Free Rad. Res. Comms., Vol. 1, No. 2, pp. 79–88
8755-0199/85/0102-0079 $25.00/0

Printed in Great Britain

TRANSITION METALS POTENTIATE PARAQUAT TOXICITY

RON KOHEN and MORDECHAI CHEVION

Department of Cellular Biochemistry, The Hebrew University of Jerusalem, Jerusalem 91 010, Israel

(Received April 1st 1985)

The involvement of transition metal ions in paraquat toxicity was studied in bacterial model system. We show that the addition of micromolar, or lower, concentrations of copper dramatically enhanced the rate of bacterial inactivation. In contrast, the addition of chelating agents totally eliminated the killing of *E. coli*. No inactivation was observed under anaerobic exposure to paraquat, both in the absence and presence of copper. However, in the presence of copper, the anaerobic addition of hydrogen peroxide resulted in complete restoration of inactivation as under aerobiosis.

Paraquat either produces superoxide ions or directly reduces bound copper ions in a catalytic mode. The reduced cuprous complexes react with hydrogen peroxide to locally form hydroxyl radicals ($OH^{\cdot}$) which are probably responsible for the deleterious effects.

This study indicates the involvement of a site-specific metal-mediated Haber-Weiss mechanism in paraquat toxicity. It is in agreement with earlier observations that copper unusually enhance biological damage induced by either superoxide or ascorbate.

Key words: paraquat toxicity/transition metal ions/copper/chelators/superoxide/superoxide dismutase/hydroxyl radical.
Abbreviations: Detapac — diethylene-triamine pentaacetate; SOD — superoxide dismutase.

INTRODUCTION

Considerable biochemical understanding of the mechanism of paraquat toxicity in bacterial systems has been gained by Fridovich and his colleagues[1-3]. They showed that superoxide radical is the major causative agent responsible for its toxicity[2]. Recent studies, using chemical or biological systems, demonstrated the frequent enhancement, by copper or iron, on the deleterious effects exerted by superoxide radicals.[4-8]. Based on the rationale that paraquat toxicity may also be mediated by transition metals[9, 19, 20], we examined their role in paraquat toxicity in laboratory mice[10]. The results clearly showed that treatment of animals with a chelating agent provided significant protection against the poisonous effects of paraquat, while intraperitoneal introduction of iron markedly exacerbated its toxicity[10].

Since evaluation of the detailed mechanism of toxicity in a whole animal is a rather complex procedure, we decided to elucidate the molecular role of transition metals in paraquat toxicity in a simpler *E. coli* system.

MATERIALS AND METHODS

Escherichia coli B (SR-9) was used throughout the experiment. The cells were grown at 37°C in a shaking incubator in medium containing KH_2PO_4 0.7% (Merck), K_2HPO_4 0.3% (Merck), Na-citrate 0.5% (AR Mallinckrodt), $(NH_4)_2SO_4$ 0.1% (Baker analytical reagent) at pH 7.0. Magnesium sulphate 1 mg/ml (Merck) was added after sterilization in an autoclave, and glycerol 1% (Frutarom, Israel) was used as carbon source. The bacteria were washed three times in phosphate buffer (1 mM) containing glucose (0.5%). The washed cells were suspended in phosphate buffer (1 mM, pH 7.14) containing glucose (0.5% w/v) and magnesium sulphate (1 mM) to a density of 3×10^9 cell/ml. Variation in the pH of the cell suspension during 30 min reaction was less that 0.05 pH units. Copper salicylate was prepared by mixing copper sulphate and salicylic acid solutions in a ratio of 1:2. Samples from the reaction mixture were taken at various times and the reaction was terminated by diluting the samples in a phosphate buffer (1 mM) containing diethylenetriamine pentaacetate (Detapac) (0.01 mM) and gelatin 0.5% (Difco Laboratories). The samples were diluted by a factor of $10^3 - 10^6$ and were plated on agar disks containing: agar 2%, bacto tryptone 1% (Difco Laboratories) and sodium chloride 0.5% (Frutarom). Each sample was plated at least four times. All the materials were prepared fresh before every experiment. Triply distilled water was used for the preparation of paraquat and copper solutions in glass tubes coated with silicone. To achieve anaerobic conditions, high purity nitrogen (>99.999%) was bubbled through the bacterial suspension for 20 min before the reaction and flushed over the suspension during the reaction.

The survival curves were evaluated from colony counts after an overnight incubation at 37°C. All the results displayed represent an average of at least three different experiments for each system.

RESULTS

The effect of copper on bacterial inactivation induced by paraquat

Exposure of *E. coli* B for 30 min to paraquat in the concentration range 0.1–1.0 mM caused inactivation of 36–83% of bacteria, depending on paraquat concentration (Figures 1 and 2a). Similarly, incubation of these cells with *copper alone* for 30 min showed only marginal inactivation. For example, copper concentrations of 0.8 or below 1 and 2 μM produced inactivations of 10% or less, 20% and 50% respectively (not shown).

By contrast, exposure of *E. coli* B cells to a *combination* of paraquat (0.25 mM) and copper(II) (1.0 μM) led to a rapid, exponential inactivation of the cells, that, after 20 min, exceeded 3 decimal logarithmic units. This dramatic enhancement was dependent on copper concentration; increasing [Cu(II)], using constant paraquat concentration, led to a higher rate of bacterial killing (Figure 1).

When Detapac was added to the incubation mixture that contained *E. coli* B cells and paraquat (no copper added), the residual inactivation was very small (2–3% after

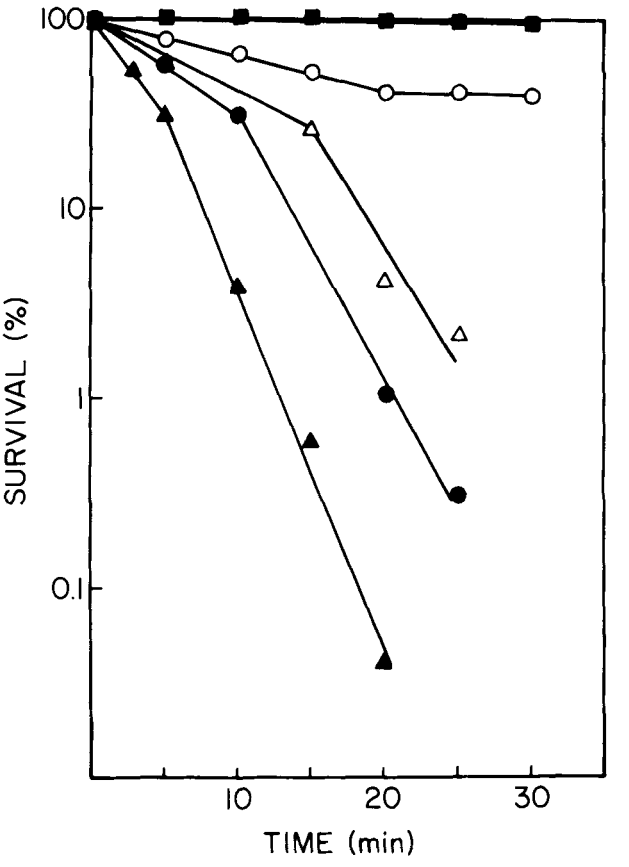


FIGURE 1 *The effect of copper on paraquat-induced bacterial inactivation.* All the incubation mixtures contained 3 × 10^7 E. coli B cells/ml, paraquat (0.25 mM), glucose (0.5% w/v) in phosphate buffer (1 mM, pH 7.4). Copper concentrations were 1 μM (▲), 0.5 μM (●), 0.1 μM (△), no added copper (o), and no added copper in the presence of desferrioxamine (0.1 mM) (■).

30 min). Other chelating agents like EDTA or desferrioxamine were also highly efficient in preventing the damage induced by paraquat. The rate (slope of the curve) of inactivation in the presence of paraquat (0.25 mM), and desferrioxamine was less than 1% of that exhibited for the reaction mixture containing *both* paraquat (0.25 mM) and copper (1 μM) (figure 1).

Figure 2b shows the increased rate of bacterial killing by increasing concentrations of paraquat, at a constant copper concentration (1 μM). All the results of bacterial inactivation in the presence of both copper and paraquat (figures 1 and 2b) demonstrated two phases. A shoulder which is characterized by a moderate rate with variable slope followed by a linear and steeper rate.

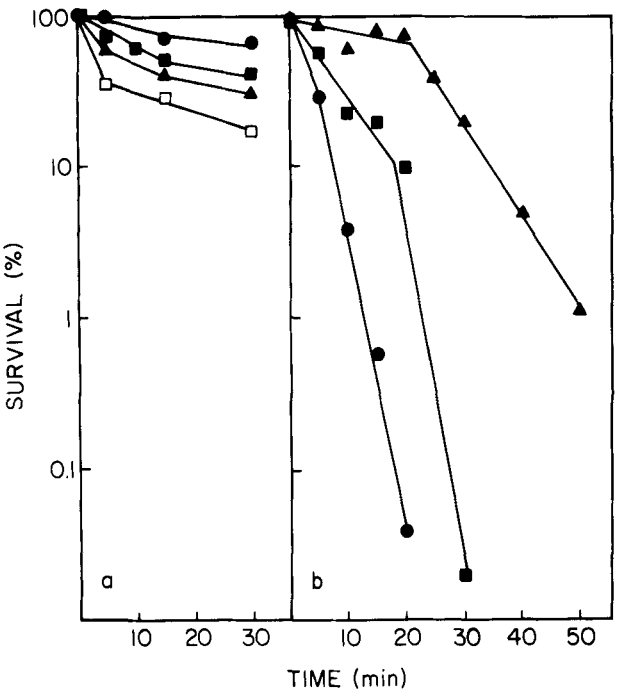


FIGURE 2 *The effect of increasing concentrations of paraquat on bacterial inactivation in the absence of added copper (a) and in the presence of added 1 μM copper (b).* All the incubation systems contained 3×10^7 E. coli B cells/ml, glucose (0.5% w/v) in phosphate buffer (1 mM, pH 7.4).
a. cupric sulfate = 0; 0.10 mM paraquat (●); 0.25 mM paraquat (■); 0.50 mM paraquat (▲); 1.00 mM paraquat (□).
b. cupric sulfate = 1 μM; 0.01 mM paraquat (▲); 0.10 mM paraquat (■); 0.25 mM paraquat (●).

Anaerobic and aerobic inactivation

The same concentrations of paraquat and copper ions which had caused an inactivation of 3 logarithmic units of *E. coli* B in 17 min, under aerobic conditions (figure 1), caused bacterial inactivation of only 0.1 logarithmic units under anaerobic conditions (fig. 3a). Hydrogen peroxide (0.25 mM), itself, innocuous to the cells (not shown), was added to the reaction mixture that contained *both* paraquat and copper, and led to a marked increase in the rate of inactivation (fig. 3a). When paraquat and hydrogen peroxide were anaerobically introduced into another reaction mixture, the rate of inactivation was increased only slightly (fig. 3a). Hydrogen peroxide acted on the cells under aerobic atmosphere as well (fig. 3b); it raised the killing to 1.2 logarithmic units after 30 min. The addition of desferrioxamine to the reaction mixture containing hydrogen peroxide and paraquat almost completely prevents the bacterial inactivation (fig. 3b).

The effect of superoxide dismutase, catalase and other proteins

Superoxide dismutase (SOD) (100 μg/ml) prevents the damage induced by paraquat

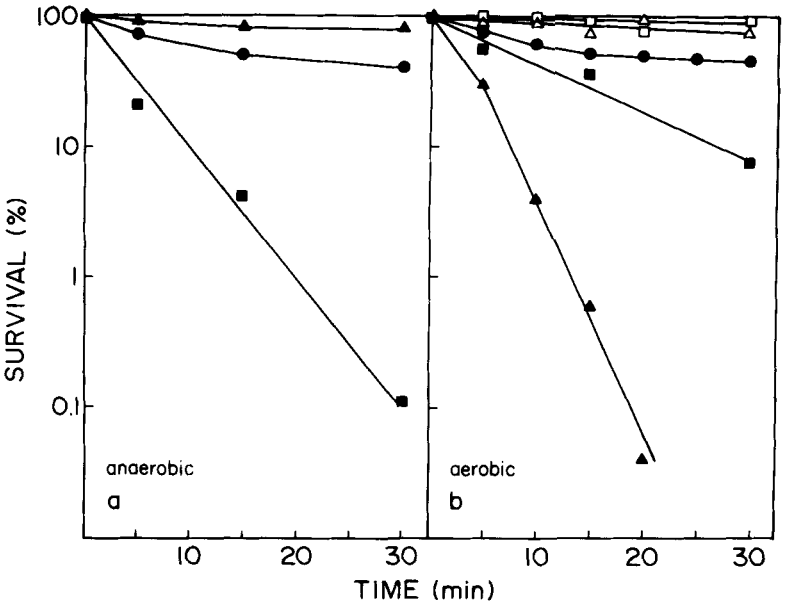


FIGURE 3 *The effect of the combination of hydrogen peroxide and copper on paraquat-induced bacterial inactivation under anaerobic (a) or aerobic (b) conditions.* All the incubation systems contained 3 × 10^7 E. coli B cells/ml, paraquat (0.25 mM), glucose (0.5% w/v) in phosphate buffer (1 mM, pH 7.4).
a. Anaerobic. cupric sulfate (1 μM) (▲); H_2O_2 (0.1 mM) (●); cupric sulfate (1 μM) together with H_2O_2 (0.1 mM) (■).
b. Aerobic. no additions (●); cupric sulfate (1 μM) (▲); H_2O_2 (0.1 mM) (■); H_2O_2 (0.1 mM) together with desferrioxamine (0.1 mM) (□); desferrioxamine (0.1 mM) (△).

and copper ions, *only* when all the reaction components were added simultaneously (fig. 4a). Heat-inactivated SOD was also similarly efficient in reducing cellular killing (fig. 4a).

By contrast, when the cells were pre-incubated with copper alone for 12 mins and *then* paraquat and superoxide dismutase were added, SOD could not stop the damage induced by paraquat and copper (fig. 4a).

Copper salicylate (1 μM), which has been shown to possess SOD activity[1, 12] failed to provide protection against paraquat toxicity. In fact, the slope of the bacterial inactivation curve in its presence was similar to that exhibited in the presence of copper sulphate (1 μM) and paraquat.

Like SOD, catalase prevents the damage induced by paraquat and copper, *only* when all the components were added together (fig. 4b). Similarly, even heat-inactivated catalase reduced the rate of bacterial inactivation (figure 4b). By contrast, when the cells were first loaded with copper ions, and only after 12 min of pre-incubation, catalase and paraquat were added, catalase failed to prevent bacterial killing (figure 4b).

When paraquat and copper together with either hexokinase or collagenase were added *simultaneously* to *E. coli* B cells, the rate of bacterial inactivation was very low. The protection provided by each of these proteins to the cells was similar to that of catalase and SOD (figure 4b). Even heat-inactivated hexokinase or collagenase exhi-

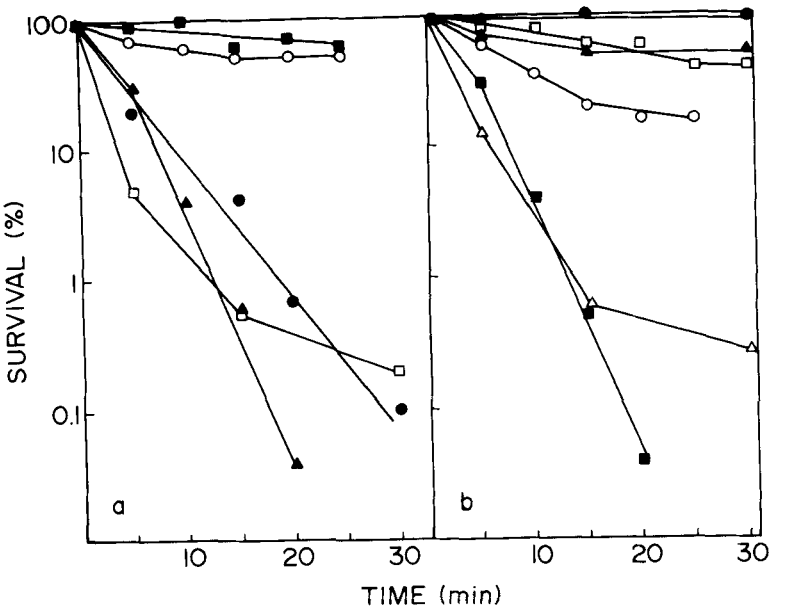


FIGURE 4 *The effects of added proteins on paraquat-induced bacterial inactivation.* All the incubation systems contained 3 × 10^7 E. coli B cells/ml, paraquat (0.25 mM), glucose (0.5% w/v) in phosphate buffer (1 mM, pH 7.4).

a. cupric sulfate (1 μM) (▲); cupric sulfate (1 μM) added simultaneously with SOD (100 micro g/ml (■); cupric sulfate (1 μM) added simultaneously with heat-inactivated SOD (o); preincubation of the bacteria with copper (1 μM, 12 min) followed by addition of SOD (100 μg/ml) (□); copper salicylate (1 μM) (●).

b. cupric sulfate (1 μM) (■); simultaneous addition of a combination of cupric sulfate (1 μM) and catalase (100 μg/ml) (□); simultaneous addition of cupric sulfate (1 μM) and heat-inactivated catalase (100 μg/ml) (o); preincubation of the bacteria with cupric sulfate (1 μM, 12 min) followed by addition of catalase (100 μg/ml) (△); simultaneous addition of cupric sulfate (1 μm) and hexokinase (100 μg/ml) (▲) or collagenase (100 μg/ml) (●).

bited a significant protective effect; after 30 min only 35% of the cells (0.55 logarithmic unit) were killed.

The effect of free radical scavengers

Mannitol (100 mM) or tert-butyl alcohol (100 mM) provided a small decrease in the rate of cellular inactivation (fig. 5). Unlike these scavengers, low concentrations of histidine (1 mM), which is also an effective chelating agent, reduced the rate of inactivation more efficiently and proved to be a better protectant (fig. 5). The bacterial killing was completely stopped after 15 min of incubation with histidine.

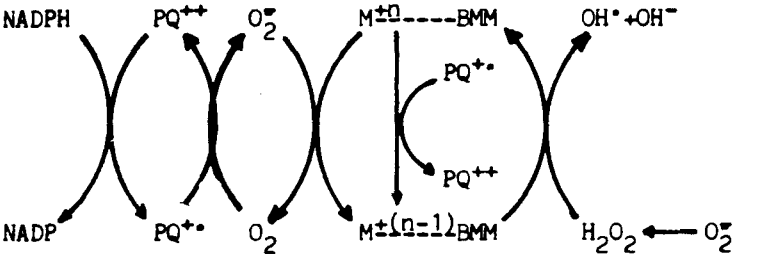
DISCUSSION

The involvement of transition metals in enhancing bacterial killing induced by paraquat was shown in two ways: The effect of chelators which minimized the damage and the effect of added copper ions which enhanced it dramatically. It is possible that the residual bacterial killing in the basic system (no copper added) is due to contamination of transition metal ions originating from either paraquat[13], medium or other reagents[13].

A trivial and inadequate explanation of these results is that added copper enhances the enzymatic reduction of paraquat to the corresponding radical. However, analogous effects were also observed in other systems, in which the reducing capacity did not depend on enzymatic reaction, but rather was carried out either chemically (by ascorbate[4, 5]) or radiolytically (by superoxide radical[7]). Alternatively, it has been suggested that the reduction of paraquat is carried out by NADPH-Cyt C reductase[14, 15], where its maximal activity has no requirement for copper or iron[14, 15].

The Detailed Mechanism

Copper ions can participate in a mechanism undergoing cyclic oxidation-reduction in chemical as well as in biological systems[16]. Reduction of paraquat to the stable monocation radical has been shown in various biological systems[1, 2, 17]. This stable radical reacts very quickly with oxygen to generate superoxide[17, 18]. The superoxide radical can reduce cupric complexes into the corresponding cuprous ones (5 and scheme). Analogously, the paraquat radical can also directly reduce biological complexes of transition metal ions[19, 20]. The superoxide radical thus formed can dismute, enzymatically or spontaneously, to produce hydrogen peroxide. Subsequently, the reduced metal complexes can react with hydrogen peroxide to produce hydroxyl radicals in a site specific manner[21], which may be responsible for the biological damage (scheme).



BMM = Biological Macro Molecules

The component reactions mediating this suggested mechanism were substantiated by several sets of experiments performed in our laboratory. When paraquat was introduced in the absence of oxygen, no superoxide radical could be formed and no bacterial killing was recorded even in the presence of copper ions (fig. 3). However, when 0.1 mM hydrogen peroxide was added to this anaerobic system (containing

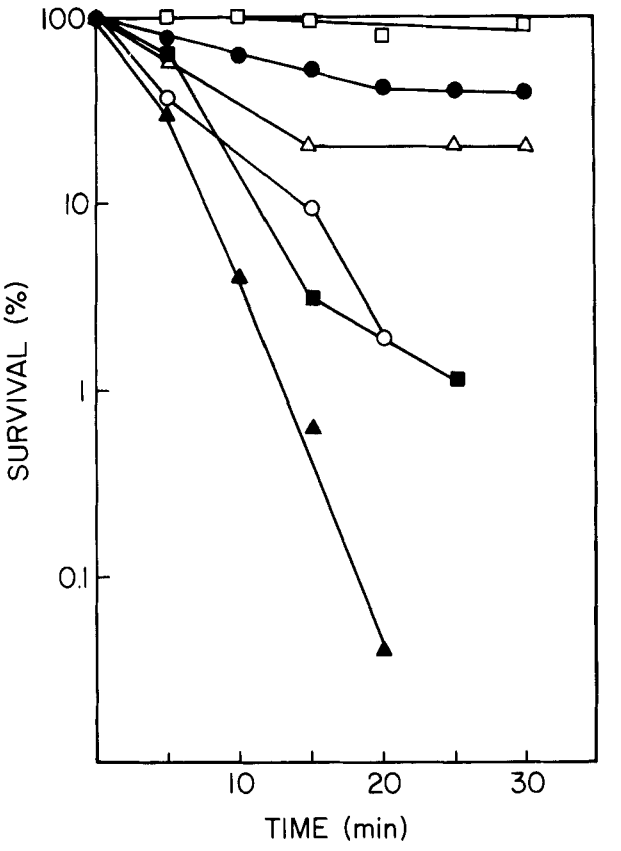


FIGURE 5 *The effects of free radicals scavengers on the paraquat-induced bacterial inactivation.* All the incubation systems contained 3 × 10^7 E. coli B cells/ml, paraquat (0.25 mM), glucose (0.5% w/v) in phosphate buffer (1 mM, pH 7.4). Addition of cupric sulfate (1 μM) (▲); cupric sulfate (1 μM) with tert-butyl alcohol (100 mM) (■); cupric sulfate (1 μM) with mannitol (100 mM) (o); cupric sulfate (1 μM) with histidine (1 mM) (△); desferrioxamine (0.1 mM) (□); and no additions (●).

paraquat and copper), the rate of bacterial killing was *similar* to that observed under aerobic conditions, in the absence of exogenous hydrogen peroxide. Oxygen formed in the anaerobic system by the catalatic decomposition of added hydrogen peroxide is estimated to be at least an order of magnitude lower than that in air-saturated solutions. This is because the catalase is low in un-induced *E. coli* cells[22] and because apparent Km for hydrogen peroxide is very high. In addition, the continuous flushing of the reaction vessel with nitrogen would remove the liberated oxygen.

When SOD or other proteins (which do not penetrate into the cells), native or heat-inactivated, were added to the system they prevented bacterial killing (figs. 4a, 4b). This indicates that the protective effect was due to something other than the dismutation of superoxide — possibly the chelation of metals[23, 24, 25]. Copper-salicylate, which possesses SOD activity *in vitro*[11, 22] failed to lower the bacterial killing, but rather

enhanced the rate of inactivation (fig. 4a). This model is in accordance with the lowering of the killing curves shown in figs. 4a, 4b, when the bacteria were pre-incubated with copper ions.

Hydroxyl radicals are suggested as the primary cause for the biological damage observed. In principle, it is expected that $OH^{\cdot}$ scavengers should afford protection. However, in the case of the recently proposed site specific metal mediated Haber-Weiss reaction[4, 6, 21], hydroxyl radical scavengers were shown to protect weakly, if at all[26]. In such reactions $OH^{\cdot}$ radicals are formed on biological macromolecules or in close proximity to biological targets, and they react predominantly at the site of their formation, to which the scavenger may not have free access. Two different scavengers, tertiary butyl alcohol and mannitol that were at a high concentration (100 mM) reduced the rate of inactivation only by a factor of about 2 (fig. 5). Histidine, on the other hand, a potent transition metal chelator[27], and a known scavenger of $OH^{\cdot}$, provided much higher protection at the lower concentrations of 1 mM (fig. 5). Thus, it seems that the effect of histidine arises from the chelation of metal ions, or repair by hydrogen donation.

In summary, based on these findings with copper and with chelators we suggest that some form of labile, redox active transition metal complexes mediate paraquate toxicity, and thus, chelation therapy should be tried in treatment of paraquat poisoning. The experiments with mice support this approach[10].

Acknowledgement

This research has been supported by a grant from the United States–Israel Binational Foundation (BSF).

References

1. M.H. Hassan and I. Fridovich, *J. Biol. Chem.*, **253**, 8143, (1978).
2. M.H. Hassan and I. Fridovich, *J. Biol. Chem.*, **254**, 10846, (1979).
3. M.H. Hassan and I. Fridovich, *Arch. Biochem. Biophys.*, **196**, 385, (1979).
4. A. Samuni, M. Chevion, G. Czapski, *J. Biol. Chem.*, **256**, 12632, (1981).
5. E. Shinar, T. Navok, M. Chevion, *J. Biol. Chem.*, **258**, 14778, (1983).
6. T. Navok and M. Chevion, *Biochem. Biophys. Res. Commun.*, **122**, 297, (1984).
7. A. Samuni, M. Chevion, G. Czapski, *Rad. Res.*, **99**, 562, (1984).
8. R.L. Levine, C.N. Oliver, M.R. Fulks, E.R. Stadtman, *Proc. Natl. Acad. Sci. USA*, **78**, 2120, (1980).
9. R. Kohen, P. Korbashi, M. Chevion "Paraquat toxicity is mediated by transition metal ions". Abstracts of the First Joint Meeting of the Israel Societies of Life Sciences, Jerusalem, October 1983.
10. R. Kohen and M. Chevion, *Biochem. Pharmacol.*, **34**, 1841 (1985).
11. R.J.Y. Youngman, A.D. Dodge, E. Lengfelder, E.C. Elstner, *Experimenta*, **35**, 1295, (1979).
12. E. Lengfelder, M. Saran, W. Bors, Abstract Nr. L-3. International Conference on Singlet Oxygen and Related Species in Chemistry and Biology, 1977.
13. D.C. Borg, K.M. Shaich, A. Forman, *Proc. Conf. on Oxygen Radicals in Chemistry and Biology*, (Walter de Gruyer and Co., Berlin), 1983, (in press).
14. H.J. Forman, J. Nelson, A.B. Fisher, *J. Biol. Chem.*, **255**, 9879, (1980).
15. J.S. Bus, S.Z. Cagen, M. Olgaard, J.E. Gibson, *Toxicol. Appl. Pharmacol.*, **35**, 501, (1976).
16. H. Sigel in *Metal Ions in Biological Systems Properties of Copper*, (Marcel Dekker Inc. N.Y. and Basel, 1981), Vol. 12, p. 4.
17. M.R. Montgomery, *Res. Commun. Chem. Pathol. Pharmacol.*, **16**, 155, (1979).
18. T.A. Farrington, N. Ebert, E.J. Land, K. Fletcher, *Biochim. Biophys. Acta.*, **314**, 372, (1973).
19. H.C. Sutton, C.C. Winterbourn, *Arch. Biochem. Biophys.*, **235**, 106, (1984).
20. C.C. Winterbourn, H.C. Sutton, *Arch. Biochem. Biophys.*, **235**, 116, (1984).
21. A. Samuni, J. Aronovitch, D. Godinger, M. Chevion, G. Czapski, *Eur. J. Biochim.*, **137**, 119, (1983).

22. H.E. Richter and P.C. Loewen, *Arch. Biochem. Biophys.*, **215**, 72, (1982).
23. M. Anbar and A. Levitzki, *Radiat. Res.*, **37**, 32, (1966).
24. E.K. Hodgson and I. Fridovich, *Biochemistry*, **14**, 5294, (1975).
25. N.R. Bachur, S.L. Gordon, M.V. Gee, *Cancer Res.*, **38**, 1745, (1978).
26. G. Czapski, *Israel J. Chem.*, **24**, 29, (1984).
27. J. Peisach, P. Aisen, W.E. Blumberg, (eds) "The Biochemistry of Copper", Academic Press., N.Y., 1966, p. 1.

Accepted by G. Czapski