Lehner, C. F., Stick, R., Eppenberger, H. M., & Nigg, E. A. (1987) *J. Cell Biol. 105*, 577–587.
Lin, J. J. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2335–2339.
Lin, J. J. C. & Feramisco, J. R. (1981) *Cell (Cambridge, Mass.) 24*, 185–193.
Lischwe, M. A., & Ochs, D. (1982) *Anal. Biochem. 127*, 453–457.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maul, G. G., Schatten, G., Jimenez, S. A., & Carrera, A. E. (1987) *Dev. Biol. 121*, 368–375.
McKeon, F. D., Kirschner, M. W., & Caput, D. (1986) *Nature (London) 319*, 463–468.
Mendelson, E., & Bustin, M. (1984) *Biochemistry 23*, 3459–3466.
Newmeyer, D. D., & Ohlson-Wilhelm, B. M. (1985) *Chromosoma 92*, 297–303.
Nikodem, V., & Fresco, J. R. (1979) *Anal. Biochem. 97*, 382–386.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell (Cambridge, Mass.) 12*, 1133–1142.
Ottaviano, Y., & Gerace, L. (1985) *J. Biol. Chem. 260*, 624–632.
Piszkiewicz, D., London, M., & Smith, E. L. (1970) *Biochem. Biophys. Res. Commun. 40*, 1173–1178.
Pruss, R. M., Mirsky, R., & Raff, M. C. (1981) *Cell (Cambridge, Mass.) 27*, 419–428.
Raymond, Y., & Suh, M. (1986) *J. Immunol. Methods 93*, 103–106.
Schweizer, J., & Goerttler, K. (1980) *Eur. J. Biochem. 113*, 243–249.
Steinert, P. M., & Parry, D. A. D. (1985) *Annu. Rev. Cell Biol. 1*, 41–65.

Cytoplasmic Membrane Is the Target Organelle for Transition Metal Mediated Damage Induced by Paraquat in *Escherichia coli*†

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ABSTRACT: Bacterial survival indicates that copper or iron is an essential mediator in paraquat toxicity in *Escherichia coli* [Kohen, R., & Chevion, M. (1985) *Free Radical Res. Commun. 1*, 79–88; Korbashi, P., Kohen, R., Katzhendler, J., & Chevion, M. (1986) *J. Biol. Chem. 261*, 12472–12476]. In this study we have identified the cytoplasmic membrane as a target organelle in metal-mediated paraquat toxicity and have demonstrated the complete correlation of the membrane damage with the levels of adventitious copper (or iron). The extent of membrane damage was related by use of four parameters: (a) the level of cellular ATP, (b) the level of cellular potassium, (c) the cellular capacity to accumulate and retain radiolabeled leucine, and (d) the cellular integrity as reflected by transmission electron microscopy (TEM). Exposure of bacterial cells to a combination of paraquat and copper caused a marked decline in parameters a, b, and c. This decline was found to occur in parallel with, or even to precede, the sharp loss of survival of *E. coli* under the same conditions. Likewise, TEM micrographs clearly indicated alterations in cellular structure that possibly reflect sites of detachment of the cytoplasmic membrane from the bacterial capsule. In contradistinction, copper alone or paraquat alone could not bring about similar changes in cellular structure. These findings are in accord with the suggested site-specific metal-mediated Haber–Weiss mechanism for paraquat toxicity and support our notion that specific chelators of transition metals could reduce or prevent the biological deleterious effects of this herbicide.

The toxicity of the herbicide paraquat (PQ^{2+}),[1] also known as methyl viologen, has been extensively studied in a variety of biological models including *Escherichia coli* (Hassan & Fridovich, 1977, 1978, 1979a,b; Farrington et al., 1973; Kohen & Chevion, 1985a,b; Bagley et al., 1986). Fridovich and Hassan (1977, 1978, 1979a,b) showed that paraquat is concentrated by the bacterial cells and is reduced enzymatically to the monocation radical ($PQ^{\cdot+}$) and subsequently reacts with molecular oxygen to produce the superoxide radical within the cell. They also showed that its bactericidal effect requires molecular oxygen and a carbon source and that induction of endogenous superoxide dismutase and catalase provided considerable cellular protection.

In recent studies in both *E. coli* cells (Kohen & Chevion, 1985a,b; Korbashi et al., 1986) and mice (Kohen & Chevion, 1985c), we have demonstrated that the transition metals copper

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[1] Abbreviations: PQ^{2+}, paraquat dication, methyl viologen; $PQ^{\cdot+}$, paraquat radical monocation; detapac, diethylenetriaminepentaacetate; EDTA, ethylenediaminetetraacetate.

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and iron are essential mediators in paraquat toxicity. We have suggested a mechanism whereby paraquat serves as an intracellular generator of the superoxide radical (Kohen & Chevion, 1985a). This radical, in turn, plays a dual role; it serves as a source of hydrogen peroxide (Halliwell & Gutteridge, 1984) and also reduces copper or iron in the complex with biological molecules. The corresponding Cu(I) or Fe(II) can subsequently react with hydrogen peroxide in the site-specific Fenton reaction (Samuni et al., 1983) to *locally* produce the very reactive hydroxyl radical ($OH^{\bullet}$) which may be directly responsible for the biological damage (Bus & Gibson, 1979; Gutteridge & Halliwell, 1982; Brown & Fridovich, 1980). The ability of $OH^{\bullet}$ to cause DNA breaks (Fridovich, 1983; Kohen et al., 1986) and protein inactivation, as well as membrane function impairment, has been demonstrated (Ross et al., 1979). Similarly, there are other systems in which free radicals and various active-oxygen species damage specific subcellular components or organelles, such as DNA, cytoplasmic membrane, and specific proteins (Rawley & Halliwell, 1983; Lavelle et al., 1973; Shinar et al., 1983); therefore, one or more of these organelles have been suggested as the cellular target of paraquat toxicity, and the $OH^{\bullet}$ has been suggested as the immediate deleterious agent (Richmond & Halliwell, 1982; Youngman & Elstner, 1981). Paraquat can induce single- and double-strand breaks in DNA (Ross et al., 1979), inhibit DNA synthesis (Van Osten & Gibson, 1975), and affect DNA conformation. We have shown that it can cause a low incidence of mostly single-strand but also a still much lower incidence of double-strand breaks in bacterial DNA in vivo. Since the level and the rate of formation of these breaks could not account for the observed massive bacterial killing (Kohen et al., 1985), we have looked for possible damage induced to another organelle.

While in previous studies the paraquat-induced damage to the alveolar membrane in the lungs was studied in the absence of adventitious metals, we have, in the present study, investigated the *metal-mediated* effects of paraquat on *E. coli* and shown that the cytoplasmic membrane is indeed a target organelle for the deleterious effect of paraquat in the presence of copper or iron.

Materials and Methods

Chemicals. Paraquat, chloramphenicol, cytochrome *c* (type IV), diethylenetriaminepentaacetic acid (detapac), ethylenediaminetetraacetic acid (EDTA), luciferine lucifirase, and ATP were all from Sigma (St. Louis, MO).

Ferrous sulfate, glucose, leucine, magnesium sulfate, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were from Merck. Bacto agar, lacto tryptone, potassium chloride, protein peptone, and yeast extracts were from Difco. Glycerol, hydrogen peroxide, and sodium chloride were from Frutarom (Israel). Copper sulfate, magnesium sulfate, and sodium arsenate were from Mallinckrodt. Scintillation liquids were from United Technologies-Packard, and [^{14}C]leucine was from Amersham.

Bacterial Growth and Survival. E. coli B (SR-9), used throughout the experiments, were grown at 37 °C in a shaking incubator in medium containing KH_2PO_4 (0.07%), K_2HPO_4 (0.3%), sodium citrate (0.51%), and $(NH_4)_2SO_4$ (0.1%), at pH 7.0. Magnesium sulfate (1 mg/mL) was added after sterilization in an autoclave, and glycerol (1%) was added as a carbon source. The cells were washed 3 times in phosphate buffer (1 mM), pH 7.4, containing glucose (0.5%). The washed cells were suspended in phosphate buffer (1 mM, pH 7.4) containing glucose (0.5%) and $MgSO_4$ (1 mM) to a density of 3×10^9 cells/mL. The cells were then exposed to paraquat and other additives and sampled at various times. The reaction was terminated by dilution of the samples in phosphate buffer (1 mM) containing diethylenetriaminepentaacetate (detapac) (0.01 mM) and gelation (0.5%). The cell suspensions were diluted by a factor of 10^3–10^6 and were subsequently plated on agar disks containing agar (2%), bacto tryptone (1%), and sodium chloride (0.5%). Each sample was plated at least 4 times. Survival curves were plotted from colony counts following overnight incubation at 37 °C.

Cellular ATP Content. Samples (0.2 mL) were taken out at various times into preheated test tubes containing 1.8 mL of water and boiled for 5 min. After cooling to room temperature, 1 mL of the contents was placed into a scintillation vial containing 1 mL of sodium arsenate buffer (0.1 M, pH 7.4) with magnesium sulfate (4 mM) and 1 mL of phosphate buffer (10 mM, pH 7.4) with magnesium sulfate (4 mM). Fifty microliters of firefly lantern extract reagent was added, and the chemiluminescence was determined immediately in the ^{3}H channel with a Packard Tri-carb liquid scintillation spectrometer in which the coincidence circuit had been disconnected. Standard ATP curves were constructed and used to convert the average of duplicate count values to ATP concentration (Cole et al., 1967).

Accumulation of [^{14}*C*]*Leucine.* The initial rate of transmembrane transport and retention (accumulation) of leucine was determined with [^{14}C]leucine in the presence of chloramphenicol (0.1 mg/mL): samples (1 mL) were placed in test tubes containing chloramphenicol (0.1 mg) and 4 μM [^{14}C]-leucine (3.7 Ci/mol). Cells were collected at various times and filtered on a Schleicher & Schüll membrane filter and washed twice with 2 mL of distilled water at 37 °C. Filters were dried at 90 °C for 20 min and were put into scintillation vials containing 2.5 mL of liquid. The vials were measured in the ^{14}C channel on a Packard Tri-carb liquid scintillation spectrometer (Arraku, 1968).

Induction of Catalase. The cells were grown at 37 °C on a shaking incubator in an LB medium containing protease peptone (1%), yeast extract (0.5%), and sodium chloride (0.5%). Catalase was induced by the addition of hydrogen peroxide. The *E. coli* were washed at 0 °C by centrifugation for 5 min at 6900*g* and resuspended in potassium phosphate buffer (50 mM, pH 7.0) to a density of 5×10^8 cells/mL. Catalase was assayed by spectrophotometric analysis of the rate of decomposition of hydrogen peroxide at 240 nm (Aebi, 1985).

Determination of Potassium. The cells were washed first with saline solution and then with doubly distilled water. Twenty milliliters of the washed cells (8×10^8 cells/mL) were exposed to different reactants in the presence of glucose (0.5%). The reaction was stopped at various times by filtration. The filter was washed 6 times with 10 mL of distilled water, and the cells were resuspended in water. The cell suspension was heated to 70 °C for 5 min, cooled, centrifuged (10 000 rpm × 10 min), and kept at 4 °C overnight. The supernatant was separated and divided among three vials, and distilled water was added to make up 4 mL in each sample. Potassium concentration in each sample and in the standard vials was determined by atomic absorption with a Perkin-Elmer spectrometer (Model 403) and a Perkin-Elmer K-lamp (Model 383).

Transmission Electron Microscope. Samples of *E. coli* cells (10^7 cells/mL), which had been treated with various substances, were put on carbon-covered grids. Cells were fixed by negative staining with phosphotungstic acid (pH 6.0, 2%) as described by Huxley and Zubay (1960). The preparations

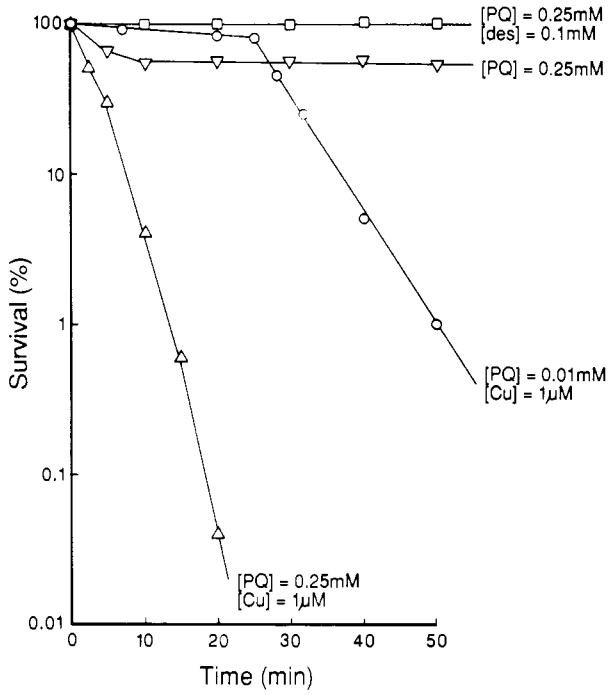

FIGURE 1: Effect of copper on paraquat-induced bacterial inactivation. All the inactivation mixtures contained *E. coli* B (2×10^7 cells/mL), paraquat (0.25 mM), and glucose (0.5% w/v), in phosphate buffer (1 mM, pH 7.4). Paraquat (0.25 mM) alone (▽); paraquat (0.25 mM) and copper (1 μM) (△); paraquat (0.01 mM) and copper (1 μM) (○); paraquat (0.25 mM) and desferrioxamine (0.01 mM) (□).

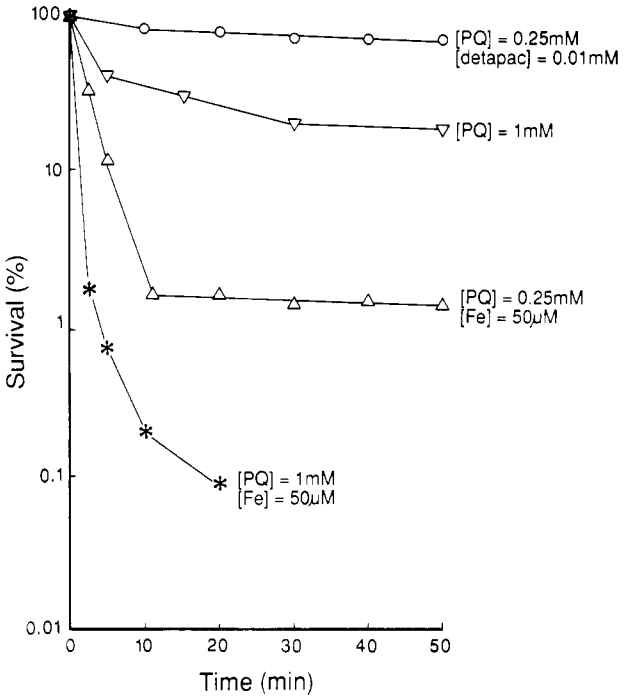

FIGURE 2: Effect of iron on paraquat-induced bacterial inactivation. All the reaction mixtures contained *E. coli* B (2×10^7 cells/mL) and glucose (0.5% w/v) in phosphate buffer (1 mM, pH 7.4). Paraquat (1 mM) alone (▽); paraquat (1 mM) and ferrous sulfate (50 μM) (*); paraquat (0.25 mM) and ferrous iron (50 μM) (△); paraquat (0.25 mM) and detapac (0.01 mM) (○).

were examined at 60 kV by a Philips transmission electron microscope (E. M. Model 300) (Holland).

Anaerobic Conditions. Anaerobic conditions were obtained by bubbling high-purity nitrogen (>99.999%) through the preparation 20 min before the experiment, followed by continuous flushing with nitrogen during the experiment.

RESULTS

Bacterial Inactivation. Exposure of *E. coli* (2×10^7 cells/mL) to paraquat (0.25 mM) caused inactivation of 36–46% of the bacteria in aerobic conditions (Figure 1). Addition of the chelating agent desferrioxamine (100 μM) to the reaction mixture prevented cell killing (Figure 1). Other chelating agents (EDTA, NTA, and histidine) were also effective in preventing the inactivation induced by paraquat (not shown). By contrast, exposure of *E. coli* to a combination of paraquat (0.25 mM) and copper(II) (1 μM) led to a rapid exponential inactivation of the cells that, after 20 min, exceeded 3 orders of magnitude (Figure 1). This dramatic enhancement was dependent on the copper concentration and on the paraquat concentration (Figure 1) (Kohen & Chevion, 1985a). Analogous results were obtained when iron ions were introduced into the cell suspension containing paraquat; however, the shape of the inactivation curve was different from that obtained when copper ions were used (Figure 2). The enhancement in the killing rate of the cells was dependent on both iron concentration (Figure 2) and paraquat concentration (not shown) (Korbashi et al., 1986; Kohen, 1985).

Cellular ATP Levels. The ATP content of cells is a sensitive parameter of cellular function and metabolic activity (Gelvan & Samuni, 1986). Damage to the cytoplasmic membrane that leads to leakage of the cell contents and to interference with the normal proton gradient will cause an activation of energy-demanding pumps. A rapid dissipation in the cellular energy expressed as a decrease in ATP content should occur. Therefore, when the *E. coli* cell membrane is damaged, it is expected that its cellular ATP level will be rapidly and markedly lowered as compared to that of an intact cell. As seen in Figure 3, the ATP content of the *E. coli* cells (10^8 cells/mL) has significantly decreased following exposure to a combination of paraquat (0.5 mM) and copper (5 μM) for 90 s under the same experimental conditions. Thus, the loss of cellular ATP preceded the inactivation of bacteria, and the cell survival was found to remain as high as 80% (0.8 decimal logarithmic unit) after 90 s of exposure (Figure 3).

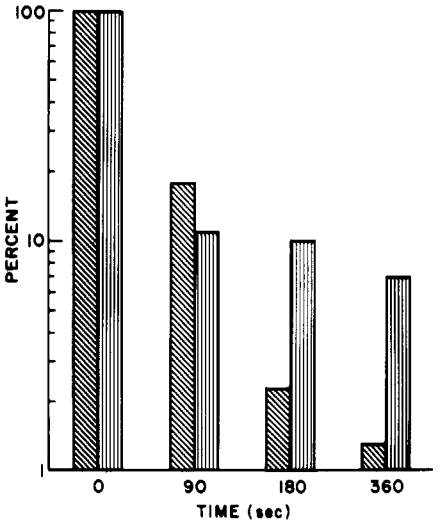

FIGURE 3: Survival and cellular ATP levels in *E. coli* B cells exposed to the combination of paraquat and copper. The incubation mixture contained *E. coli* B cells (1×10^8 cells/mL, 100% value), copper sulfate (5 μM), glucose (0.5%), and paraquat (0.5 mM) in phosphate buffer (1 mM, pH 7.4). Cell survival (▨); ATP level (▥).

Figure 4 shows both bacterial survival and residual cellular ATP following various treatments. When only copper (0.6 μM) was employed, bacterial killing reached 30% after 15 min, while no loss in cellular ATP was recorded. Likewise, no loss

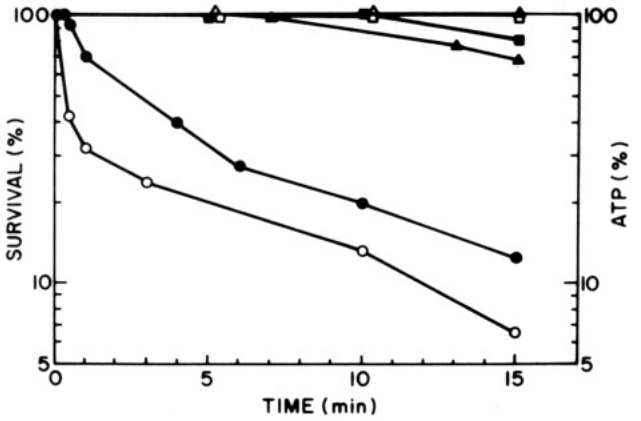

FIGURE 4: Survival and cellular ATP levels in *E. coli* B cells exposed to the combination of paraquat and copper. The incubation mixture contained *E. coli* B cells (1×10^8 cells/mL, 100% value) and glucose (0.5%) in phosphate buffer (1 mM, pH 7.4). Survival of cells following treatment with paraquat (0.5 mM) and copper 0.6 μM (●); ATP level of the cells following treatment with paraquat (0.5 mM) and copper (0.6 μM) (○); survival of cells in a reaction mixture containing copper alone (▲); ATP level of cells in a reaction mixture containing copper alone (△); survival of cells in a test system containing paraquat (0.25 mM) and detapac (0.1 mM) (■); ATP level of cells in a reaction mixture containing paraquat (0.25 mM) and detapac (0.1 mM) (□).

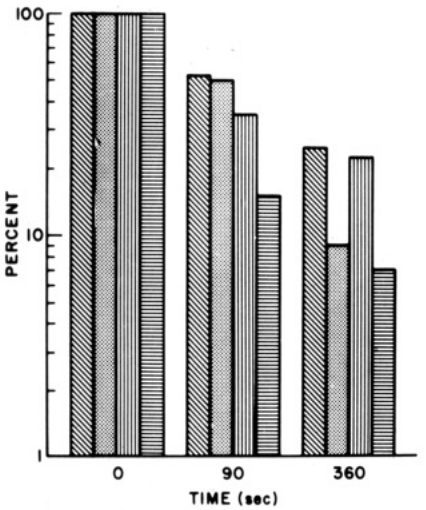

FIGURE 5: Effect of endogenous catalase on the paraquat-induced inactivation of *E. coli* B cells and on cellular ATP in the presence of copper ions. The *E. coli* B cells were grown in a rich medium (LB) in the presence of H_2O_2 (0.44 mM) as an inducer. The cells were harvested at midlog phase and were treated with paraquat (0.25 mM) and copper sulfate (5 μM). Survival of the uninduced cells (▨); survival of the induced cells (▩); ATP level of the uninduced cells (▥); ATP level of induced cells (▤).

in ATP took place when the bacterial cells were exposed to the combination of paraquat (0.25 mM) *and* detapac (0.1 mM). The bacterial killing under these conditions was only 20%. In contradistinction, when a combination of paraquat *and* copper was employed, a marked decrease in both parameters was observed. The decrease in cellular ATP was characterized by an initial fast phase (1–2 min), followed by a moderate loss in energy content, while bacterial killing trailed behind. Following its initial burst, the long second phase of bacterial killing was characterized by a slope similar to that of the curve representing ATP loss (Figure 4). It is important to note that under the various conditions both parameters are similarly affected (Figure 4).

Endogenous catalase had been induced 8-fold in *E. coli* cells by growing the bacteria in rich medium containing hydrogen peroxide (0.44 mM). These catalase-rich cells displayed a

Table I: ATP Level and Survival in *E. coli* B Cells Which Were Treated with Copper and Paraquat in an Anaerobic Atmosphere[a]

expt	[PQ] (mM)	[Cu(II)] (μM)	[H_2O_2] (mM)	[detapac] (mM)	residual [ATP] (%)	residual survival (%)
1	0.25	5			115	66
2		5	0.1		100	90
3	0.25		0.1	0.1	90	80
4	0.25	5	0.1		2.4	0.1

[a] ATP levels and survival measurements were carried out following 15-min incubation. Determinations were done as described under Materials and Methods. The reaction mixture contained *E. coli* B (1×10^8 cells/mL, 100% value), paraquat, and copper in phosphate buffer (1 mM, pH 7.4), glucose (0.5%), and magnesium sulfate (1 mM).

Table II: ATP Level and Survival in *E. coli* B Treated with Paraquat and Copper at 37 and 0 °C[a]

expt	time of exposure (min)	temp (°C)	residual [ATP] (%)	residual survival (%)
1	0	37	100	100
	5		12	5.3
	10		10	0.1
	15		5	0.01
2	0	0	100	100
	5		86	94
	15		84	91

[a] The reaction mixture contained *E. coli* B cells (1×10^8 cells mL, 100% value) in phosphate buffer (1 mM, pH 7.4), glucose (0.5%), magnesium sulfate (1 mM), paraquat (0.25 mM), and copper sulfate (5 μM).

lower sensitivity to damage induced by the combination of paraquat and copper (Figure 5). After 6 min, the catalase-rich cells showed only close to 80% inactivation (less than 1 order of magnitude of cellular killing) when exposed to a combination of copper (5 μM) and paraquat (0.25 mM). After 6 min, the residual cellular ATP level of these cells was above 20% (loss of 77%) (Figure 5). In comparison, uninduced cells suffered both a more severe inactivation *and* a loss in ATP (Figure 5).

Under anaerobic conditions, the cells were not affected by the exposure to paraquat (0.25 mM) and copper (5 μM) (Table I); no loss was recorded in cellular survival or in ATP content. Only when H_2O_2 was added to the anaerobic reaction mixture containing both paraquat and copper, the cells were markedly damaged, as evidenced by the loss of cellular ATP level and the decrease in survival. When the inactivation reaction was carried out at 0 °C, no cell killing was recorded, and almost no decrease in the ATP level was found (Table II).

Accumulation of Leucine. The uptake and retention of [^{14}C]leucine was used as an additional functional marker for monitoring damage to membranes (Davis et al., 1980). Active transport across the cytoplasmic membrane is known to be highly sensitive to membrane integrity. *E. coli* cells (2×10^8 cells/mL) were incubated with either paraquat, copper, or both. Samples were taken at various times and pulsed with [^{14}C]leucine in the presence of chloramphenicol (0.1 mg/mL). The level of cellular leucine was determined as described above. In the presence of chloramphenicol, the residual protein synthesis accounted for less than 10% of the leucine uptake. The results presented in Figure 6 show that untreated cells accumulate leucine until a plateau has been reached, i.e., after 5 min. When the cells (2×10^8 cells/mL) were exposed to both paraquat (0.5 mM) and copper (5 μM), a marked decrease in both the accumulation and the retention of leucine by the cells was observed (Figure 6). Under the same conditions, their survival was found to be 5, 1.5, and 0.1% after 5, 7, and

Table III: Cellular Potassium Level in *E. coli* following Exposure to Paraquat and Copper[a]

expt	[PQ] (mM)	[Cu(II)] (μM)	temp (°C)	[detapac] (mM)	cells/mL (100% value)	residual potassium level (%)	survival (%)
1			37		8×10^8	100	100
2		5	37		8×10^8	80	97
3	2.5		37		8×10^8	81	64
4	2.5	5	37		2×10^9	38	2.4
5	2.5	5	37		8×10^8	19	0.09
6	2.5		37	0.1	8×10^8	81	97
7	2.5		0		2×10^9	76	111
8	2.0	5	37		8×10^8	31	0.5

[a] The reaction mixture contained *E. coli* B cells, glucose (0.5% w/v), magnesium sulfate (1 mM), paraquat, and copper. The determination of potassium levels and the survival of the cells were carried out following 15 min of incubation.

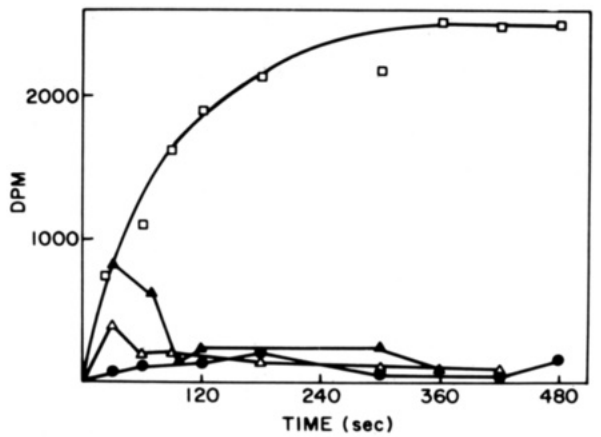

FIGURE 6: Accumulation of [^{14}C]leucine by *E. coli* B cells following exposure to paraquat and copper ions. The reaction mixture contained *E. coli* B (2×10^8 cells/mL), paraquat (0.5 mM), and copper (5 μM) in phosphate buffer (1 mM, pH 7.4). Determination of cellular content of [^{14}C]leucine was carried out as described under Materials and Methods. Control (untreated) cells (□); following 5-min incubation (▲); following 7-min incubation (△); following 15-min incubation (●).

15 min, respectively. Addition of the chelating agent detapac (0.1 mM), to the reaction mixture which contained paraquat, showed a pattern of leucine accumulation identical with that of the untreated cells (not shown). Similarly, exposure of the cells to copper sulfate alone (5 μM) did not alter the normal accumulation or retention of leucine by control cells.

Additional Supportive Evidence. In our search for additional evidence to substantiate the membrane damage in cells that have been exposed to paraquat and copper, two other parameters tested were (1) the effect on the intracellular level of potassium ions (K^+) and (2) the alterations of cellular patterns in the transmission electron micrographs (TEM). Exposure of *E. coli* cells for 15 min to a combination of paraquat and copper led to a marked lowering in the cellular potassium content. This lowering was in parallel to the bacterial inactivation (Table III). When the exposure was carried out at 0 °C or when detapac was added to the incubation mixture, no loss in potassium and no reduction in bacterial survival were recorded.

Figure 7 shows the TEM pictures of untreated, control, and treated cells. Panel A depicts a typical picture of an untreated cell. Indistinguishable pictures can be seen when the cells are exposed to a combination of paraquat and detapac (not shown). In panel B, a cell that had been incubated with copper alone is presented. This cell, like that in panel A, seems intact; this is in agreement with the absence of bacterial inactivation under these conditions. In contrast, panels C–F show typical examples of cells that had been exposed to the combination of paraquat and copper, under conditions where the survival proved to be less than 1%. Characteristic low-density regions (white domains) are easily seen in these panels, and these may

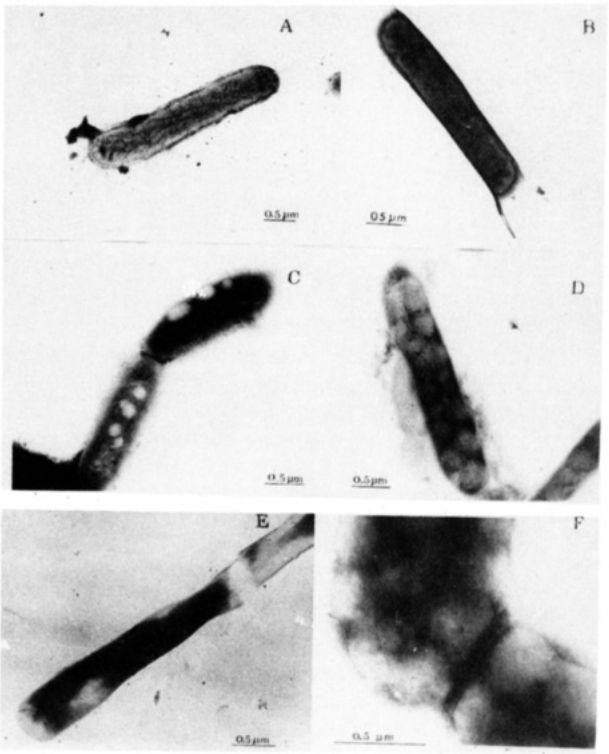

FIGURE 7: Transmission electron microscope pictures of intact and treated bacterial cells. *E. coli* B cells (2×10^7 cells/mL) were treated with a combination of paraquat and copper for 15 min in phosphate buffer (1 mM, pH 7.4) which contained glucose (0.5%). The cells were stained with phosphotungstic acid as described under Materials and Methods. (Panel A) Control cells; (panel B) cells exposed to copper alone (1 μM); (panels C–F) cells exposed to a combination of paraquat (0.25 mM) and copper (1 μM), under conditions where the survival of the cells was less than 1%.

represent sites of detachment of the cytoplasmic membrane from the bacterial capsule.

Discussion

The essential mediatory role of transition metals in damage induced to the *E. coli* cytoplasmic membrane by paraquat was indicated by studying changes in the levels of three cellular parameters: the cellular content of ATP, the cellular level of potassium, and the uptake and retention of radioactive leucine. Additional direct evidence for alterations in cellular structure indicating damage to the membrane was obtained from observations by transmission electron microscopy (TEM).

The dramatic enhancement in the rate of microbial killing, when copper or iron was added to the reaction mixture containing *E. coli* B cells and paraquat (Figures 1 and 2), was similarly reflected in the other parameters of cellular functions that were studied. A marked loss in cellular ATP and potassium and an inability to accumulate and retain leucine were

linked to the presence of *both* paraquat and copper; in itself, methyl viologen or the transition metal alone could not bring about these changes. However, addition of a chelating agent that had prevented cell killing (Figures 1 and 2) also prevented changes in these parameters. The dramatic effect on the three parameters is consistent with severe structural damage to the membrane as was seen by TEM.

This structural damage, as yet unidentified, may result in a leakage of protons, metal cations, or other low molecular weight components through the cytoplasmic membrane. In order to maintain its membrane or its chemical gradient, the cell would utilize ATP energy by a variety of pumps such as the Ca^{2+}/K^{+}-ATPase (Gelvan & Samuni, 1986). Similarly, the active transport of leucine into the cell is highly sensitive to changes in membrane potential, pH, and the protomotive force (Kohen & Chevion, 1985b). Changes in these parameters would immediately be reflected by alterations in the rate of leucine uptake (Kohen & Chevion, 1985b).

Induction of catalase led to a decrease in the rate of inactivation when the cells were exposed to paraquat and copper (Figure 5). Decrease in cell killing was associated with an analogous decrease in the dissipation of the intracellular ATP level.

These results with the cytoplasmic membrane are in accordance with the previously suggested "site-specific metal-mediated Haber–Weiss reaction" as a possible mechanism for metal-mediated paraquat-induced toxicity (Kohen, 1985). In this mechanism, paraquat is reduced to the paraquat radical, $PQ^{\bullet+}$, by a soluble reducing enzyme. $PQ^{\bullet+}$ then reacts with O_2 to produce the superoxide radical ($O_2^{\bullet-}$ that can dismute spontaneously or enzymatically (by superoxide dismutase) to hydrogen peroxide. When the efflux of $O_2^{\bullet-}$ increases, as is the case with paraquat, the superoxide radical per se is able to reduce a bound transition metal (copper or iron) in its complex to the corresponding cuprous or ferrous state. Paraquat radical itself was shown capable of reducing bound metals (Kohen, 1985; Sutton & Winterbourn, 1984; Winterbourn & Sutton, 1984). Subsequently, the reduced bound metal can react locally with hydrogen peroxide to produce the hydroxyl radical ($OH^{\bullet}$). When the enzymatic reduction of paraquat to its monocation radical form ($PQ^{2+} \rightarrow PQ^{\bullet+}$) did not take place (at 0 °C) or when the experiment was carried out under anaerobic conditions, where no superoxide radical could be formed and subsequently no hydrogen peroxide was available for the Fenton reaction, no bacterial inactivation was recorded.

While free radical induced damage to the cytoplasmic membrane has been reported by Hassan and Fridovich, the results reported here add the important focus of essential mediatory role of transition metal to membrane damage induced by active oxygen derived species (Hassan & Fridovich, 1977b).

It is worthwhile to mention that the enhancement of the paraquat-induced bactericidal effect by copper is different than that by iron. Copper is 24-fold more effective (on a molar basis), and there is a time delay in its effect (see Discussion; Korbashi et al., 1986). In addition, after its initiation, the copper effect is linear (for 3–4 orders of magnitude), while that of iron is characterized by two phases: a first linear rapid one (1–2 orders of magnitude) followed by a second slower one, that is probably associated with the precipitation or removal of available iron from the system (Korbashi et al., 1986).

In summary, the present study shows that the combination of paraquat and copper ions can cause damage to the cytoplasmic membrane of *E. coli* B and that the kinetics of formation of the damage is parallel to, or precedes, the kinetics of bacterial killing. These results are in accord with our earlier suggestion for the development of clinical procedures designed to interfere with paraquat toxicity by use of specific chelators for copper and iron, lipid-soluble antioxidants such as β-carotenes, and vitamin E.

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Registry No. PQ^{2+}, 4685-14-7; Cu, 7440-50-8; Fe, 7439-89-6.

REFERENCES

Aebi, H. (1985) *Methods Enzymol. 105*, 121–130.
Arraku, Y. (1968) *J. Biol. Chem. 243*, 3128–3135.
Bagley, A. C., Krall, J., & Lynch, B. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3189–3193.
Brown, K., & Fridovich, I. (1980) *Arch. Biochem. Biophys. 206*, 414–419.
Bus, J. S., & Gibson, J. E. (1979) *Rev. Biochem. Toxicol. 1*, 125–322.
Cole, H. A., Wimpeng, J. W. T., & Hughes, D. E. (1967) *Biochim. Biophys. Acta 143*, 445–453.
Davis, B. D., Dulbecco, R., Eisen, H. N., & Geoberg, H. S. (1980) in *Microbiology*, 3rd ed., pp 93–99, Harper and Row, Philadelphia, PA.
Farrington, J. A., Ebert, M., Land, E. J., & Fletcher, K. (1973) *Biochim. Biophys. Acta 314*, 372–381.
Fridovich, I. (1983) *Annu. Rev. Pharmacol. Toxicol. 23*, 239–245.
Gelvan, D., & Samuni, A. (1986) *Biochem. Pharmacol. 35*, 3267–3275.
Gutteridge, J. M. C., & Halliwell, B. (1982) *Biochem. Pharmacol. 31*, 2801–2803.
Halliwell, B., & Gutteridge, J. M. C. (1984) *Biochem. J. 219*, 1–14.
Hassan, H. M., & Fridovich, I. (1977a) *J. Biol. Chem. 252*, 7667–7672.
Hassan, H. M., & Fridovich, I. (1977b) *J. Bacteriol. 129*, 1579–1583.
Hassan, H. M., & Fridovich, I. (1978) *J. Biol. Chem. 253*, 8143–8148.
Hassan, H. M., & Fridovich, I. (1979a) *J. Biol. Chem. 254*, 10846–10852.
Hassan, H. M., & Fridovich, I. (1979b) *Arch. Biochem. Biophys. 196*, 385–395.
Huxley, H. E., & Zubay, G. J. (1960) *Mol. Biol. 2*, 189–196.
Kohen, R. (1985) Ph.D. Thesis, Hebrew University of Jerusalem.
Kohen, R., & Chevion, M. (1985a) *Free Radical Res. Commun. 1*, 79–88.
Kohen, R., & Chevion, M. (1985b) in *Biochemical and Inorganic Aspects of Copper Coordination Chemistry* (Karlin, K. D., & Zubieta, J., Eds.) pp 159–172, Academic, New York.
Kohen, R., & Chevion, M. (1985c) *Biochem. Pharmacol. 34*, 1841–1843.
Kohen, R., Korbashi, P., & Chevion, M. (1986a) in *Superoxide and Dismutases in Chemistry, Biology and Medicine* (Rotilio, G., Ed.) pp 349–351, Elsevier Science, New York.
Kohen, R., Szyf, M., & Chevion, M. (1986b) *Anal. Biochem. 154*, 485–491.
Korbashi, P., Kohen, R., Katzhendler, J., & Chevion, M. (1986) *J. Biol. Chem. 261*, 12472–12476.

Lavelle, F., Michelson, A. M., & Dimitrekevic, I. (1973) *Biochem. Biophys. Res. Commun. 55*, 350–357.
Richmond, R., & Halliwell, B. (1982) *J. Inorg. Biochem. 17*, 95–98.
Ross, W. E., Block, E. R., & Chang, R. Y. (1979) *Biochem. Biophys. Res. Commun. 91*, 1302–1308.
Rowley, D. A., & Halliwell, B. (1983) *Biochim. Biophys. Acta 761*, 86–90.
Samuni, A., Aronovitch, J., Godinger, D., Chevion, M., & Czapski, G. (1983) *Eur. J. Biochem. 137*, 119–124.
Shinar, E., Navok, T., & Chevion, M. (1983) *J. Biol. Chem. 258*, 14778–14783.
Sutton, H. C., & Winterbourn, C. C. (1984) *Arch. Biochem. Biophys. 235*, 106–115.
Van Osten, G. K., & Gibson, J. E. (1975) *Food Cosmet. Toxicol. 13*, 47–53.
Winterbourn, C. C., & Sutton, H. C. (1984) *Arch. Biochem. Biophys. 235*, 116–121.
Youngman, R. J., & Elstner, E. F. (1981) *FEBS Lett. 129*, 265–268.

Affinity Labeling of the P Site of *Drosophila* Ribosomes: A Comparison of Results from (Bromoacetyl)phenylalanyl-tRNA and Mercurated Fragment Affinity Reactions†

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ABSTRACT: The binding site of the peptidyl group of peptidyl-tRNA in the P site of *Drosophila* ribosomes was probed with (bromoacetyl)phenylalanyl-tRNA (BrAcPhe-tRNA). This affinity label binds specifically to the P site by virtue of its ability to participate in peptide bond formation with puromycin following its attachment to ribosomes. As many as nine ribosomal proteins may be labeled under these conditions; however, the majority of the labeling is associated with three large-subunit proteins and two small-subunit proteins. Two of the large-subunit proteins, L4 and L27, are electrophoretically very similar to the proteins labeled by the same reagent in *Escherichia coli* ribosomes L2 and L27. Reexamination by a different two-dimensional gel system of the ribosomal components labeled by a second P site reagent, the 3′ pentanucleotide fragment of *N*-acetylleucyl-tRNA which is derivatized to contain mercury atoms at the C-5 position of all three cytosine residues, shows two major and three minor labeled proteins. These proteins, L10/L11, L26, S1/S4, S13, and S20, are likely present in the binding site of the 3′ end of peptidyl-tRNA, a site that appears to span both subunits. These results have allowed us to construct a model for the protein positions in and near the peptidyl-tRNA binding site of *Drosophila* ribosomes.

Affinity labeling has proved to be an important and useful technique for establishing structure–function relationships in the ribosome. In particular, the bromoacetyl derivative of aminoacyl-tRNA has been used by several investigators to obtain information on the P site components of bacterial and eukaryotic ribosomes [reviewed by Cooperman (1980)]. The α-bromocarbonyl group is potentially reactive toward nucleophiles present in either the protein or RNA molecules in the ribosomal site. We have used this reagent to probe the binding neighborhood of the peptidyl moiety of peptidyl-tRNA located in the P site of *Drosophila* ribosomes.

We also reexamined the protein components labeled by a second P site reagent, a mercurated analogue of the 3′-terminal pentanucleotide fragment of *N*-acetylaminoacyl-tRNA. In this reagent three mercury atoms are substituted at the C-5 position of the three cytosine residues of the fragment. The mercury atoms are capable of reaction with sulfhydryl-containing protein side chains in the P site. When we first used this reagent to probe the P site of *Drosophila* ribosomes (Fabijanski & Pellegrini, 1979), we identified one ribosomal protein as the major affinity-labeled product. Subsequently, we applied the reagent to a study of rat liver ribosomes. In this system we compared two different two-dimensional (2-D)[1] gel systems for the analysis of the affinity-labeled proteins (Fabijanski & Pellegrini, 1981). The acidic first dimension, SDS second dimension gel system allowed us to identify four additional labeled proteins. Therefore, we have reexamined the *Drosophila* proteins labeled by the mercurated fragment reagent in this gel system. We now can identify two heavily labeled and three less heavily labeled proteins in that region of the P site that binds the 3′ terminus of tRNA.

Our results from the use of these two different P site reagents can be readily correlated with data on other proteins in the peptidyltransferase center. We, therefore, present a model of the arrangement of proteins present in or near this active site in *Drosophila* ribosomes.

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[1] Abbreviations: rRNA, ribosomal RNA; DTT, dithiothreitol; TCA, trichloroacetic acid; 1-D, one dimensional; 2-D, two dimensional; SDS, sodium dodecyl sulfate; BrAcPhe-tRNA, (bromoacetyl)phenylalanyl-tRNA; C(Hg)-A-C(Hg)-C(Hg)-A(Ac[^{3}H]Leu) or mercurated fragment, the 3′-terminal pentanucleotide fragment of *N*-acetylleucyl-tRNA containing a mercury atom at the three cytosine residues; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane.

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