Free Rad. Res., Vol. 21, No. 2, pp. 85-93
Reprints available directly from the publisher
Photocopying permitted by license only

NEURODEGENERATION PRODUCED BY INTRAHIPPOCAMPAL INJECTION OF PARAQUAT IS REDUCED BY SYSTEMIC ADMINISTRATION OF THE 21-AMINOSTEROID U74389F IN RATS

GIACINTO BAGETTA[1], MICHELANGELO IANNONE[2], IMMACOLATA VECCHIO[2], VINCENZO RISPOLI[3], DOMENICANTONIO ROTIROTI[3] and GIUSEPPE NISTICÓ[2]

[1]Faculty of Pharmacy, Calabria University, 87030 Cosenza (Italy); [2]Chair of Pharmacology and "Mondino-Tor Vergata" Neurobiology Centre, Department of Biology, University of Rome "Tor Vergata", 00133 Rome (Italy); [3]Faculty of Pharmacy, University of Reggio Calabria, Catanzaro (Italy)

(Received February 14th, 1994)

The behavioural, electrocortical (ECoG) and neurodegenerative effects of intrahippocampal injection of paraquat, a well-known free radical producing agent, were studied in rats. Injection of paraquat (100 nmol) into one dorsal hippocampus produced limbic motor and ECoG seizures. These effects were accompanied at 24 h by severe damage to CA1, CA3 and CA4 hippocampal pyramidal neurones and dentate gyrus granule cells. In comparison to the cell number counted in control, untreated, side of the hippocampus, significant ($P < 0.05$) neuronal loss was observed in the CA1 and CA3 pyramidal cell layers of the treated hippocampus. A lower dose of the herbicide (10 nmol) did not produce consistent motor and ECoG effects and in no instance was significant neuronal loss observed. A pretreatment with U74389F [21-4-(2,6-di-l-pyrrodinyl-4-pyridinyl)-1-piperazinyl-pregna-1,4,9(11)triene-3,20-dione monomethansulfonate] (30 mg/kg i.p., 15 min before paraquat) completely protected rats from motor and ECoG epileptogenic effects induced by intrahippocampal paraquat (100 nmol). This dose of U74389F also reduced the hippocampal damage typically produced by paraquat and no significant neuronal loss was reported in the CA1 and CA3 pyramidal cell layers. A lower dose of U74389F (10 mg/kg i.p.) did not afford any protection against the epileptogenic effects produced by paraquat (100 nmol); in these animals hippocampal damage was still evident though neuronal loss did not reach statistical significance. In conclusion, the present data show that systemic administration of U74389F possesses neuroprotective effects against seizures and neurodegeneration typically elicited by intrahippocampal injection of paraquat.

KEY WORDS: Paraquat, seizures, neurodegeneration, 21-aminosteroids, U74389F, free radicals.

INTRODUCTION

Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) is a potent herbicide widely used in agriculture. This herbicide produces a toxic, and often lethal effect on the pulmonary system both in human and experimental animals.[1-5] Liver and kidney damage has also been reported, the latter effect probably being responsible for the persistence of the herbicide in the body.[6-8] Cyclic reduction/reoxidation of the

Author for Correspondence: Prof. Giuseppe Nisticó, Chair of Pharmacology, Department of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Roma, Italy. Tel 39-6/72594875. Fax 39-6/2022720.

herbicide with generation of oxygen free radicals and depletion of cellular NADPH seems to be involved in the toxicity of paraquat;[1,9] free radicals would then react with membrane phospholipids to cause cell damage.[10] A Na-independent uptake mechanism for paraquat has been described in the lung epithelial cells and this may explain the high vulnerability of the pulmonary system to the toxic action of this herbicide.[11]

Evidence exists indicating that paraquat can also cause brain damage in poisoned individuals,[12,13] though studies on the direct neurotoxicity of this herbicide in humans are lacking. Indeed, intrahippocampal injections of nanomolar concentrations of paraquat have been reported to produce motor limbic seizures accompanied by severe damage to the hippocampal formation in rats.[14,15] Biochemical and neuropathological data indicate that 21-aminosteroids are effective neuroprotective agents in several models of neuronal cell death.[16-18] The beneficial effects of aminosteroids have been related to their free radical scavenging action and prevention of lipid peroxidation.[19] The aim of the present study was to ascertain whether a component of this group of scavengers, namely U74389F [21-4-(2,6-di-1-pyrrodinyl-4-pyridinyl)-1-piperazinyl-pregna-1,4,9(11)triene-3,20-dione monomethansulphonate], could protect rats from the epileptogenic and neurodegenerative effects induced by focal injection of paraquat into the hippocampus. In previous experiments it has been shown that epileptogenic effects induced by microinjection of paraquat into the substantia nigra were prevented by giving into the same site Cu-free superoxide dismutase.[20] This result was rationalized in term of chelation of redox-active copper ions by the protein, thus suggesting the involvement of an abnormal synthesis and release of superoxide anions in the neurotoxic mechanism of paraquat. Neuroprotection by U74389F would support the idea that generation of free radicals is involved in the mechanisms of paraquat neurotoxicity.

MATERIALS AND METHODS

Adult male Wistar rats (280–300 g) were housed under controlled temperature (20 ± 2 °C), humidity (65%) and a 12 h light/12 h dark cycle (light on at 8.00 a.m.); food and water were available *ad libitum*. Prior to experiment, the rats were anaesthetized with chloral hydrate (400 mg/kg i.p.) and positioned in a stereotaxic frame (D. Kopf Instruments). A stainless steel guide cannulae (25 gauge) was then implanted unilaterally into one dorsal hippocampus under stereotaxic guidance;[21] rats were allowed one week recovery before experiments were carried out. Electrocortical (ECoG) recordings were obtained as previously described.[14] Intracerebral injections were made with a 5 μl Hamilton syringe connected by a teflon tube to an injection cannula. Paraquat was dissolved in double distilled pyrogen-free H_2O (pH = 6 approximately) and the volume of infusate was 1 μl (rate 1 μl/min). Control injection were obtained by injecting animals into one dorsal hippocampus with an identical volume of the vehicle used to dissolve paraquat; U74389F was dissolved in an aqueous solution containing bovine serum albumin and dimethylsulphoxide (DMSO) 100:1 v/v. Postural and motor changes and convulsive behaviours were recorded under blind conditions during a 3 h period of observation. For histological purpose, 24 h after paraquat administration the animals were anaesthetized and 40 ml of heparinized saline infused through the left ventricle of the heart followed by 200 ml of 4% paraformaldehide in phosphate buffered (pH = 7.0) saline. The brain was removed from the skull and processed for conventional light microscopy.

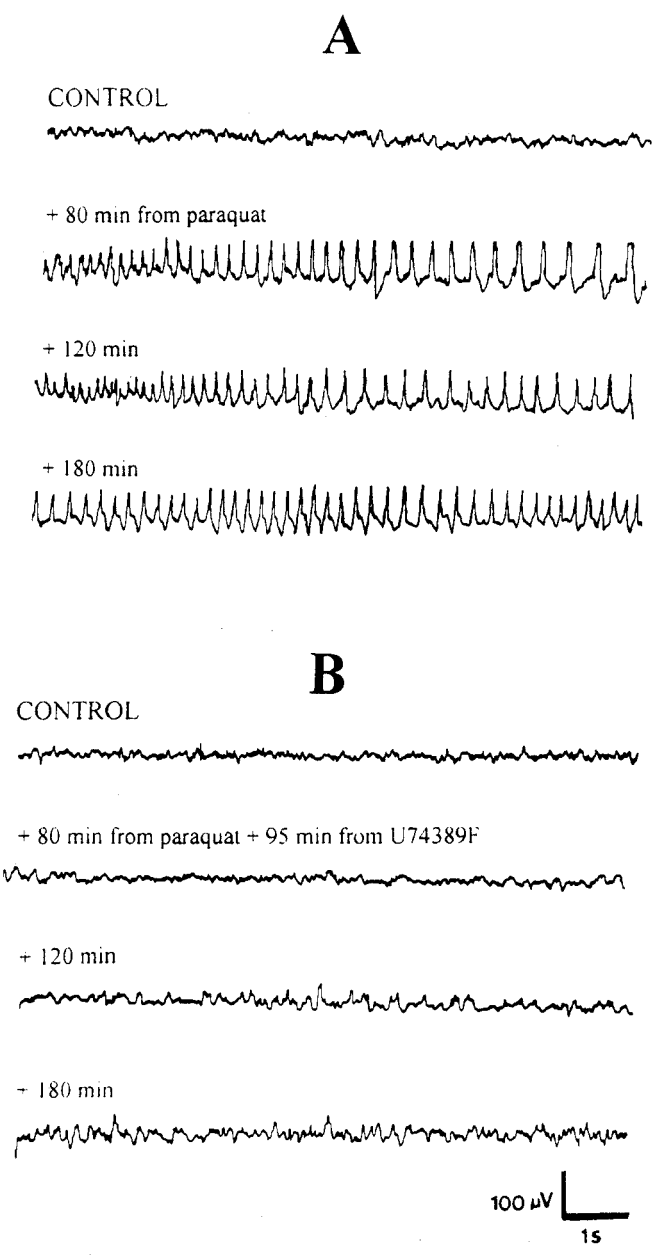

FIGURE 1 Electrocortical records showing epileptic-like, high voltage spikes recorded from a rat brain cortex (A) 80 min after a single dose (100 nmol) of paraquat injected into one dorsal hippocampus. Note that epileptogenic discharges are evident also 180 min after injection, though these were not continuous during 3 h recording session. (B) shows the protection conferred to a different rat from (A) in which the herbicide was injected 15 min after systemic administration of U74389F (30 mg/kg i.p.).

Morphological characteristics of serial brain coronal sections (10 μm) were assessed by using a Leitz Orthoplan microscope. Quantitation of the neuronal loss was performed according to the method developed by Bagetta *et al.*[22]

RESULTS

Intrahippocampal injection of paraquat (100 nmol; 12 rats) produced a pattern of behavioural stimulation consisting of increased exploratory activity, circling contralateral to the site of injection, vibrissal movements, blinking, myoclonic movements of the forelimbs, rearing and eventually falling. This symptomatology started 80 ± 12 min after treatment and was accompanied by ECoG epileptogenic spikes (Figure 1A). Several episodes of motor seizures and ECoG epileptogenic discharges were observed for all of the observation time (3 h), though these were not continuous. At 24 h, histological examination of brain sections ($n = 6$ sections per brain; $n = 6$ rats) revealed severe damage to the hippocampal formation accompanied by neuronal cell loss in the CA1, CA3 and CA4 pyramidal cell layers and dentate gyrus granule cell layer. A typical example of the damage caused to the CA1 pyramidal cell layer of the hippocampus is reported in Figure 2B. The cell loss ranged from 20–30% decrease in the cell number counted in the CA3 and CA4 areas and dentate gyrus, whereas it reached 70–80% decrease in the CA1 area; the cell loss was statistically significant ($P < 0.05$) in the CA1 and CA3 regions of the hippocampus (Table 1). Microinjection of lower doses of paraquat (10 and 50 nmol; $n = 3$ rats per dose) did not produce consistent behavioural and ECoG changes. By contrast, these doses evoked neuronal damage in all of the hippocampal areas examined, though the neuronal loss reached statistical significance only in the CA1 area of rats injected with 50 nmol of paraquat (Table 1). In rats pretreated with U74389F (30 mg/kg i.p.; $n = 6$) the following (15 min after) intrahippocampal injection of paraquat (100 nmol) failed to produce motor convulsion and ECoG epileptogenic discharges (Figure 1B). In addition, no neurodegenerative effects typically observed after injection of this dose of the herbicide were observed (Figure 2C; Table 1). By contrast, a dose of 10 mg/kg i.p. of U74389F ($n = 3$) did not prevent motor and ECoG epileptogenic phoenomena produced by paraquat (100 nmol) whilst it decreased the hippocampal neuronal loss (Table 1). In control experiments systemic administration of U74389F (30 mg/kg i.p.) did not produce *per se* behavioural, ECoG and neuropathological changes (data not shown).

DISCUSSION

The present results are in agreement with previous data demonstrating that intrahippocampal injection of paraquat, a well known generator of free oxygen radicals, produces motor and ECoG seizures accompanied by severe damage to the hippocampal formation.[14,15] In the present study, the damage produced by the herbicide has been quantified and this revealed neuronal loss in all subsectors of the dorsal hippocampus. The most important finding of the present research work is, however, that systemic administration of U74389F, a free radical scavenger, is able to prevent seizures and hippocampal damage. There is ample evidence showing that 21-aminosteroids prevent the neuropathological effects evoked by cerebral ischaemia,[23] a syndrome in which free oxygen radicals are generated during the

TABLE 1
Neuronal loss produced by intrahippocampal injection of paraquat: reversal by systemic administration of U74389F.

TREATMENT	HIPPOCAMPAL AREA							
	CA1		CA3		CA4		DG	
	C	T	C	T	C	T	C	T
Vehicle (1 μl)	53.4±7.2	53.0± 5.6	52.0±1.6	51.7± 2.3	38.6±3.9	37.9±5.1	80.6± 6.5	78.3± 6.2
PARAQUAT 10 nmol	61.4±5.7	41.9± 5.0	51.9±4.7	51.0± 3.7	43.1±5.7	40.4±5.4	75.0± 4.0	62.9± 7.9
PARAQUAT 50 nmol	52.5±4.1	36.4± 3.5*	47.9±4.5	39.9± 0.7	43.3±4.3	37.3±6.0	75.3± 0.8	64.2± 2.0
PARAQUAT 100 nmol	40.6±9.3	9.6± 2.0*	49.4±3.7	31.4± 2.5*	32.7±3.9	26.4±1.1	67.0±12.4	53.3± 7.2
PARAQUAT 100 nmol + U74389F 10 mg/kg	41.6±5.0	27.6±11.3	50.4±5.9	31.6±11.9	43.8±4.9	29.0±9.9	70.3± 7.7	63.7± 9.9
PARAQUAT 100 nmol + U74389F 30 mg/kg	45.6±9.0	32.9± 8.2	40.7±5.3	36.9± 9.0	35.0±5.7	30.3±7.0	62.2±18.0	65.2±15.4

The data represent the mean ± s.e.m. cell number counted in the control (C) and in the contralateral, treated (T) side of the hippocampus of rats receiving injections of paraquat into one CA1 area (T). U74389F was given systemically (10 and 30 mg/kg i.p.) 15 min before paraquat. Control experiments were carried out with intrahippocampal injections of the vehicle used to dissolve paraquat (double distilled pyrogen free H_2O; 1 μl). In each experimental group (3–6 rats per group; $n = 6$ slices per rat brain) the mean cell numbers counted in the treated (T) and control (C) side of the hippocampal areas studied (CA1, CA3 and CA4 pyramidal cell layer and dentate gyrus granule cell layer, DG) were evaluated statistically for differences by using Student's "t" test. *Denotes $P < 0.05$.

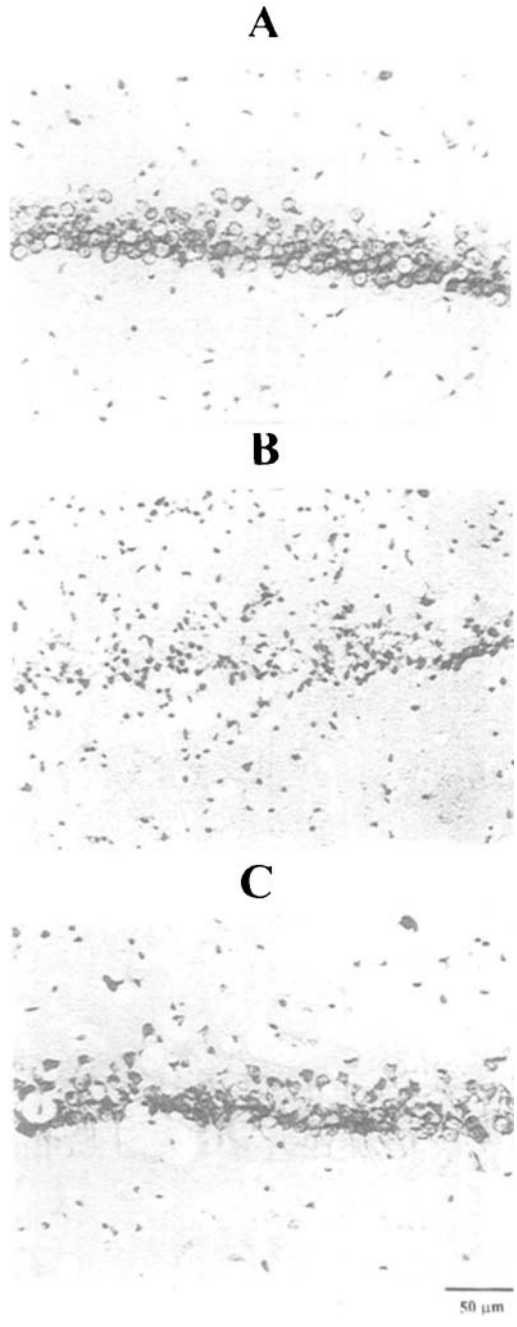

FIGURE 2 Light microphotographs of rat brain coronal sections (10 μm) showing (B) neuronal cell death produced in the CA1 pyramidal layer 24 h after a single injection of paraquat (100 nmol) into one dorsal hippocampus; (C) shows the neuroprotection afforded by systemic administration of U74389F (30 mg/kg i.p., given 15 min before the herbicide). (A) brain section taken from a control rat.

reperfusion period; these radical species are known to play a major role in the mechanisms of ischaemia-induced brain damage.[24-26] During brain ischaemia, the synaptic levels of the excitatory amino acid glutamate increase[27] leading to N-methyl-D-aspartate (NMDA) receptor-gated Ca^{2+} entry into the neurones.[28] The latter event would lead the cell to metabolic stress with consequent excessive production of free oxygen radicals, cytoplasmic membrane lipid peroxidation and neuronal death.[29] Recent reports demonstrate that the NMDA receptor complex comprises a redox sensitive site that, in the presence of free radicals, decreases NMDA receptor-mediated neuronal responses.[30-32] This system would protect neurones from abnormal excitatory receptor stimulation, though it deranges when free radicals are highly concentrated.[33] In line with this mechanism, NMDA receptor antagonists confer neuroprotection when administered before or soon after the excitotoxic stimulus has been delivered,[34-35] thus indicating that they may decrease the production of free radicals but are unable to counteract the detrimental effects of the radicals already formed. Furthermore, free radicals have been reported to cause excitatory amino acid release from hippocampal slices maintained *in vitro*.[36] Interestingly, it has recently been shown that paraquat stimulates lipid peroxidation in mouse brain microsomal preparations at greater extent than in pulmonary microsomes and this has been correlated to the different lipid composition of the two systems.[37] The mechanism through which paraquat produces the neuropathological effects here described remains to be elucidated. However, it can be assumed that this herbicides produces seizures and brain damage via production of free oxygen radicals which in turn would stimulate their own accumulation through the release of the endogenous excitatory transmitter, glutamate. This hypothesis fits well with the "excitotoxic" effects of paraquat and is supported by the neuroprotection afforded by U74389F (this study) and by MK801,[14] a use-dependent NMDA receptor antagonist.[38] Further support to this idea comes from the evidence that the damage caused by intranigral infusion of paraquat in rats can be prevented by injection into the same site of superoxide dismutase,[20] an enzyme which convertes superoxide anions into hydrogen peroxide, subjected to detoxication for intervention of catalase.[39]

In conclusion, the present experiments show that U74389F is able to prevent seizures and brain damage caused by focal injection of paraquat into the dorsal hippocampus in rats. In addition, they suggest that neuronal degeneration caused by intrahippocampal injection of this herbicide is a valid experimental model to study mechanisms of neuronal cell death due to abnormal formation of oxygen free radicals and to evaluate the activity of neuroprotective agents in this model.

Acknowledgements

Partial financial support from the Ministry for University and Scientific Research and Technology (40% fund) and from CNR (Rome) is gratefully acknowledged. Our thanks to Dr. D.C. Zimmermann and E.D. Hall (The Upjohn Company, Kalamazoo, MI) for reading the manuscript and valuable suggestions. We also thank Ms. Alessandra Recchia for valid collaboration and Mr. Giovanni Politi for excellent technical assistance. U74389F was a kind gift from Upjohn Research Laboratoires (Kalamazoo, MI).

References

1. P. Smith and D. Heath (1974) Paraquat lung: a reappraisal. *Thorax*, **29**, 643–653.
2. D.B. Greenberg, K.M. Reiser and J.A. Last (1978) Correlation of biochemical and morphologic manifestation of acute pulmonary fibrosis in rats administered paraquat. *Chest*, **74**, 421–425.

3. A.G. Nerlich, M.L. Nerlich, I. Langer and R.H. Demling (1984) Release of amino-terminal procollagen peptides in paraquat-induced acute pulmonary fibrosis. *Experimental & Molecular Pathology*, **40**, 311–319.
4. C.I. Schoenberger, S.I. Rennard, P.B. Bitterman, Y. Fukuda, V.J. Ferrants and R.G. Crystal (1984) Paraquat-induced pulmonary fibrosis. Role of the alveolitis in modulation the development of fibrosis. *American Review Respiratory Disease*, **129**, 168–173.
5. Y. Fukuda, V.J. Ferrans, C.I. Schoenberger, S.I. Rennars and R.G. Crystal (1985) Patterns of pulmonary structural remodelling after experimental paraquat toxicity. The morphogenesis of intra-alveolar fibrosis. *American Journal of Pathology*, **118**, 452–475.
6. R.E. Murray and J.E. Gibson (1974) Paraquat disposition in rats, guinea pigs and monkeys. *Toxicology Applied & Pharmacology*, **27**, 283–289.
7. S.Z. Cagen, A.F. Janoff, J.S. Bus and J.E. Gibson (1976) Effect of paraquat on liver function in mice. *Journal of Pharmacology and Experimental Therapeutics*, **198**, 222–228.
8. J.E. Gibson and S.Z. Cagen (1977) Paraquat induced functional changes in Kidney and liver. In: *Biochemical Mechanisms of Paraquat Toxicity*. Ed. Autor AP. Academic Press, 1977, London, pp. 117–136.
9. A. Farrington, M. Ebert, E.J. Land and K. Fletcher (1973) Bypiridilium quaternary salts and related compounds. V. Pulse radiolysis studies of the action of paraquat radical with oxygen. Implication for the mode of action of BH. *Biochemical & Biophysical Acta*, **314**, 372–381.
10. G.L. Plaa and H. Witschi (1976) Chemical drugs and lipid peroxidation. *Annual Review of Pharmacology and Toxicology*, **16**, 125–141.
11. M.S. Rose and L.L. Smith (1977) The relevance of paraquat accumulation by tissues. In: Autor AP ed Biochemical mechanisms of paraquat toxicity. London: Academic Press 1977; 71–91.
12. H.C. Grant, P.L. Lantos and P. Parkinson (1980) Cerebral damage in paraquat poisoning. *Histopathology*, **4**, 185–195.
13. J.T. Hughes (1988) Brain damage due to paraquat poisoning: a fatal case with neuropathological examination of the brain. *Neurotoxicology*, **2**, 243–248.
14. G. Bagetta, M.T. Corasaniti, M. Iannone, G. Nisticó and J.D. Stephenson (1992) Production of limbic motor seizures and brain damage by systemic and intracerebral injections of paraquat in rats. *Pharmacology and Toxicology*, **71**, 443–448.
15. M.T. Corasaniti, G. Bagetta, P. Rodinó, S. Gratteri and G. Nisticó (1992) Neurotoxic effects induced by intracerebral and systemic injection of paraquat in rats. *Human and Experimental Toxicology*, **11**, 535–539.
16. E.D. Hall (1988) Effects of the 21-aminosteroid U74006F on post-traumatic spinal cord ischemia. *Journal of Neurosurgery*, **68**, 462–465.
17. E.D. Hall, K.E. Paszara and J.M. Braughler (1988) 21-Aminosteroid lipid peroxidation inhibitor protects against cerebral ischemia in gerbils. *Stroke*, **18**, 997–1002.
18. W. Young, J.C. Wojak and V. De Crescito (1988) 21-Aminosteroid reduces ion schifts and oedema in the rat middle cerebral artery occlusion model of regional ischemia. *Stroke*, **19**, 1013–1019.
19. J.M. Braughler and J.F. Pregenzer (1989) The 21-aminosteroid inhibitors of lipid peroxidation: reactions with lipid peroxyl and phenoxy radicals. *Free Radicals in Biology and Medicine*, **7**, 125–130.
20. M. Iannone, M.R. Ciriolo, G. Rotilio and G. Nisticó (1991) Intra-nigral infusion of Cu-free superoxide dismutase prevents paraquat-induced behavioural stimulation and ECoG epileptogenic discharges in rats. *Neuropharmacology*, **30**, 893–898.
21. G. Paxinos and C. Watson (1982) *The rat brain in stereotaxic coordinates*. Academic Press, New York.
22. G. Bagetta, G. Nisticó and N.G. Bowery (1990) Prevention by the NMDA receptor antagonist MK801 of neuronal loss produced by tetanus toxin in the rat hippocampus. *British Journal of Pharmacology*, **101**, 776–770.
23. E.D. Hall and K.E. Pazara (1989) Effects of novel 21-aminosteroid antioxidants on post-ischemic neuronal degeneration. In: Cerebrovascular Diseases (Ginsberg E.D. Ed.) Raven Press, New York, pp. 387–391.
24. H. Hagberg, A. Lehman, M. Sandberg, B. Nystrom, I. Jacobson and A. Hamberger (1985) Ischemic-induced shifts of inhibitory and excitatory amino acids from intra-to extra-cellular compartments. *Journal of Cerebral Blood Flow and Metabolism*, **5**, 413–419.
25. M.Y.-T. Globus, R. Busto, W.D. Dietrich, E. Martinez, I. Valdez and M.D. Ginsberg (1988) Effect of ischemia on the in vivo release of striatal dopamine, glutamate and GABA studied by intracerebral microdialysis. *Journal of Neurochemistry*, **51**, 1455–1464.
26. W. Cao, J.M. Carney, A. Duchon, R.A Floyd and M. Chevion (1988) Oxygen free radical involvement in ischemia and reperfusion injury to brain. *Neuroscience Letters*, **88**, 233–238.

27. H. Beneviste, J. Prejer, A. Schousboe and N.H. Diemer (1984) Elevation of extracellular concentration of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *Journal of Neurochemistry*, **43**, 1369–1374.
28. D.W. Choi (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, **1**, 623–634.
29. S.W. Werns and B.R. Lucchesi (1990) Free radicals and ischemic tissue injury. *Trends in Pharmacological Sciences*, **11**, 161–166.
30. E. Aizenmann, S.A. Lipton and R.H. Loring (1989) Selective modulation of NMDA responses by reduction and oxidation. *Neuron*, **2**, 1257–1263.
31. D.I. Levy, N.J. Sucher and S.A. Lipton (1990) Redox modulation of NMDA-mediated toxicity in mammalian central neurones. *Neuroscience Letters*, **110**, 291–296.
32. D.L. Tauck and G.A. Ashbeck (1990) Glycine synergistically potentiates the enhancement of LTP induced by a sulfhydryl reducing agent. *Brain Reserach*, **519**, 129–132.
33. E. Aizenmann, K.A. Hartnett and I.J. Reynolds (1990) Oxygen free radicals regulate NMDA receptor function via a redox modulatory site. *Neuron*, **5**, 841–846.
34. R. Gill, A.C. Foster and G.N. Woodruff (1987) Systemic administration of MK801 protects against ischaemia-induced hippocampal neurodegeneration in the gerbil. *Journal of Neuroscience*, **7**, 3343–3349.
35. R.P. Simon, J.H. Swan, T. Griffith and B.S. Meldrum (1984) Blockade of N-methyl-D-aspartate receptors may protect against ischaemic damage to the brain. *Science*, **226**, 850–852.
36. D.E. Pellegrini-Giampietro, G. Cherici, M. Alesiani, V. Carla and F. Moroni (1990) Excitatory amino acid release and free radical formation may cooperate in the genesis of ischemia-induced neuronal damage. *Journal of Neuroscience*, **10**, 1035–1041.
37. S. Hara, T. Endo, F. Kuriiwa and S. Kano (1991) Different effects of paraquat on microsomal lipid peroxidation in mouse brain, lung and liver. *Pharmacology and Toxicology*, **68**, 260–265.
38. E.H.F. Wong, J.A. Kemp, T. Priestly, A.R. Knight, J.N. Woodruff and K.L. Iversen (1986) The anticonvulsant MK801 is a potent N-methyl-D-aspartate antagonist. *Proceedings of the National Academy of Science, USA*, **83**, 7104–7108.
39. G. Rotilio (Ed.) (1986) Superoxide and superoxide dismutase in Chemistry, Biology and Medicine. Amsterdam: Elsevier Publ. Co.

Accepted by Professor J.V. Bannister