JOURNAL OF BACTERIOLOGY, Feb. 1990, p. 691–695
0021-9193/90/020691-05$02.00/0

Vol. 172, No. 2

Effects of pH, Glucose, and Chelating Agents on Lethality of Paraquat to *Escherichia coli*

HISANORI MINAKAMI,† JEFFREY W. KITZLER,‡ AND IRWIN FRIDOVICH*

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received 1 August 1989/Accepted 6 November 1989

Retention of paraquat by *Escherichia coli* B was greatest after exposure at pH 9.0 and was progressively less after exposure at pH 7.0 and 5.0, respectively. This retained paraquat was capable of persistent growth inhibition. Uptake and retention of paraquat by *E. coli* B was dependent upon a carbon source, such as glucose. Under comparable conditions *E. coli* K-12 did not retain paraquat. The lethality of paraquat was seen in TSY medium but not in VB medium. The addition of Soytone, tryptone, or yeast extract, to the VB medium allowed the lethality of paraquat to be seen. A variety of chelating agents, including EDTA, 8-hydroxyquinoline, and *o*-phenanthroline, prevented the lethal effect of paraquat in TSY medium. Although EDTA protected against the lethality of paraquat, it did not protect against its bacteriostatic effect.

The toxic effects of paraquat are dependent upon cycles of intracellular reduction and autoxidation with concomitant production of O_2^- and, secondarily, of H_2O_2. As expected for such a mechanism, both dioxygen and a source of electrons are required for expression of the deleterious effects of this viologen (3, 6, 16). Moreover, aerobic exposure to paraquat elicits increased biosynthesis of the manganese-containing superoxide dismutase, which acts to diminish the toxic effects of paraquat (5, 6).

This clear understanding of the mechanism of action of paraquat was obscured by the finding that paraquat was bacteriostatic but not bacteriocidal (1). While attempting to explore the basis of this apparent contradiction, we learned that salts inhibit the uptake of paraquat (9) and, moreover, that there are striking differences in the retention of paraquat by different strains of *Escherichia coli* (10). Thus, *E. coli* B retains paraquat during dilution and plating, whereas *E. coli* K does not. Since the lethal effect of paraquat takes several hours to be expressed at 37°C, relatively brief exposure leads to apparent lethality in the case of the B strain but has no effect on the K strain (10). The B strain was actually killed by paraquat, not in the exposure medium but on the TSY agar plates; whereas the K strain, having lost its paraquat during dilution and plating, grew into colonies.

Awareness of these previously unsuspected complexities in the interaction of *E. coli* with paraquat led us to examine the effects of several variables on the uptake and toxicity of paraquat. We now report on the large effects of pH, glucose, and chelating agents.

MATERIALS AND METHODS

Media. TSY medium contained 30 g of tryptic soy broth, 2.5 g of glucose, and 5 g of yeast extract (all from Difco Laboratories) per liter, adjusted to pH 7.0 with HCl. Petri plates were prepared with TSY medium solidified with 1.5% Bacto-Agar (Difco). VB salts contained 0.8 mM $MgSO_4$, 9.5 mM citric acid, 44 mM K_2HPO_4, and 17 mM $Na(NH_4)HPO_4$ at pH 7.0. VB medium was VB salts plus 0.5% glucose and 1 μg of vitamin B_{12} per ml.

Strains and manipulations. *E. coli* B (ATCC 29682) and *E. coli* K-12 (ATCC 23716) were cultured for 13 to 16 h at 37°C in VB medium aerated at 200 rpm. These overnight cultures were diluted to 1×10^7 to 3×10^7 CFU/ml in the specified exposure medium; after incubation in Delong flasks at 200 rpm in air at 37°C, with a flask volume/medium volume ratio of 10:1, under the specified conditions, the cell suspension was diluted by a factor of 10^4 to 10^5 with VB salts at 25°C and plated onto TSY-agar plates. Enumeration of colonies was performed after 12 to 18 h at 37°C. Plates were incubated either aerobically or in a Coy chamber in an atmosphere of 85% N_2, 10% H_2, and 5% CO_2.

Effects of paraquat. The effects of prior exposure to paraquat on subsequent behavior were explored as follows.

(i) Growth effects. Overnight cultures in VB medium were centrifuged at 4°C, suspended in 0.2 volume of 25 mM phosphate buffer at pH 7.0 and 25°C, and then diluted into the specified exposure medium with or without 10 μM paraquat at 37°C in air at 200 rpm. After 30 min of exposure the cells were collected by centrifugation in the cold, washed by suspension and centrifugation in the same medium lacking paraquat and glucose, and then suspended in VB medium containing 1 μg of thiamine per ml to an A_{600} of 0.08 to 0.12. These cell suspensions were then incubated at 37°C in Delong flasks shaken in air at 200 rpm, and the A_{600} was monitored.

(ii) Measurement of uptake of paraquat. ^{14}C-labeled paraquat with a specific radioactivity of 10 mCi/mmol was obtained from Sigma Chemical Co., and its radiochemical purity was confirmed by thin-layer chromatography. Samples (0.5 ml) of mid-log-phase cultures of *E. coli* in TSY medium were sedimented for 2 min in a Fisher microfuge. The cell pellet was suspended in 0.5 ml of the specified solutions containing 10 μM ^{14}C-labeled paraquat (0.1 μCi/ml), followed by aerobic incubation at 37°C. At intervals the cells were collected by centrifugation for 2 min and washed once by suspension in 25 mM neutral sodium phosphate buffer, followed by centrifugation. These washed cells were suspended in 0.4 ml of water and then mixed with 4.5 ml of Aquasol, and the level of retained ^{14}C was estimated by scintillation counting.

* Corresponding author.

† Permanent address: Department of Obstetrics and Gynecology, Jichi Medical School, Minami Kawachi-machi, Tochigi, 329-04, Japan.

‡ Present address: Biochemistry Department, University of California, Berkeley, CA 94720.

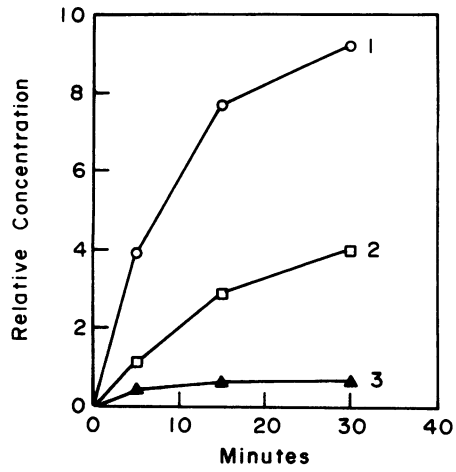

FIG. 1. Effects of pH on the retention of paraquat by *E. coli* B. *E. coli* B was incubated with 10 μM ^{14}C-labeled paraquat in 25 mM potassium phosphate–0.5% glucose at pH 9.0 (curve 1), 7.0 (curve 2), or 5.0 (curve 3). At intervals samples were removed; after washing, the level of retained paraquat was measured by scintillation counting as described in Materials and Methods.

RESULTS

Effects of pH on uptake and retention of paraquat. As the pH was raised from 5 to 7 to 9, *E. coli* B took up and retained progressively greater amounts of paraquat. Figure 1 presents the results of exposing *E. coli* B to 10 μM ^{14}C-labeled paraquat plus 0.5% glucose in 25 mM phosphate buffers at pH of 5, 7, or 9 for different times at 37°C. These data represent paraquat retained by the cells after washing. Since saturation behavior was evident, they reflect an effect of pH upon the capacity for retention of paraquat within *E. coli* B, rather than an effect upon an initial rate of paraquat uptake.

Since *E. coli* B has previously been shown to retain paraquat for long periods of time (10), it appeared possible to demonstrate the effect of pH on paraquat retention in terms of growth inhibition after exposure and washing (Fig. 2). Thus *E. coli* B was incubated for 30 min at 37°C in 0.5% glucose–10 μM paraquat–25 mM phosphate buffer at pH 5, 7, or 9. The cells were then washed and suspended in VB medium containing 1 μg of thiamine per ml, and aerobic growth was monitored at 37°C. Growth was most severely inhibited in cells that had been loaded with paraquat at pH 9.0 and was least affected by prior loading with the viologen at pH 5.0 (Fig. 2). Prior incubation at pH 9.0 in the absence of paraquat did not prevent subsequent growth (Fig. 2, curve 1). Prior loading with paraquat at pH 9.0 was more inhibitory of subsequent growth than was prior loading at pH 7.0, which, in turn, caused more inhibition than did loading at pH 5.0 (Fig. 2, curves 2 through 4). Figure 2, curve 5, demonstrates the inhibitory effect of 5 μM paraquat upon cells not previously exposed to this viologen.

E. coli K-12 was previously shown not to retain paraquat (10). That being the case, prior exposure of the K-12 strain to paraquat at pH 5, 7, or 9 should have little effect upon subsequent growth in paraquat-free VB medium. This expectation was fulfilled (Fig. 3); 5 μM paraquat in the medium caused greater inhibition of growth of *E. coli* B than of *E. coli* K-12 (compare curves 5 in Fig. 2 and 3). This growth inhibition exhibited a lag in the B strain (Fig. 2) but not in the K-12 strain (Fig. 3). This difference probably reflects gradual accumulation of paraquat within *E. coli* B because of its ability to retain this compound.

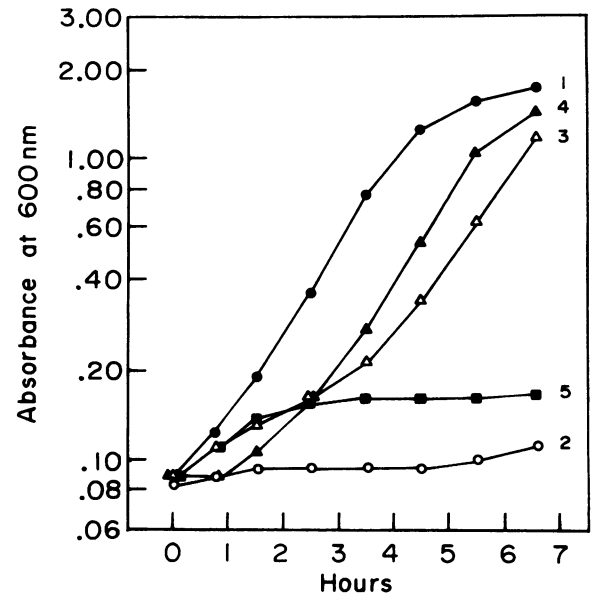

FIG. 2. Effects of retained paraquat on the growth of *E. coli* B. *E. coli* B was incubated for 30 min in 25 mM potassium phosphate at pH 5.0, 7.0, or 9.0, with or without 5 or 10 μM paraquat. The cells were washed and suspended in VB medium containing 1 μg of thiamine per ml, and growth was followed in terms of A_{600}. Curves: 1, pH 9.0, no paraquat; 2, pH 9.0, with paraquat; 3, pH 7.0, with paraquat; 4, pH 5.0, with paraquat; 5, pH 9.0 with 5 μM paraquat added to the growth medium.

Effects of glucose. *E. coli* B has been shown to be capable of taking up paraquat against a concentration gradient, and mutants defective in this uptake have been described (7). Active uptake requires energy. We therefore anticipated that uptake and retention of paraquat by *E. coli* B should depend upon the presence of glucose. The data in Fig. 4 demonstrate that this was the case.

Because of the ability of the *E. coli* B to retain paraquat,

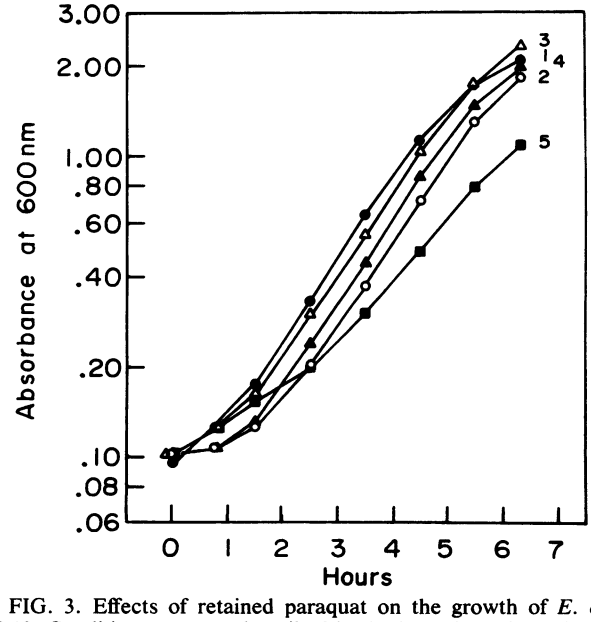

FIG. 3. Effects of retained paraquat on the growth of *E. coli* K-12. Conditions were as described in the legend to Fig. 2, but *E. coli* K-12 was used in place of *E. coli* B.

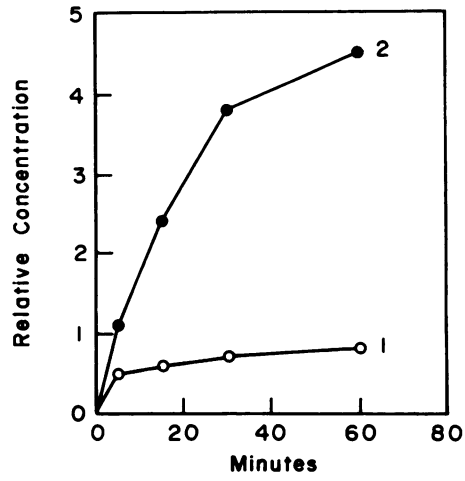

FIG. 4. Effect of glucose on the retention of paraquat by *E. coli* B. *E. coli* B was incubated with 10 μM ^{14}C-labeled paraquat in 25 mM potassium phosphate at pH 7.0 without (curve 1) or with (curve 2) 0.5% glucose. At intervals samples were removed; after washing, the level of paraquat retained by the cells was determined by liquid scintillation counting.

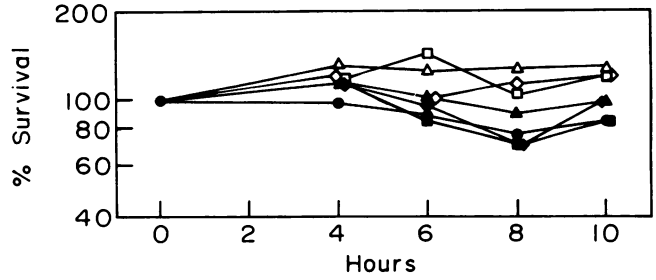

FIG. 5. Paraquat exerts no lethality on *E. coli* K-12 in VB medium with or without Cu(II). A 1.2% inoculum of *E. coli* K-12 grown in VB medium was placed into fresh VB medium containing 5.0 mM paraquat and the following levels of $CuSO_4$: ○, none; ■ and □, 1 μM; ▲ and △, 10 μM; ◆ and ◇, 100 μM. At the indicated intervals samples were removed, diluted, and plated on TSY-agar. Open symbols denote anaerobic incubation of the TSY plates, whereas closed symbols denote aerobic incubation.

it appeared possible to demonstrate the effect of glucose on paraquat uptake in terms of persistent growth inhibition after washing and suspension in fresh VB medium. The technique used was similar to that which had been applied to explore the effect of pH on paraquat uptake (Fig. 2). The results showed that for *E. coli* B, but not for *E. coli* K-12, prior exposure to paraquat in the presence of glucose imposed a more profound inhibition of growth than was the case in the absence of glucose (data not shown).

Effects of the medium on the lethality of paraquat. We previously reported that *E. coli* B did not suffer loss of viability when exposed to 2.5 mM paraquat in VB medium for up to 10 h, provided that the TSY-agar plates used for enumeration were incubated anaerobically (10). In *E. coli* K-12, which does not retain paraquat, no lethality was observed whether the TSY-agar plates were incubated aerobically or anaerobically (Fig. 5). The addition of Cu(II) to the VB medium did not make *E. coli* K-12 susceptible to the lethality of paraquat (Fig. 5).

That *E. coli* B failed to succumb to paraquat in aerobic VB medium or on anaerobic TSY-agar, but did so on aerobic TSY-agar, suggested that some component of the TSY medium was required for expression of the lethality of paraquat. *E. coli* K-12 did not lose viability during 8 h of incubation with 2.5 mM paraquat in VB medium (Fig. 6, curve 1). The addition of 0.3% Soytone (Difco) (curve 2), 1.7% tryptone (curve 3), or 0.5% yeast extract (curve 4) supported a progressively greater lethality. Some component present in the yeast extract, and to a lesser degree also present in the protein hydrolysates, was clearly able to allow expression of the lethality of paraquat.

The work of Chevion and associates (11–13) suggested that the essential component might be a transition metal cation. *E. coli* B was therefore exposed to 2.5 mM paraquat in TSY medium with or without chelating agents. At intervals samples were taken, diluted, and spread onto TSY-agar for enumeration of surviving cells. These plates were incubated anaerobically to avoid lethality on the plates caused by retained paraquat. Lethality became apparent after a lag of approximately 2 h (Fig. 7, curve 1), and EDTA (curve 2) or *o*-phenanthroline (curve 3) protected against lethality. A similar experiment was performed with *E. coli* K-12 with very similar results. With *E. coli* K-12, it was not necessary to incubate the TSY-agar plates anaerobically, because this K-12 strain does not retain paraquat. Figure 8 presents the results obtained with *E. coli* K-12. Once again there was a 2-h lag before lethality became apparent (curve 1), whereas curves 2, 3, 4 and 5 illustrate the progressively greater protection provided by desferrioxamine, 8-hydroxyquinoline, EDTA, and *o*-phenanthroline, respectively.

We have previously shown that the bacteriostatic and the

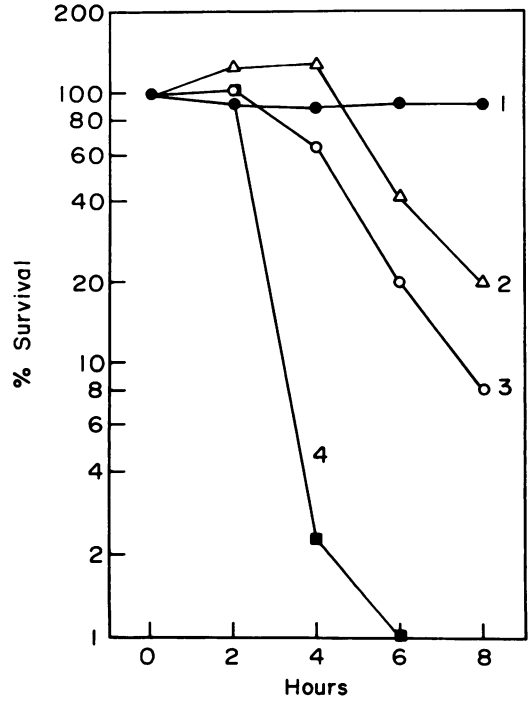

FIG. 6. Effects of complex medium components on the lethality of paraquat to *E. coli* K-12. *E. coli* K-12 was exposed to 2.5 mM paraquat in VB medium containing the following additions (curves): 1, none; 2, 0.3% Soytone; 3, 1.7% tryptone; 4, 0.5% yeast extract. At intervals samples were removed, diluted, and plated on TSY agar. Enumeration was done after plates were incubated for 16 h.

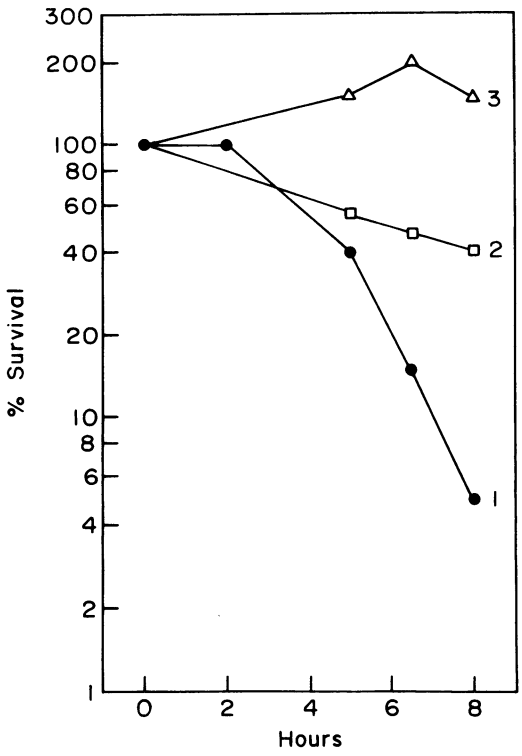

FIG. 7. Effects of chelating agents on the lethality of paraquat to *E. coli* B in TSY medium. *E. coli* B was exposed to 2.5 mM paraquat in TSY medium containing the following additions (curves): 1, none; 2, 0.5 mM EDTA; 3, 0.5 mM *o*-phenanthroline. At intervals samples were removed, diluted, and plated onto TSY-agar plates, which were incubated overnight anaerobically before enumeration.

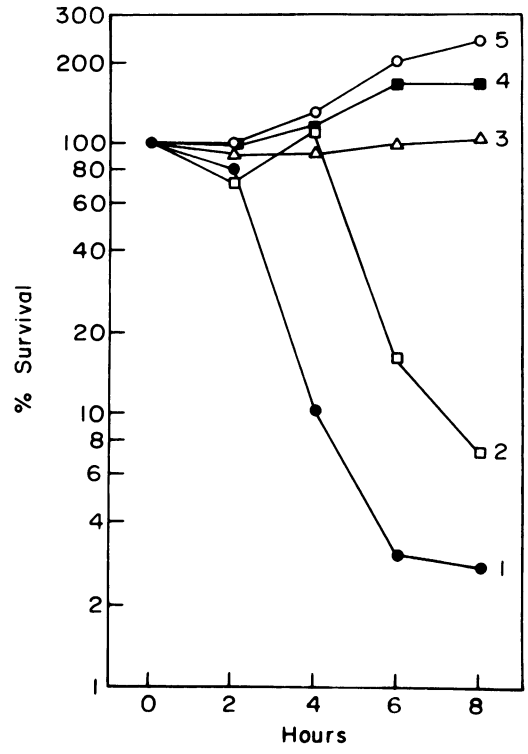

FIG. 8. Effects of chelating agents on the lethality of paraquat to *E. coli* K-12 in TSY medium. E. coli K-12 was exposed to 5.0 mM paraquat in TSY medium containing the following additions (curves): 1, none; 2, 0.5 mM desferrioxamine; 3, 0.5 mM 8-hydroxyquinoline; 4, 0.5 mM EDTA; 5, 0.5 mM *o*-phenanthroline. At intervals samples were removed for plating and counting. The TSY-agar plates were incubated overnight aerobically before enumeration.

bactericidal effects of paraquat on *E. coli* are distinct phenomena, reflecting attack on different targets (8). We therefore inquired whether the protective effects of chelating agents applied to both of these effects of paraquat. *E. coli* B was exposed to 2.5 mM paraquat in TSY medium. At intervals samples were taken and, after dilution, were plated onto TSY-agar plates with or without 500 μM EDTA. These plates were incubated first aerobically for 16 h and then anaerobically for an additional 30 h. Colonies were enumerated, without opening the plates, after both the aerobic and the subsequent anaerobic incubations. Exposure of *E. coli* B to paraquat led to cell death on the aerobically incubated plates lacking EDTA, since subsequent anaerobic incubation of these plates did not increase enumeration (Fig. 9, curves 1 and 2). In contrast, the paraquat-exposed cells failed to grow on the aerobic EDTA-containing plates, but they did not lose viability, since subsequent anaerobic incubation allowed growth into visible colonies (curves 3 and 4). It follows that EDTA protected against the lethality of retained paraquat but not against its bacteriostatic effect, both of which are, of course, dioxygen dependent. EDTA at 0.5 mM in TSY medium at 37°C did not strongly inhibit growth. Indeed, it increased the generation time from 27 min to 36 min (data not shown).

Effect of paraquat: intracellular or extracellular. Repeated cycles of reduction of paraquat, followed by its autoxidation, will result in increased intracellular production of O_2^- and secondarily of H_2O_2. Since the paraquat monocation radical is more able to cross the cell envelope than is the oxidized dicationic form (4) and since H_2O_2 should be able to diffuse across the cell envelope, we wondered whether the lethal effects of paraquat were due, at least partially, to extracellular events.

In that case, the addition of superoxide dismutase or catalase to the suspending medium might provide some protection. An inoculum of *E. coli* K-12 was transferred from an overnight culture in VB medium into TSY medium to which 0.25% glucose and 5.0 mM paraquat had been added. At intervals during aerobic incubation at 37°C, samples were taken for dilution, plating, and counting. The results reflected the lethality of paraquat after a lag of 1 to 2 h. Neither catalase at 10 μg/ml nor superoxide dismutase at 1 μg/ml in the exposure medium afforded any protection (data not shown). It thus appears that the lethality of paraquat is overwhelmingly due to intracellular events.

DISCUSSION

The uptake of paraquat into *E. coli* B was markedly augmented by the availability of glucose, as expected for an energy-dependent active transport process. We previously noted that paraquat had no effect upon *E. coli* B in the absence of a readily metabolizable carbon source (6). This result was interpreted in terms of the need for a source of electrons to support the redox cycling of paraquat, which is the basis of its oxygen-dependent toxicity. Since these earlier studies involved enumeration on TSY-agar plates and used *E. coli* B, it is now clear that the effect of the carbon source was really an effect on the active uptake of paraquat;

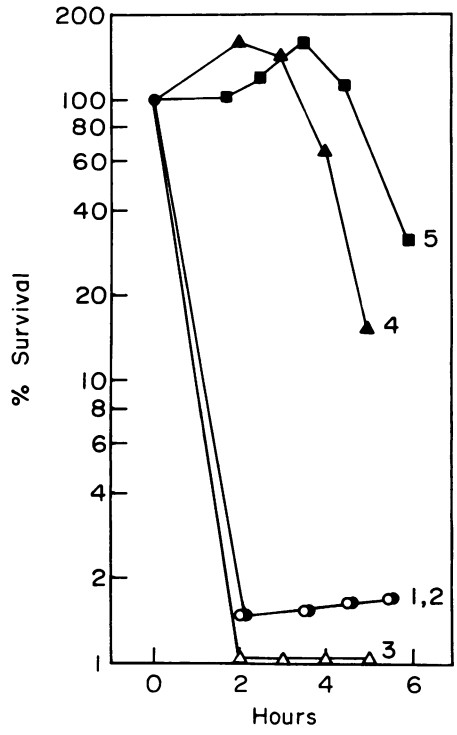

FIG. 9. Effects of EDTA on the bacteriostatic and bactericidal effects of paraquat on *E. coli* B. *E. coli* B was exposed to 2.5 mM paraquat in TSY medium. At the indicated intervals samples were taken for dilution and plating. The conditions applied before enumeration were as follows (curves): 1, TSY-agar after 16 h of aerobic incubation; 2, as in curve 1, but after 30 h of additional incubation under anaerobic conditions; 3, TSY–0.5 mM EDTA–agar after 16 h of aerobic incubation; 4, as in curve 3, but after 30 h of additional anaerobic incubation; 5, TSY-agar after 16 h of anaerobic incubation.

if this were not the case, *E. coli* B which had taken up sufficient paraquat would then have suffered lethality on the TSY-agar.

Elevation of pH in the range of 5.0 to 9.0 increased the amount of paraquat retained by *E. coli* B. This effect of pH was seen either in terms of retention of ^{14}C-labeled paraquat or in terms of inhibition of growth by retained paraquat. *E. coli* possesses homeostatic mechanisms that diminish the effect of changes of external pH on the internal pH (15). Moreover, elevation of external pH increases the membrane electrochemical gradient (2, 14, 17), which provides energy for active uptake. The increasing uptake and retention of paraquat by *E. coli* B with elevation of pH (Fig. 1) thus probably reflects an effect of pH on the membrane electrochemical gradient.

Some component of the TSY medium is as important for supporting the lethality of paraquat as are dioxygen and an electron source. This essential component is present in yeast extract, tryptone, and Soytone. Since chelating agents sharply diminished the lethality of paraquat in TSY medium but did not prevent its bacteriostatic effect, we suppose that a metal cation is a factor in the former but not in the latter. It appears that the agonist of paraquat lethality is a product of the interaction of a transition metal cation with some component of the TSY medium. The precise nature of this interaction remains to be determined.

ACKNOWLEDGMENTS

This work was supported in part by grants from The Council for Tobacco Research-U.S.A., Inc., the American Cancer Society, the National Science Foundation, and the National Institutes of Health.

LITERATURE CITED

1. **Fee, J. A., A. C. Lees, P. L. Bloch, P. L. Gilliland, and O. R. Brown.** 1981. Oxygen stasis of bacterial growth: analogy between the stasis of *E. coli* by hyperbaric oxygen and by aerobic paraquat. Biochem. Int. **1**:304–311.
2. **Felle, H., J. S. Porter, C. L. Slayman, and H. R. Kaback.** 1980. Quantitative measurement of membrane potential in *Escherichia coli*. Biochemistry **19**:3585–3590.
3. **Fisher, H. K., and G. Williams.** 1976. Paraquat is not bacteriostatic under anaerobic conditions. Life Sci. **19**:421–426.
4. **Jones, R. W., T. A. Gray, and P. B. Garland.** 1976. A study of the permeability of the cytoplasmic membrane of *Escherichia coli* to reduced and oxidized benzyl viologen and methyl viologen cations. Biochem. Soc. Trans. **4**:671–673.
5. **Hassan, H. M., and I. Fridovich.** 1977. Regulation of the synthesis of superoxide dismutase in *E. coli*: induction by methyl viologen. J. Biol. Chem. **252**:7667–7672.
6. **Hassan, H. M., and I. Fridovich.** 1978. Superoxide radicals and the oxygen enhancement of the toxicity of paraquat in *Escherichia coli*. J. Biol. Chem. **253**:8143–8148.
7. **Kao, S. M., and H. M. Hassan.** 1985. Biochemical characterization of a paraquat-tolerant mutant of *Escherichia coli*. J. Biol. Chem. **260**:10478–10481.
8. **Kitzler, J., and I. Fridovich.** 1986. The effects of paraquat on *Escherichia coli*: distinction between bacteriostasis and lethality. J. Free Rad. Biol. Med. **2**:245–248.
9. **Kitzler, J., and I. Fridovich.** 1986. Effects of salts on the lethality of paraquat. J. Bacteriol. **167**:346–349.
10. **Kitzler, J. W., H. Minakami, and I. Fridovich.** 1990. Effects of paraquat on *Escherichia coli*: differences between B and K-12 strains. J. Bacteriol. **172**:686–690.
11. **Kohen, R., and M. Chevion.** 1985. Transition metals potentiate paraquat toxicity. Free Rad. Biol. Med. **1**:79–88.
12. **Kohen, R., and M. Chevion.** 1988. Cytoplasmic membrane is the target organelle for transition metal-mediated damage induced by paraquat in *Escherichia coli*. Biochemistry **27**:2597–2603.
13. **Korbashi, P., R. Kohen, J. Katzhendler, and M. Chevion.** 1986. Iron mediates paraquat toxicity in *Escherichia coli*. J. Biol. Chem. **261**:12472–12476.
14. **Padan, E., D. Zilberstein, and S. Schuldiner.** 1981. pH homeostasis in bacteria. Biochim. Biophys. Acta **650**:151–166.
15. **Slonczewski, J. L., B. P. Rosen, J. R. Alger, and R. M. Macnab.** 1981. pH Homeostasis in *Escherichia coli*: measurement by ^{31}P nuclear magnetic resonance of methyl phosphonate and phosphate. Proc. Natl. Acad. Sci. USA **78**:6271–6275.
16. **Yonei, S., A. Noda, A. Tachibana, and S. Akasaka.** 1986. Mutagenic and cytotoxic effects of oxygen free radicals generated by methylviologen (paraquat) on *Escherichia coli* with different DNA-repair capacities. Mutat. Res. **163**:15–22.
17. **Zilberstein, D., S. Schuldiner, and E. Padan.** 1979. Proton electrochemical gradient in *Escherichia coli* cells and its relation to active transport of lactose. Biochemistry **18**:669–73.