# Bacteriostasis of *Escherichia coli* by the Herbicide Paraquat (35264)

CLAIRE L. DAVISON AND BRUNO PAPIRMEISTER

(Introduced by Morris N. Green)

*Department of Experimental Medicine, Medical Research Laboratory, Research Laboratories, Edgewood Arsenal, Maryland 21010*

The herbicide paraquat, 1,1′-dimethyl-4, 4′-bipyridilium dichloride (Gramoxone W and Weedol), has been cited as causing a number of human poisonings with pathologic manifestations in the lung, liver, and central nervous system (1). A significant decrease in pulmonary surfactant material of lungs of rats exposed to paraquat has been reported (2). In addition to the human, plant, or animal problem, there is concern for the effect of herbicides on the microscopic members of the ecological chain, such as bacteria (3). These considerations prompted us to study the action of paraquat at the cellular level, especially since little work has been done in this area.

*Material and Methods. Cells and media.* *Escherichia coli* B/r and *E. coli* Bs-1, representing a radiation-resistant and a radiation-sensitive derivative of *E. coli* B (4), respectively, were studied. Cells were either grown overnight or to log phase at $5 \times 10^8$/ml in Hershey's medium (HM) (5). $T_1$ bacteriophage was a laboratory strain originally obtained from Dr. R. M. Herriott of the Johns Hopkins University. HeLa cells were obtained from a cell line kept in continuous culture in our laboratory in Eagle's minimum essential medium (EM) (6).

*Chemicals.* Paraquat, supplied by Dr. J. A. Clements of the University of California Medical Center, San Francisco, was dissolved in distilled water at $10^{-1}$ *M* and kept at —20° until use.

Carrier-free $H_3{}^{32}PO_4$ and L-leucine-1-$^{14}$C (20 mCi/mmole) were purchased from New England Nuclear Corp., uridine-$^3$H (2 Ci/mmole) from I.C.N. Corp; and glucose-6-$^{14}$C (23 mCi/mmole) from Nuclear Chicago Corp.

*Growth and viability measurements.* The bacterial cells were diluted to approximately $2 \times 10^7$/ml in HM containing concentrations of paraquat at $10^{-5}$, $10^{-4}$, $10^{-3}$, and $10^{-2}$ *M*. Growth at 37° was measured turbidimetrically at 650 m$\mu$ in a Coleman Jr. spectrophotometer. The titer of viable cells was obtained by plating diluted aliquots on nutrient agar plates.

$T_1$ bacteriophage were treated by exposing viral suspensions of $1 \times 10^9$ particles/ml in 0.1 *M* Tris buffer, pH 7.6, to $10^{-4}$, $10^{-3}$, and $10^{-2}$ *M* paraquat for 1 hr at room temperature (24°). Virus and agent were next diluted in saline, and samples were plated with *E. coli* B as the indicator strain.

For comparative purposes, one experiment utilizing HeLa cells was conducted by gently shaking cells in 37° EM supplemented with paraquat at $10^{-5}$, $10^{-4}$, $10^{-3}$, and $10^{-2}$ *M*. Aliquots were removed at 1, 4, 6, and 24 hr and checked for viability by the Erythrosin B dye exclusion method (7).

*Stability of the cellular phosphate pool.* The procedure used was adapted from a technique of Rye and Wiseman (8) as follows: log phase cells were filtered, washed three times in phosphate-free HM minus glucose that had been warmed to 37°, and then suspended in phosphate-free HM at 37°. Carrier-free neutralized $H_3{}^{32}PO_4$ was added at 0.025 $\mu$Ci/ml and after 30-sec incubation, 0.1 *M* $KH_2PO_4$ was added to reduce the specific activity. After 1 min, cells were washed several times with $5 \times 10^{-3}$ *M* phosphate buffered saline, pH 7.6, and added to 20° HM minus glucose containing paraquat at $10^{-5}$, $10^{-4}$, and $10^{-3}$ *M*. At 10 and again at 20 min, duplicate 2-ml samples were taken for measurement of total $^{32}$P and acid-

insoluble $^{32}P$. For total $^{32}P$ the sample was filtered through a 25-mm, 0.45 $\mu$ Millipore filter, washed twice with $5 \times 10^{-3}$ *M* phosphate-buffered saline, pH 7.6, and dried. The filter containing the sample was then agitated in the scintillation solution of Goldstein and Brown (9) and the $^{32}P$ activity was counted in a Packard scintillation spectrometer. For acid insoluble $^{32}P$, the sample was added to 2 ml of 10% trichloroacetic acid at 4° for 30 min to extract the acid-soluble pool. The sample was then Millipore-filtered, dried, and counted as above. The metabolic pool is the difference between total $^{32}P$ and acid-insoluble $^{32}P$.

*Macromolecular syntheses determination.* *E. coli* cell cultures grown overnight in HM were diluted in HM containing $10^{-5}$, $10^{-4}$, and $10^{-3}$ *M* paraquat, and supplemented with appropriate radioactive precursor. For DNA and RNA synthesis, cells were grown at 37° in the presence of 1 $\mu$Ci of $^{32}P$/ml, (1.5 $\mu$Ci/$\mu$mole), and incorporation of $^{32}P$ into each constitutent was assessed by a modified Schmidt-Thannhauser procedure (10). Protein synthesis was measured by growing cells in 37° HM containing 50 $\mu$g/ml of unlabeled L-leucine and 1 $\mu$Ci/ml of *l*-leucine-1-$^{14}C$, and measuring the incorporation of radiocarbon into a product which is insoluble in 5% trichloroacetic acid. Messenger RNA synthesis was measured by the extent to which cells incorporated tritium from HM supplemented with 50 $\mu$Ci/ml of uridine-$^{3}H$ during a 1-min incubation at 37°.

Radioactive samples were counted in a Packard scintillation spectrometer using a cocktail described by Goldstein and Brown (9).

*Glucose utilization.* The metabolism of glucose was measured by shaking bacteria in the presence of $10^{-5}$, $5 \times 10^{-5}$, and $10^{-4}$ *M* paraquat and measuring the production of $^{14}CO_2$ from labeled glucose. The HM employed for this experiment was supplemented with 5 mg/ml of unlabeled glucose and 2 $\mu$Ci/ml of glucose-6-$^{14}C$. The technique followed was based on Buhler's method of entrapment of $^{14}CO_2$ (11).

*Results.* Most studies reported herein were performed with both *E. coli* B/r and Bs-1. Since these cells possess different abilities to repair damage to the DNA by various agents (*e.g.*, UV, mustard, mitomycin, etc.) (12), we studied them to assess possible production of genetic damage by paraquat. The results obtained with both strains were practically identical; therefore, only the findings obtained with strain B/r are reported.

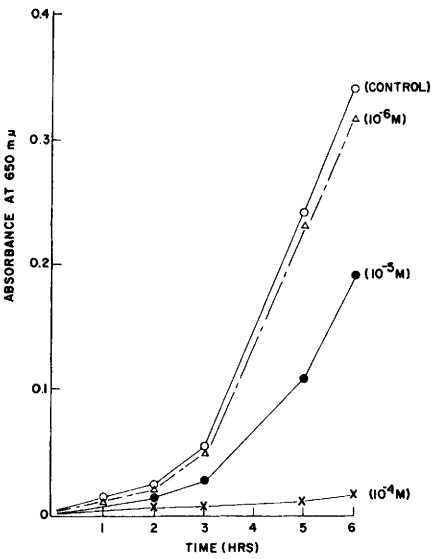


FIG. 1. Effect of paraquat on cell growth of *E. coli* B/r as measured by the absorbance at 650 m$\mu$.

*Effect of paraquat on growth and viability.* Paraquat caused a dose-dependent slowing of bacterial growth (Fig. 1). As little as

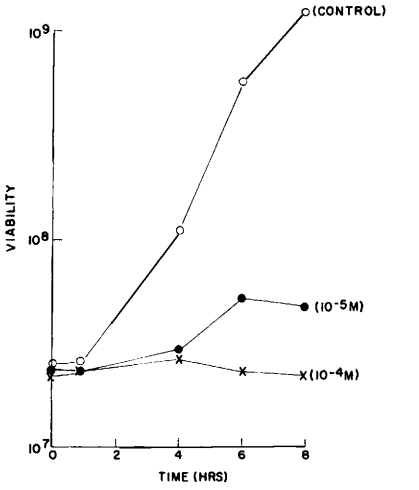


FIG. 2. Effect of paraquat on viability of *E. coli* B/r as measured by the number of colony forming cells/ml.

TABLE I. Effect of Paraquat on the Viability of HeLa Cells.

| Exposure time (hr) | Survival[a] in the presence of paraquat | | | | |
|---|---|---|---|---|---|
| | Control[b] | $M$: $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | $10^{-2}$ |
| 1 | 1.0 | 1.1 | 1.0 | 1.0 | 0.95 |
| 4 | 1.0 | 0.95 | 0.89 | 0.82 | 0.42 |
| 6 | 1.0 | 0.93 | 1.0 | 0.83 | 0.32 |
| 24 | 1.0 | 1.1 | 0.35 | 0.15 | <0.01 |

[a] Survival = (% viable in presence of paraquat)/(% viable in absence of paraquat); calculations based on at least 100 or more cells/sample.

[b] The viability of control cells at the start of the experiment was greater than 80% and dropped to approximately 50% after 24 hr.

$10^{-5}$ $M$ paraquat (2.5 μg/ml) significantly decreased the rate of growth while a $10^{-4}$ $M$ concentration produced almost complete inhibition. The action of paraquat on *E. coli* is bacteriostatic rather than bactericidal; so that upon diluting out the paraquat, exposed cells recovered fully, forming appropriate numbers of colonies of normal size and morphology (Fig. 2). A $10^{-4}$ and $10^{-3}$ $M$ concentration (not shown) produced complete but reversible inhibition of cell multiplication. However, a 1-hr exposure of *E. coli* or $T_1$ bacteriophage to $10^{-2}$ $M$ paraquat killed approximately 90% of the organisms.

For comparative purpose, one experiment with paraquat was performed with HeLa cells (Table I). Results indicate that cytotoxicity of paraquat on the mammalian cell is a function of both dose and time of exposure. At the highest concentration of $10^{-2}$ $M$, 6 hr of exposure caused the killing of 68% of the cells as noted by dye uptake (7). However, at a concentration of $10^{-4}$ $M$, 24 hr of exposure was needed to produce a similar killing.

The concentration range of paraquat found to be effective against HeLa cells was similar to that for the bacterial systems.

*Effect of paraquat on the stability of the phosphate pool.* To test for possible damaging effects of paraquat on cellular membranes, we studied the loss of phosphate from cells which had been radiolabeled during a 30-sec exposure to high specific activity $^{32}P$. In this assay, a typical bactericidal compound like chlorhexidine caused a dose-dependent loss of the $^{32}P$ from *E. coli* cells in 5 min or less (8). On the other hand, paraquat was relatively inert. Results shown in Fig. 3 indicate approximately 20% loss of labeled phosphate at $10^{-5}$ $M$ but no further loss at $10^{-4}$ or $10^{-3}$ $M$ when cells were exposed for 20 min. Therefore, this limited loss appears to be related to paraquat effect on surface-bound phosphate components rather than on intracellular phosphate pools.

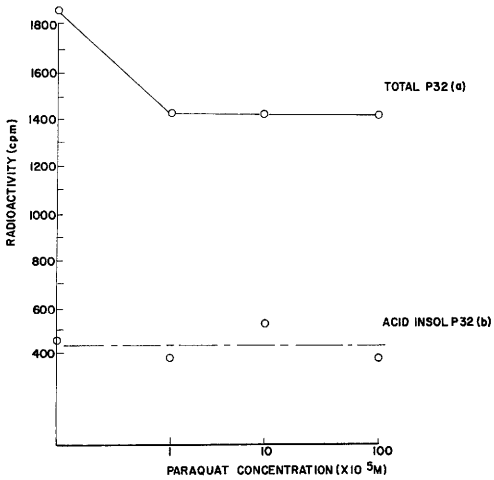


FIG. 3. Effect of paraquat on the intracellular phosphate pool of *E. coli* B/r. Cells were pulse-labeled with $^{32}P$ and exposed to paraquat for 20 min at 20°. The intracellular pool is the difference between curves (a) and (b).

*Effect of paraquat on metabolism.* Paraquat severely inhibited the synthesis of DNA, RNA, messenger RNA, and protein. A dose–response study showed quantitatively similar inhibitions for cell macromolecules studied at $10^{-5}$ and $10^{-4}$ $M$ concentrations (Figs. 4A–D). Glucose utilization also showed a similar pattern of sensitivity, a

$10^{-4}$ *M* concentration of paraquat being sufficient for 70% inhibition of $CO_2$ production (Fig. 5).

*Discussion.* These data demonstrate that the principal action of the herbicide paraquat dichloride on bacteria is bacteriostasis since *E. coli* B/r can recover its ability for colony formation after the paraquat is diluted from the growth medium. This bacteriostatic action is consistent with the failure of paraquat to damage the bacterial membrane, as was shown by the stability of the intracellular phosphate pools. Also, bacteriostasis is supported by the observation that paraquat sim-

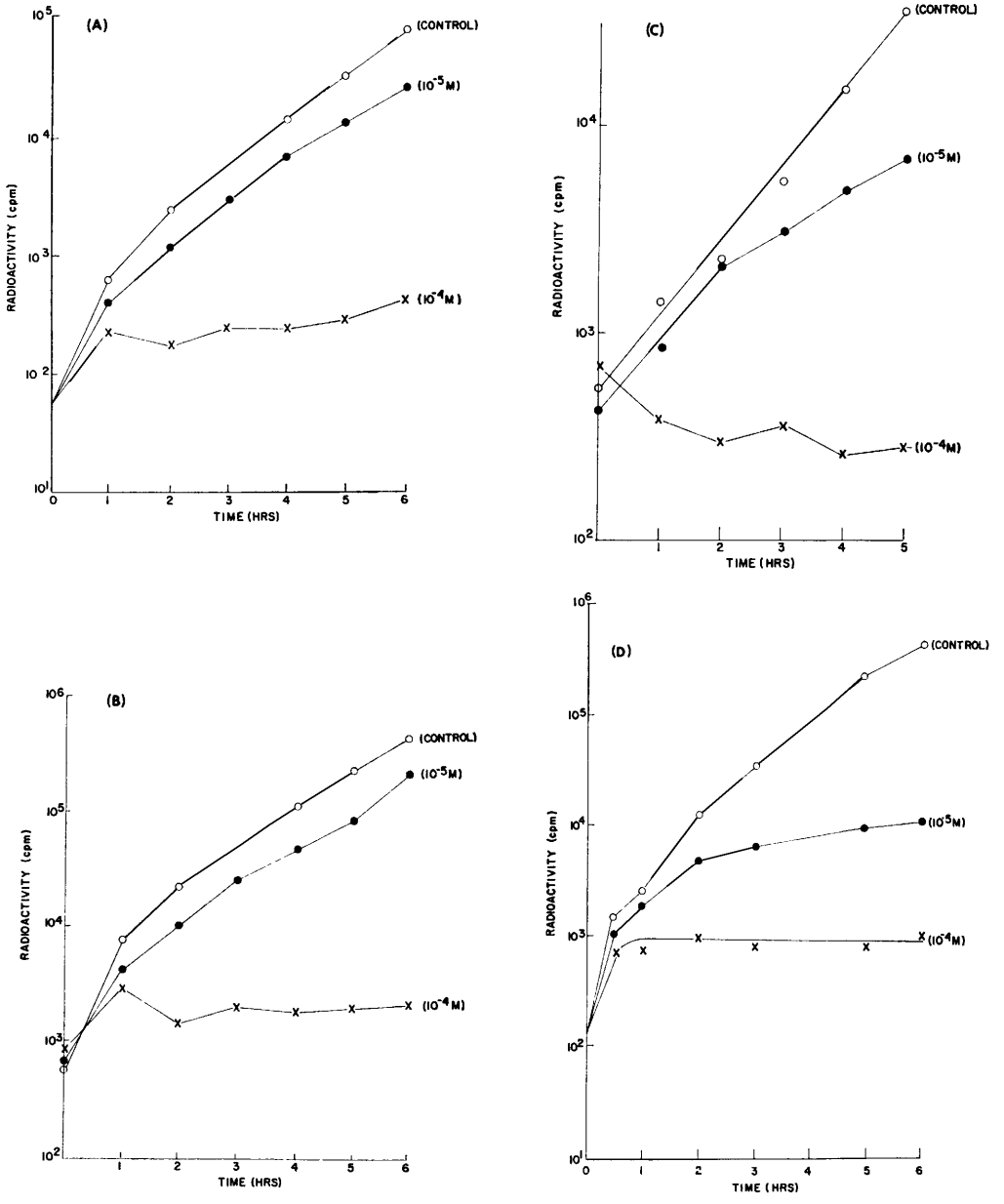


FIG. 4A–D. Action of paraquat on the synthesis of some cellular constituents of *E. coli* B/r. (A) DNA synthesis, $^{32}P$ incorporation; (B) RNA synthesis, $^{32}P$ incorporation; (C) messenger RNA synthesis, 1-min uridine-$^{3}H$ pulse; (D) protein synthesis, L-leucine-1-$^{14}C$ incorporation.

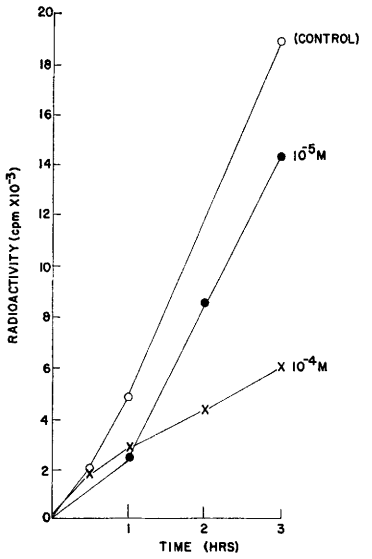


FIG. 5. Action of paraquat on the utilization of glucose-6-$^{14}$C by *E. coli* B/r as measured by $^{14}CO_2$ production.

ultaneously inhibits syntheses of macromolecular constituents such as DNA, RNA, messenger RNA, proteins, as well as glucose utilization. Such massive metabolic inhibition most likely precludes lethality due to unbalanced growth.

Since many metabolic functions were similarly affected, it appears likely that the primary action of paraquat is on some central process, such as energy metabolism. The sensitivity of $CO_2$ production to paraquat supports this view as well as Gage's observation of the paraquat effect on the biological redox reactions (13). Additionally, it may act bacteriostatically by forming inhibitory metal chelates similar to a number of 2,2-bipyridine hydrochlorides (14).

The sensitivity of mammalian cells (HeLa) to paraquat did not differ greatly from that found with microorganisms. For both cell types paraquat at $10^{-5}$, $10^{-4}$, and $10^{-3}$ *M* produced stasis effects. Extended exposure to these concentrations or exposure to a concentration of $10^{-2}$ *M* was required for bactericidal or cytotoxic effects.

The lack of damage to DNA by this herbicide is suggested by the identical sensitivities of *E. coli* B/r and Bs-1. If DNA damage were a factor, we would have expected the two strains to have different sensitivities since repair capable and incapable strains differ in their ability to recover from DNA damage by various agents (12). In addition, unpublished findings show that paraquat does not act as an inhibitor of DNA repair since it does not increase the killing of sulfur mustard-treated cells as do typical DNA-repair inhibitors, such as caffeine and acriflavine (12).

Under the conditions of these experiments, the recovery of exposed cells by dilution and the apparent lack of DNA damage argues for the relative safety of paraquat on short exposure. However, the long-term effects of a bacteriostatic compound on ecological systems are open to conjecture. Also, the potential of paraquat for mutagenicity, would have to be evaluated by different protocols.

*Summary.* The effects of the herbicide paraquat, 1,1′-dimethyl-4,4′-bipyridilium dichloride (Gramoxone W and Weedol), were studied in *Escherichia coli* B/r and *E. coli* Bs-1, which are, respectively, radiation-resistant and radiation-sensitive derivatives of *E. coli* B.

It was found that paraquat is powerfully bacteriostatic against *E. coli*. In the range of $10^{-5}$ to $10^{-3}$ *M*, paraquat showed similar dose-dependent inhibitory effects on growth rate, synthesis of DNA, RNA, messenger RNA, protein, and glucose utilization. At these concentrations, no significant effect on the bacterial membrane was observed as measured by the release of intracellular phosphate pools. However, approximately 90% of the exposed bacteria, both the radiation-resistant and-sensitive strain and $T_1$ bacteriophage were killed at $10^{-2}$ *M*. A limited study on the HeLa cell indicated that these mammalian cells were also more sensitive to this latter concentration.

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