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MPP^+ TOXICITY IN *E. COLI* UNDER AEROBIC AND ANAEROBIC CONDITIONS

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MPP^+ and paraquat (PQ^{+2}) are two structurally analogous and highly toxic pyridinium compounds. The mechanism of PQ^{+2} toxicity is best understood in the bacterial model system. While numerous studies, in a variety of systems, have indicated the causative role of free radicals and other oxygen-derived active species in PQ^{+2} toxicity, this question is yet unresolved in the case of MPP^+. In this study we have used the *E. coli* model and have demonstrated that MPP^+ is toxic to bacterial cells in a dose and time dependent modes. Additionally, it is shown that only in the presence of molecular oxygen, bacterial inactivation occurred. The protective effects of the chemical scavenger – mannitol – and of histidine are presented. These results are in complete accord with a free radical mechanism for MPP^+ toxicity.

KEY WORDS: HPTP, free radicals, pyridinium compounds, hydrogen peroxide, E. coli.

ABBREVIATIONS: MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+ – 1-methyl-4-phenyl pyridinium iodide; PQ^{+2} - paraquat, 1,1'-dimethyl-4-4'-bipyridinium dichloride; DETAPAC — diethylenetriamine pentaacetic acid.

INTRODUCTION

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is known as one of the most selective CNS toxins, causing clinical symptoms indistinguishable from Parkinson's disease in man and animal models.[1] It has been shown that the biotransformation of MPTP to MPP^+, which is a necessary step for toxicity to occur, is mediated by monoamine oxidase type B.[2] The mechanism of MPTP-induced parkinsonism is yet not fully understood.

It has been proposed[1,2] that the MPP^+ may produce free radicals which, in turn, may induce selective destruction of dopaminergic neurons in the substantia nigra. This suggestion is supported by the structural similarity between MPP^+ and the paraquat (PQ^{+2}) molecule. The two pyridinium compounds might act in a similar toxic mechanism. It is already well established that paraquat can undergo cyclic oxidation/reduction, and react quickly with oxygen to yield the superoxide radical ($\cdot O_2^-$) and hydrogen peroxide. Consequently, the hydroxyl radical could ultimately be formed and cause the biological damage.[3-5] While PQ^{+2} is known to produce free radicals intermediates and is an active redox cycling agent for the formation of toxic

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oxygen species,[6] very little is known about MPP^+ in this regard. Recent studies have shown that possible inhibition of mitochondrial enzymes or interaction of MPP^+ with neuromelanin are involved in the toxic effect.[1]

It is generally considered that superoxide, hydrogen peroxide as well as a redox active transition metal, either iron or copper, are involved.[3,7-11] Superoxide and hydrogen peroxide could, along with a catalytic amount of transition metal ions, participate in reactions which produce hydroxyl free radicals[3] via the metal-catalyzed Haber-Weiss reactions. This highly reactive and transient oxygen species are normally present in biological systems only at very low levels.[3,12]

Based upon the assumption that the toxic effect of MPP^+ is carried out via oxygen derived free radical formation, we expect that this effect will be reduced in the absence of oxygen.

In the present study, we propose that MPP^+ does exert its toxicity by producing active oxygen species via the above mentioned reactions. This was accomplished by substantiating that under anaerobic conditions, the killing effect of MPP^+ on *E. coli* is markedly reduced. Free radical scavengers, histidine and mannitol, were added to the system prior to the cells' exposure to MPP^+. Each scavanger was shown to have a protecting effect against MPP^+, however, histidine was shown to be more efficient than mannitol in this regard.

MATERIALS AND METHODS

E. coli B (SR-9) was used throughout. The experimental conditions for bacterial growth and treatment with MPP^+ and other compounds were identical to those earlier described.[13] The cells were cultured at 37° C using a shaking incubator (G-24 Environmental incubator shaker, New Brunswick Scientific Corp.) at 200 rpm in a medium which contained KH_2PO_4, 0.7% (Merck), K_2HPO_4, 0.3% (Merck), sodium citrate, 0.5% (AR-Mallinckrodt), $(NH_4)_2SO_4$, 0.1% (Baker analytical reagent) at pH 7.0. $MgSO_4 \cdot 7H_2O$, 1 mg/ml (Merck), was added after sterilization in an autoclave, and glycerol 1% (Frutarom, Israel) was added as carbon source. The washing was carried out by centrifugation (Sorval RC-5) 5 min 0° C using a total volume of 5 ml/wash. The washed cells were suspended in phosphate buffer (1 mM, pH 7.4) containing glucose (0.5% w/v) and $MgSO_4$ (1 mM) to a density of 1×10^9 cells/ml. Inactivation of cells was determined according to the following procedure: The total volume of each system was 1 ml, containing 1×10^7 cells. The following materials were added to the systems at different concentrations and combinations: 1-methyl-4-phenyl pyridinium iodide (MPP^+), histidine (Sigma), hydrogen peroxide (Frutarom, Israel). Samples from the reaction mixture were taken at pre-determined times and were diluted with a phosphate buffer (1 mM, pH 7.4) containing DETAPAC (10 μM) and gelatin (0.5% w/v) (Sigma) in order to stop the reaction. The samples were additionally, diluted in this manner two more times to reach a factor of 10^3–10^6 and were then plated in quadruplicate on agar dishes containing agar (2%), bacto-tryptone (1%) (Difco Laboratories), and sodium chloride (0.5%) (Frutarom). The survival curves were evaluated from colony count after overnight incubation at 37° C. Anaerobic conditions were obtained by bubbling high purity nitrogen (> 99.999%) through the reaction buffer for 20 min before the reaction and continuously flushing over the bacteria suspension during the reaction.

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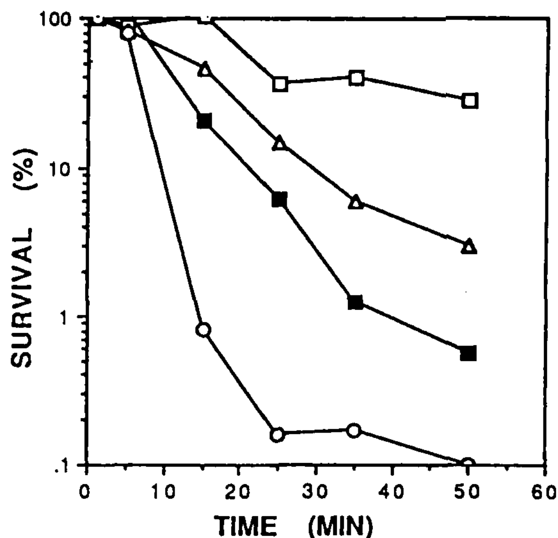

FIGURE 1. Effect of increasing concentrations of MPP^+ on bacterial survival. All the incubation systems contained 1×10^7 *E. coli* B. cells/ml and glucose (0.5 w/v) in phosphate buffer (1.0 mM, pH 7.4): □ (0.4 mM) MPP^+; Δ (0.5 mM) MPP^+; ■ (0.8 mM) MPP^+; O (1.0 mM) MPP^+.

RESULTS

Figure 1 demonstrates the dose-response of the deleterious effects of MPP^+ (0.4–1.0 mM) on the survival curves of *E. coli*. At low concentrations of the pyridinium compound (0–0.3 mM), the slope is rather small ($s \ll 1\%$ min) stemming from only marginal bacterial killing. In contrast, above 0.4 mM the slope changes to a markedly higher value. This phenomenon may suggest that the MPP^+-induced cellular injury is a threshold reaction that only above ~ 0.4 mM becomes important.

Exposure of *E. coli* to MPP^+ at 0–4° C or in the absence or presence of a carbon source (glucose) completely cancels its toxicity. Likewise, exposure at 37° C in the absence of a carbon source gives rise to a marginal inactivation, and the bacterial killing was found to be 22 fold *smaller* when compared to exposure at 37° C in the presence of glucose (not shown). This indicates that the toxicity of MPP^+ is dependent on the metabolism of the organism.

The requirement for oxygen in the injurious process was demonstrated by the markedly reduced rate of cellular killing under nitrogen (Figure 2). For example, under 0.6 and 0.8 mM MPP^+, the inactivation was reduced by 15 and 300 fold respectively, under anaerobiosis. In contrast, exposure of *E. coli* to MPP^+ under 100% oxygen did not alter the rate cellular killing when compared to air (not shown).

In the presence of mannitol (50 mM), a small protective effect (less than 2-fold) was noticed. The survivals following 50 min exposure were 8% and 15% in the absence and presence of this free radical-scavenger, respectively (Figure 3). An even smaller protection was recorded for 10 and 25 mM mannitol (not shown).

Histidine proved a more efficient protector. With 2 mM histidine the survival leaped

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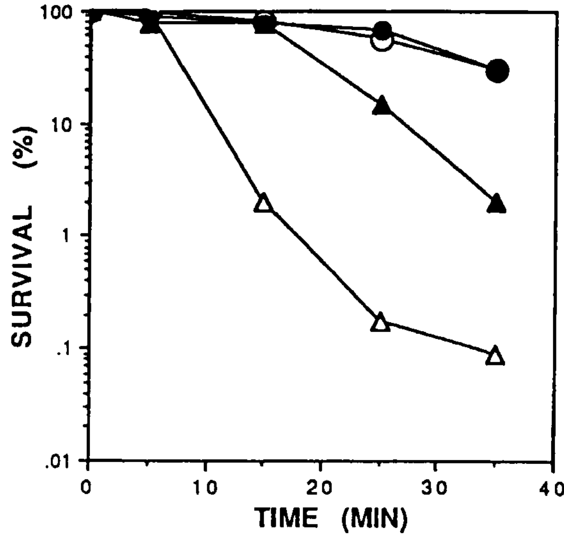

FIGURE 2. Effect of MPP⁺ on bacterial survival under anaerobic versus aerobic conditions. All the incubation systems contained 1×10^7 *E. coli* B cells/ml and glucose (0.5% w/v) in phosphate buffer (1.0 mM, pH 7.4): ● (0.6 mM) MPP^+ in anaerobic conditions; O (0.8 mM) MPP^+ in anaerobic conditions; ▲ (0.6 mM) MPP^+ in aerobic conditions; △ (0.8 mM) MPP^+ in aerobic conditions.

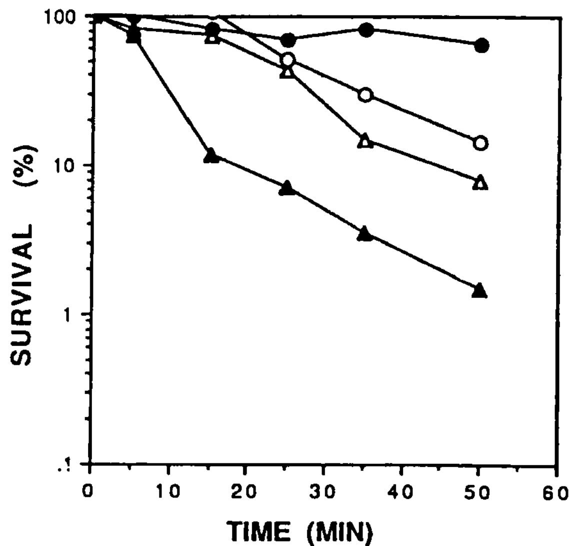

FIGURE 3. Effect of MPP^+ on bacterial survival in the absence and presence of added histidine (2 mM) or mannitol (10 mM). All the incubation systems contained 1×10^7 *E. coli* B cells/ml and glucose (0.5 w/v) in phosphate buffer (1.0 mM, pH 7.4): △ (0.5 mM) MPP^+; ▲ (0.6 mM) MPP^+; O (0.5 mM) MPP^+ and (50 mM) mannitol; ● (0.6 mM) MPP^+ and (2 mM) Histidine.

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from 1.5% (in its absence) to 65% in its presence (Figure 3). The effect of histidine could be attributed to a combination of its characteristics,[14] as an efficient hydroxyl radical scavenger, an efficient chelator for copper, an effective donor of hydrogen atoms resulting in a chemical repair, and as a molecule that forms a tight complex with hydrogen peroxide yielding an apparent lower hydrogen peroxide concentration.

DISCUSSION

The close structural similarity of paraquat (PQ^{+2}) and MPP^+ led to the assumption that their mechanism toxicity could be analogous. PQ^{+2} toxicity has been extensively investigated in a variety of systems including bacterial, mammalian and plants.

In recent years we have clearly demonstrated the essential mediatory role of the transition metals – iron and copper – in catalyzing the free radical-induced damage in PQ^{+2} toxicity in bacterial cells ([8,15]and the Schemes therein). The studies in *E. coli* have stimulated other studies in mammals which were in complete accord with those in *E. coli*. Since then, the results with PQ^{+2} have been reconfirmed in other laboratories, and served as the basis for a recent, successful clinical treatment of two cases of intoxicated humans.[16]

The results of this present study indicate an analogous requirement for MPP^+ and PQ^{+2} toxicities. There is an absolute requirement for oxygen and for a cellular metabolism which is identical to that found for PQ^{+2}. At 20% oxygen there is evidently saturation with oxygen with respect to MPP^+ toxicity so that elevation of oxygen tension to 100% does not further exacerbate the poisonous effect of this pyridinium compound.

The series of steps leading to the toxic effects of MPP^+ that can be postulated involve the single electron reduction of MPP^+ to $MPP^{\cdot}$, which could subsequently react with molecular oxygen to produce superoxide radical. Trials by others,[17] as well as by us, to directly identify $MPP^{\cdot}$ by electron spin resonance (ESR) proved unsuccessful, although the OH and $\cdot O_2^-$ spin adducts have been identified in microsomal systems[18-21] and in our system (not shown). Thus, it suggests that the life span of $MPP\cdot$ is rather short. This has been verified recently by a pulse radiolysis study.[22] We have initiated a complete chemical study involving the use of fast kinetics in order to follow the steps associated with the reactions of MPP^+ with solvated electrons and with univalent oxidizing agents.

The role of iron and copper in this toxicity is not clear yet and could prove a focal point for the difference between the mechanism of actions of these two pyridinium compounds. While some chelators proved effective protectors (histidine) while others such as EDTA, DETAPAC and desferrioxamine were ineffective (not shown). This could be attributed to the poor permeability of the latter group, and may indicate that the target-site and organelle in MPP^+ – toxicity is found within the cell and is not restricted to the cytoplasmic membrane, as with paraquat.[15]

Trying to further elucidate this point by adding copper to the reaction mixture proved ineffective, as well. This could also be due to the lack of infiltration of copper into *E. coli* cells. Further clarification of this point should be sought.

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