al., 1991), transport routes are likely to exist in mammals for this substance to reach its sites of action in the brain, and we are currently investigating this issue. Pathological activation of NMDA receptors has been implicated in various CNS disorders (Meldrum and Garthwaite, 1990). Therefore, it is possible that PQQ supplementation may be useful in their treatment. These results lead us to propose a novel class of therapeutic agents based on a PQQ prototype that act by decreasing NMDA receptor function via its redox modulatory site. In fact, a recent report of a neuroprotective action by the catalytic quinone drug idebenone may in fact be explainable by an action on the redox site of the NMDA receptor (Koizumi et al., 1990).

The evidence that PQQ may be an essential nutrient also raises interesting possibilities regarding the normal function of PQQ in the brain, and the possibility that PQQ might itself play a role in an endogenous system modulating the redox site on the NMDA receptor. Such a system would be expected to have an important role in the normal physiology of processes that make use of the NMDA receptor, such as long-term potentiation. In addition, PQQ deficiency might be associated with loss of central neurons by an NMDA receptor–mediated mechanism. Such a deficiency might occur due to inadequate exogenous supply or transport from the gut, inadequate endogenous biosynthesis, if it exists, or impairment of transport of this anionic quinone across the blood–brain barrier.

References

Aizenman E, Hartnett KA (in press) The action of CGS-19755 on the redox enhancement of NMDA toxicity in rat cortical neurons *in vitro*. Brain Res, in press.

Aizenman E, Lipton SA, Loring RE (1989) Selective modulation of NMDA responses by reduction and oxidation. Neuron 2:1257–1263.

Aizenman E, Hartnett KA, Reynolds IJ (1990) Oxygen free radicals regulate NMDA receptor function via a redox modulatory site. Neuron 5:841–846.

Aizenman E, Hartnett KA, Zhong C, Gallop P, Rosenberg PA (1991) Oxidation of the NMDA receptor redox modulatory site by pyrroloquinoline quinone (PQQ). Soc Neurosci Abstr 17:791.

Ameyama M, Shinagawa E, Matsushita K, Adachi O (1985) Growth stimulating activity for microorganisms in naturally occurring substances and partial characterization of the substance for the activity as pyrroloquinoline quinone. Agric Biol Chem 49:699–709.

Bottenstein JE (1983) Defined media for dissociated neural cultures. In: Current methods in cellular neurobiology, Vol IV, Model systems (Barker JL, McKelvy JF, eds), pp 107–130. New York: Wiley.

Choi DW, Koh J-Y, Peters S (1988) Pharmacology of glutamate neurotoxicity in cortical cell culture: attenuation by NMDA antagonists. J Neurosci 8:185–196.

Dichter MA (1978) Rat cortical neurons in cell culture: culture methods, cell morphology, electrophysiology and synapse formation. Brain Res 149:279–293.

Flueckiger R, Paz MA, Bergethon PR, Henson E, Gallop PM (in press) Glycine-dependent redox cycling and other methods for PQQ and quinoprotein detection. In: The principles and applications of quinoproteins (Davidson VL, ed), in press. New York: Dekker.

Gallop PM, Paz MA, Flueckiger R, Kagan HM (1989) PQQ, the elusive coenzyme. Trends Biochem Sci 14:343–346.

Gallop PM, Paz MA, Flueckiger R (1990) Biological significance of quinonoid compounds in, on, and out of proteins. Chemtracts-Biochem Mol Biol 1:357–374.

Hamagishi Y, Murata S, Kamei H, Oki T, Adachi O, Ameyama M (1990) New biological properties of pyrroloquinoline quinone and its related compounds: inhibition of chemiluminescence, lipid peroxidation and rat paw edema. J Pharmacol Exp Ther 255:980–985.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391:85–101.

Harris KM, Rulf D, Rosenberg PA (1989) Ultrastructural relationships between neurons and glia modulating glutamate toxicity in cortical cultures. Soc Neurosci Abstr 15:764.

Johnson JW, Ascher P (1987) Glycine potentiates the NMDA response in cultured brain mouse neurons. Nature 330:529–531.

Killgore J, Smidt C, Duich L, Romero-Chapman N, Tinker D, Reiser K, Melko M, Hyde D, Rucker RB (1989) Nutritional importance of pyrroloquinoline quinone. Science 245:850–852.

Kleckner NW, Dingledine R (1988) Requirement for glycine in activation of NMDA receptors expressed in *Xenopus* oocytes. Science 241:835–837.

Koizumi S, Kataoka W, Shigematsu K, Niwa M, Ueki S (1990) Evaluation of the neuroprotective action of WEB 1881 FV on hypoglycemia/hypoxia-induced neuronal damage using rat striatal slices. Jpn J Pharmacol 53:175–183.

Meldrum B, Garthwaite J (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol Sci 11:379–387.

Murphy DE, Hutchison AJ, Hurt SD, Williams M, Sills MA (1988) Characterization of the binding of [^{3}H]CGS-19755: a novel *N*-methyl-D-aspartate antagonist with nanomolar affinity in rat brain. Br J Pharmacol 95:932–938.

Nishigori H, Yasunaga M, Mizumura M, Lee JW, Iwatsuru M (1989) Preventive effects of pyrroloquinoline quinone on formation of cataract and decline of lenticular and hepatic glutathione of developing chick embryo after glucocorticoid treatment. Life Sci 45:593–598.

Paz MA, Flueckiger R, Boak A, Kagan HM, Gallop PM (1991) Specific detection of quinoproteins by redox cycling staining. J Biol Chem 266:689–692.

Paz MA, Flueckiger R, Gallop P (in press) The biomedical significance of PQQ. In: The principles and applications of quinoproteins (Davidson VL, ed), in press. New York: Dekker.

Pixley SK, Cotman CW (1985) Laminin supports short-term survival of rat septal neurons in low-density, serum-free cultures. J Neurosci Res 15:1–17.

Reynolds IJ, Rush EA, Aizenman E (1990) Reduction of NMDA receptors with dithiothreitol increases [^{3}H]MK 801 binding and NMDA-induced Ca^{2+} fluxes. Br J Pharmacol 101:178–182.

Rosenberg PA (1988) Catecholamine toxicity in cerebral cortex in dissociated cell culture. J Neurosci 8:2887–2894.

Rosenberg PA (1991) Accumulation of extracellular glutamate and neuronal death in astrocyte-poor cortical cultures exposed to glutamine. Glia 4:91–100.

Rosenberg PA, Aizenman E (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. Neurosci Lett 103:162–168.

Rosenberg PA, Zhong C, Gallop P, Aizenman E (1991) The putative essential nutrient PQQ protects against NMDA neurotoxicity in astrocyte-poor cortical cultures. Soc Neurosci Abstr 17:791.

Rothman SM, Thurston JH, Hauhart R (1987) Delayed neurotoxicity of excitatory amino acids *in vitro*. Neuroscience 22:471–480.

Salisbury SA, Forrest HS, Cruse WB, Kennard O (1979) A novel coenzyme from bacterial primary alcohol dehydrogenases. Nature 280:843–844.

Smidt CR, Steinberg FM, Rucker RB (1991) Physiologic importance of pyrroloquinoline quinone. Proc Soc Exp Biol Med 197:19–26.

Walicke P, Varon S, Manthorpe M (1986) Purification of a human red blood cell protein supporting the survival of cultured CNS neurons, and its identification as catalase. J Neurosci 6:114–121.

Watanabe A, Hobara N, Tsuji T (1988) Protective effect of pyrroloquinoline quinone against experimental liver injury in rats. Curr Ther Res Clin Exp 44:896–901.