Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 2851–2855, April 1990
Microbiology

Paraquat toxicity is increased in *Escherichia coli* defective in the synthesis of polyamines

(spermidine/putrescine/superoxide/oxygen-dependent toxicity/methyl viologen)

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ABSTRACT **We have shown that toxicity of paraquat for *Escherichia coli* is increased over 10-fold in strains defective in the biosynthesis of spermidine compared to isogenic strains containing spermidine. The increased sensitivity of these spermidine-deficient mutants to paraquat is eliminated by growth in medium containing spermidine or by endogenous supplementation of spermidine by the use of a *speE*$^+$*D*$^+$ plasmid. No paraquat toxicity is seen in the absence of oxygen, even in amine-deficient strains, indicating that superoxide is the agent responsible for the increased toxicity. However, the specific mechanisms responsible for the increased paraquat toxicity in the spermidine-deficient mutants remain to be determined. The marked sensitivity to paraquat of *E. coli* deficient in spermidine is of particular interest, since such mutants have no other phenotypic properties that can be easily assayed. This increased sensitivity has been used as the basis of a convenient method for scoring for mutants in polyamine biosynthesis and for the detection of plasmids containing the biosynthetic genes.**

Paraquat (methyl viologen; 1,1′-dimethyl-4,4′-bipyridinium) is a redox-cycling agent widely used as a source of superoxide in a variety of experimental systems (1–4). In this communication we report that the toxicity of paraquat for *Escherichia coli* is markedly increased in mutants defective in the biosynthesis of polyamines.

Polyamines are widely distributed in biological materials, and have been extensively investigated. The genes for the biosynthetic pathways for putrescine and spermidine have been defined, and deletion mutants have been obtained (reviewed in refs. 5 and 6). *E. coli* mutants lacking *speA* (encoding arginine decarboxylase), *speB* (encoding agmatine ureohydrolase), and *speC* (encoding ornithine decarboxylase) do not contain any putrescine or spermidine; these mutants show a 70% decrease in growth rate when grown in purified media without polyamines (7). Mutants that lack the *speED* operon (8–10) (i.e, do not contain spermidine synthase and *S*-adenosylmethionine decarboxylase) contain putrescine but have no spermidine; these mutants show only a small decrease in growth rate (≈15%) and have no other phenotypic changes (8). The increased paraquat toxicity in the latter mutants is of particular interest both for consideration of the function of spermidine in the cell and for the development of convenient methods for the differentiation of polyamine-plus and polyamine-minus strains.

MATERIALS AND METHODS

The *E. coli* strains used are listed in Table 1. The preparation of plasmids pSPD16 and pSPD16D has been described previously (9). pSPD16 is a pBR322 derivative that carries a 1795-base-pair (bp) insert of *E. coli* DNA that expresses the *speE*$^+$ and *speD*$^+$ genes. pSPD16D, identical to pSPD16 except for a deletion of approximately 145 bp at the 5′ end of the insert, does not express the *speED* genes.

Table 1. Strain list

Strain	Relevant genotype*	Source
71.18		S. Tabor
N99		S. Garges and S. Adhya
HT551	Δ(*speED*)	Derivative of 71.18 (8, 9)
HT653	Δ(*speA–speB*) Δ(*speC–glc*)	Derivative of N99†
HT654		Derivative of N99‡

*With respect to the *speA*, *speB*, *speC*, *speD*, and *speE* genes. These markers are wild type except as indicated.
†Strain HT653 was prepared by transducing the Δ(*speA–speB*) and Δ(*speC–glc*) mutations into strain N99 with the help of a *galP*::Tn*10* transposon (originally present in strain JM2071; JM2071 was obtained from the H. Kornberg laboratory via B. Bachmann of the Yale Genetics Center). The Δ(*speA–speB*) and Δ(*speC–glc*) deletions were described in ref. 7.
‡Strain HT654 was used as a control for strain HT653. It has the same history as strain HT653, except that a transductant was selected that did not have either the Δ(*speA–speB*) or the Δ(*speC–glc*) deletion.

Liquid cultures were grown at 37°C with vigorous shaking in either VB medium (11) or M9 minimal medium (12) with 0.4% glucose and thiamin at 1 μg/ml. For strains 71.18 and HT551 the medium was supplemented with pantothenate at 10 μg/ml. All cultures were grown on minimal media for at least 16 generations prior to their use to deplete the endogenous polyamine pools. For the growth experiments logarithmically growing cells were diluted to a cell density of ≈2 × 10^7 cells per ml 1 hr prior to the addition of paraquat.

Anaerobic growth experiments employed M9 minimal medium supplemented as above, except that glucose was 1%. For these experiments solutions were purged with 5% CO_2/95% N_2, and growth was under this atmosphere at 37°C with shaking. For liquid cultures, growth was monitored by optical density at either 540 or 600 nm. For growth on plates the above media were solidified with 1.5% agar.

The uptake of [^{14}C]paraquat was measured by a modification of the method used by Kao and Hassan (13). The concentration of [^{14}C]paraquat (Sigma) was 0.1 μM. The bacterial count for the uptake experiments was approximately 3 × 10^8 per ml. At the times indicated, 3-ml aliquots were filtered rapidly through a Millipore filter (25 mm diameter; HA type). The filters were dried without washing and were placed in 10 ml of scintillation fluid for determination of radioactivity.

RESULTS

Polyamine-Deficient Strains Grown in Polyamine-Free Media Are Markedly Inhibited by Paraquat. In purified amine-free medium, both wild-type (71.18) and spermidine-deficient

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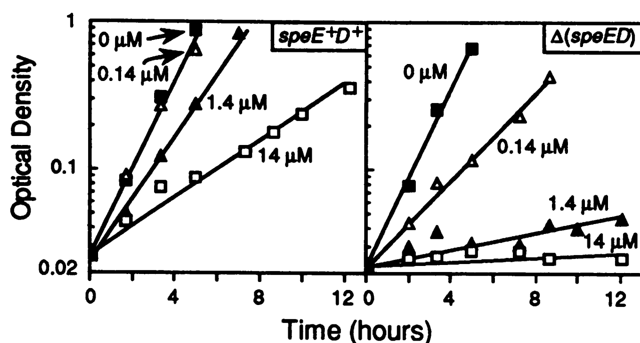

FIG. 1. Increased paraquat sensitivity of spermidine-deficient mutants. Strains were grown in minimal medium in the presence of paraquat (added at zero time) at the indicated concentrations. (*Left*) Strain 71.18 ($speE^{+}D^{+}$), which is able to synthesize both putrescine and spermidine. (*Right*) Strain HT551 [Δ(*speED*)], which is deficient in synthesis of spermidine.

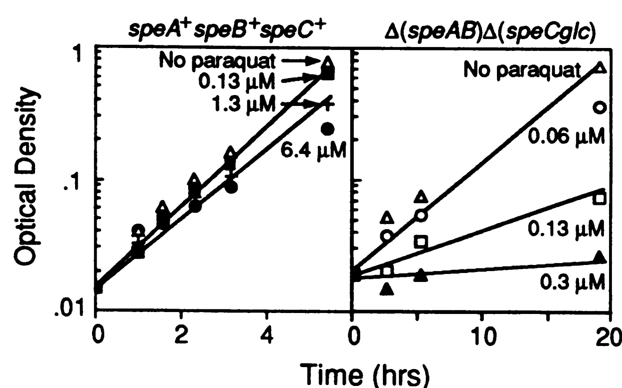

FIG. 2. Increased paraquat sensitivity of mutants deficient in both putrescine and spermidine. Strains were grown in minimal medium in the presence of the indicated concentration of paraquat. (*Left*) Strain HT654 is able to synthesize both putrescine and spermidine. (*Right*) Strain HT653 [Δ(*speA–speB*) Δ(*speC–glc*)] is deficient in the synthesis of putrescine, and therefore it contains neither putrescine nor spermidine. Note the time scales used in the two graphs are different, since the growth of strain HT653 is much slower than that of HT654.

(HT551) strains have a doubling time of 1–1.3 hr at 37°C. The addition of 1.4 μM paraquat has only a small effect on the growth rate of wild-type cells (doubling time ≈1.5 hr), but it markedly decreases the growth rate of the spermidine-deficient cells (doubling time ≈11.5 hr) (Fig. 1).

As previously reported (7), cells deficient in both putrescine and spermidine grow more slowly than nondeficient strains (Fig. 2). Thus, the doubling time of HT653 (Δ*speA–speB* Δ*speC–glc*) is over 3 hr compared to 1 hr for the nondeficient strain (HT654). The toxicity of paraquat in these deficient cells is even greater than that seen in the spermidine-deficient cells; essentially no growth is observed in 0.3 μM paraquat even after a 20-hr incubation.

Exogenous Polyamines Block Paraquat Toxicity. *E. coli* are known to actively take up putrescine and spermidine from the growth medium (6, 14, 15). Paraquat toxicity is prevented by adding polyamines to the growth medium of deficient mutants (Fig. 3); spermidine is much more effective than putrescine.

Endogenous Spermidine Restores Paraquat Resistance to Spermidine-Deficient *E. coli*. Spermidine-deficient *E. coli*, with a deletion at the *speED* locus, are restored to wild-type paraquat resistance by introduction of pSPD16, which contains and expresses the *speED* genes. Paraquat resistance of the spermidine-deficient strain is unaffected by pSPD16D, which is missing 145 bp at the 5′ end of the *speED* sequence and therefore fails to express these genes (9) (Fig. 4). Thus, endogenous supplementation with spermidine prevents enhanced paraquat toxicity due to spermidine deficiency.

Paraquat Toxicity in Polyamine-Deficient Strains Requires Oxygen. Intracellular generation of superoxide anion by the redox-cycling agent paraquat has been shown to require oxygen (1). Paraquat is reduced during metabolism and then undergoes auto-oxidation by donating electrons to molecular oxygen, producing superoxide (1, 2, 4). Consequently, paraquat toxicity has been shown to be minimal or absent under anaerobic conditions (1). In Fig. 5 we show that this requirement for oxygen is also true for the increased toxicity of paraquat in spermidine-deficient *E. coli* mutants. This observation indicates that the increased paraquat toxicity in spermidine-deficient *E. coli* is also due to superoxide formation.

Plate Assays of Paraquat Toxicity. The effect of spermidine deficiency on the toxicity of paraquat was studied on agar plates to test whether the increased toxicity represented a bacteriostatic or bacteriocidal effect. As expected from the results with liquid cultures, the spermidine-deficient mutant is more sensitive than wild-type *E. coli* on agar plates at all paraquat concentrations (Table 2); i.e., they grow more slowly. However, eventually, at paraquat concentrations ≤3.2 μM, the agar plates show the expected number of colonies of the spermidine-deficient mutant. These data show that at these levels the toxicity of paraquat is bacteriostatic rather than bacteriocidal in both the wild-type and the spermidine-deficient cells. The data with the wild-type cells are in agreement with the previous findings of Kitzler and Fridovich (16) that paraquat is bacteriostatic rather than bacteriocidal when wild-type *E. coli* are grown in VB medium.

A Method for Scoring for $speD^{+}$ and $speD^{-}$ Strains on Agar Plates Based on the Toxicity of Paraquat. As discussed earlier, the absence of spermidine in *E. coli* results in no known phenotypic effect except for a marginal decrease of growth rate, a decrease too small to be used for scoring spermidine auxotrophy in transformation or transduction experiments. The above data suggested that the increased paraquat toxicity observed on agar plates would be useful for such scoring.

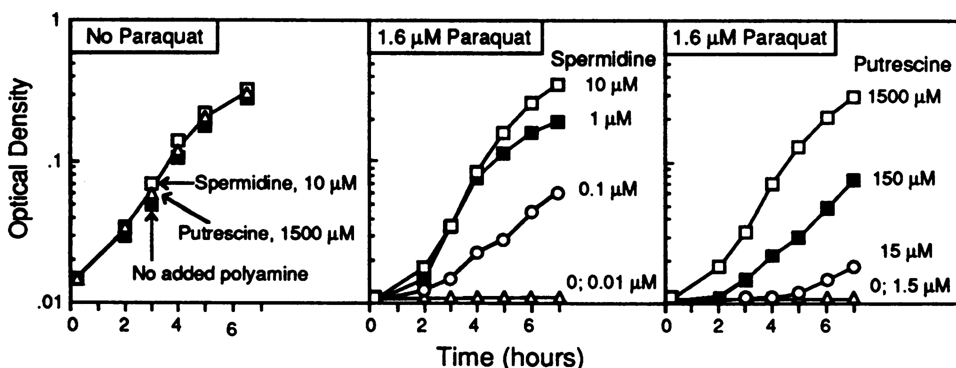

FIG. 3. Exogenous polyamines diminish paraquat sensitivity of the spermidine-deficient mutant. HT551, deficient in the biosynthesis of spermidine, was grown in minimal medium in the absence or presence of 1.6 μM paraquat. In the presence of 10 μM spermidine or 1500 μM putrescine, there was no inhibition of growth by paraquat. These concentrations of spermidine or putrescine did not detectably affect the growth rate in the absence of paraquat.

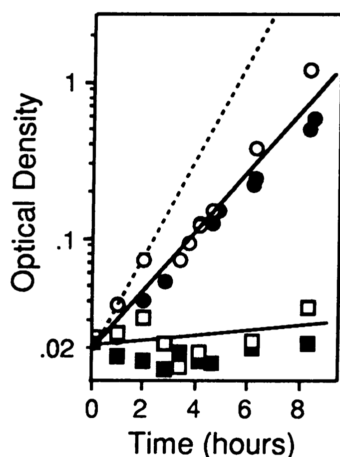

FIG. 4. Endogenous spermidine restores wild-type paraquat resistance to the spermidine-deficient mutant. Strain 71.18 (i.e., $speE^+$ $speD^+$) or strain HT551 [i.e., 71.18 Δ(*speED*)], containing either pSPD16 or pSPD16D, was grown in VB medium in the presence of 1.9 μM paraquat. Strains containing endogenously synthesized spermidine are indicated by the circles—i.e., ●, $speE^+D^+$; and ○, Δ(*speED*)/pSPD16. Strains deficient in polyamines are indicated by the squares—i.e., ■, Δ(*speED*); and □, Δ(*speED*)/pSPD16D. The dotted line indicates the growth of all of the strains in the *absence* of paraquat; i.e., all of the strains grew at approximately the same rate in the absence of paraquat.

Strain 71.18 ($speE^+D^+$ pan^+ tetracycline-sensitive) was transduced with bacteriophage P1 prepared in HT551 [Δ(*speED*) *zad*-220::Tn*10* *pan-6*], as previously described (9). Tetracycline-resistant transductants were selected and were then grown in individual wells in microtiter plates in VB medium containing pantothenate and limiting glucose (0.05%). These cells were then transferred by stamping onto an agar plate containing the same medium except for the addition of 0.3% glucose and of 14 μM paraquat (final concentration) and incubated at 37°C for 2 days. In the absence of paraquat all 63 isolates showed full growth; in the presence of 14 μM paraquat only 26 isolates grew. The cells in the microtiter plates were assayed for the presence of $speD^-$ or $speD^+$ by formation of $^{14}CO_2$ from adenosyl[*carboxyl*-^{14}C]methionine by an *in vitro* modification of the radiometric microtiter plate assay previously described (17). Each of the paraquat-resistant transductants contained the $speD^+$ gene, as shown by the formation of $^{14}CO_2$ from adenosyl[*carboxyl*-^{14}C]methionine; all of the paraquat-sensitive isolates were $speD^-$ by the same assay.

In other experiments (data not shown) this method of scoring was also found to be very useful for testing for the

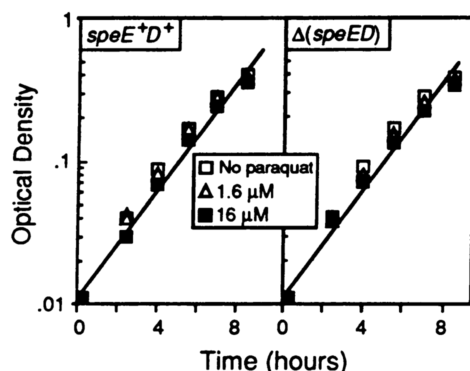

FIG. 5. Paraquat toxicity depends on the presence of oxygen. The conditions for this experiment were comparable to those in Fig. 1, except for growth under anaerobic conditions.

Table 2. Paraquat toxicity in wild type ($speE^+D^+$) and spermidine-deficient mutant [Δ(*speED*)] by plate assay

Time, days	Strain	Growth in paraquat 6.4 μM	3.2 μM	2.2 μM	1.4 μM	0 μM
1	$speE^+D^+$	0	0	±	++	++++
	Δ(*speED*)	0	0	0	0	++++
2	$speE^+D^+$	+++	++++	++++	++++	++++
	Δ(*speED*)	0	0	0	0	++++
3	$speE^+D^+$	++++	++++	++++	++++	++++
	Δ(*speED*)	0	±	++	++++	++++
4	$speE^+D^+$	++++	++++	++++	++++	++++
	Δ(*speED*)	±	+	++	++++	++++
5	$speE^+D^{+}$*	++++	++++	++++	++++	++++
	Δ(*speED*)†	+	+++	++++	++++	++++

Wild type is strain 71.18; mutant is HT551, which is 71.18 [Δ(*speED*)]. Approximately 350 cells were plated on VB agar and incubated at 37°C in air. 0 = no growth; ± = barely visible colonies; and + to ++++ indicate average colony size.

*All plates showed approximately 350 colonies.

†All plates at paraquat concentrations ≤ 3.2 μM showed approximately 350 colonies.

ability to synthesize putrescine in transductants that are obtained when a wild-type *E. coli* (strain N99) is transduced with bacteriophage P1 prepared on a Δ(*speA–speB*) Δ(*speC–glc*) donor strain. As expected from the growth curves presented in Fig. 2, transductants that completely lack the biosynthetic enzymes for putrescine (and hence lack both putrescine and spermidine) are even more sensitive to low concentrations of paraquat than the cells used in Table 2 that lack only spermidine.

Effect of Polyamine Deficiency on the Uptake (Binding) of [^{14}C]Paraquat. The results reported in this paper on the increased sensitivity to paraquat in polyamine-deficient cells cannot be explained by a direct competition between exogenous polyamines and paraquat for transport into the cell, since the difference in paraquat toxicity is observed when the wild-type strain and polyamine-deficient mutants are compared; i.e., no exogenous polyamines are present. In addition, the increase in paraquat toxicity that is observed with the spermidine-deficient strain is abolished when spermidine is supplied endogenously by the presence of a $speE^+D^+$ plasmid.

However, it seemed possible that paraquat (which is a basic compound) might bind more easily to intracellular acidic binding sites in the absence of intracellular spermidine, and therefore we measured the uptake of [^{14}C]paraquat in normal and deficient cells. The experiment presented in Fig. 6 *Left* shows that the uptake of [^{14}C]paraquat is very slow, but is definitely greater in strain HT551 [i.e., 71.18 Δ(*speED*)] than in strain 71.18. In considering the total uptake of paraquat by the cells, the values need to be corrected for the doubling of the cells that occurred in the culture during the incubation period. The large blank at zero time is largely due to adsorption on the Millipore filter, although there may be some adsorption to the surface of the cells.

We have also measured the uptake of [^{14}C]paraquat in HT653—i.e., the strain that contains no putrescine or spermidine—as well as in its isogenic control HT654. The results (Fig. 6 *Right*) show that the uptake is greater in the amine-deficient strain.

DISCUSSION

The redox-cycling agent paraquat induces marked growth inhibition of *E. coli* mutants deficient in the biosynthesis of polyamines when grown in polyamine-free media. Deficiency in spermidine alone produces a 10-fold increase in paraquat sensitivity over the toxicity observed in wild-type cells;

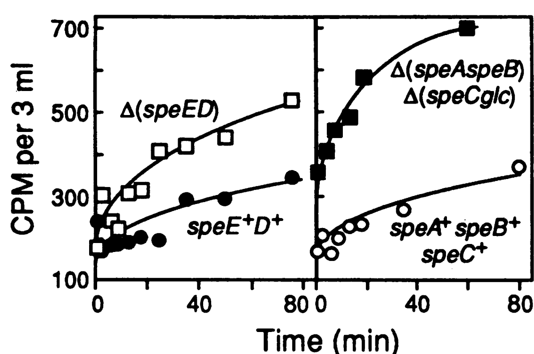

FIG. 6. Uptake of [^{14}C]paraquat. The uptake of [^{14}C]paraquat was measured in the same strains that were studied in Figs. 1 and 2. Note that the concentration of cells was 10 to 20 times higher than that used in Figs. 1 and 2. (*Left*) Strains 71.18 and HT551 [Δ(*speED*)]. (*Right*) Strains HT654 and HT653 [Δ(*speA–speB*) Δ(*speC–glc*)]. The concentration of [^{14}C]paraquat was 0.1 μM. Before filtration the cultures had 1300 cpm/ml (i.e., 3900 cpm in the 3-ml aliquot that was filtered).

deficiency in both spermidine and putrescine results in an even greater increase in sensitivity. Paraquat toxicity in both the wild-type and spermidine-deficient strains depends on the presence of oxygen, indicating that the increased toxicity in the spermidine-deficient mutant, as in the wild type (16), is due to superoxide anion.

We do not know the mechanism responsible for the increased paraquat toxicity in amine-deficient cells. Several possible explanations might be mentioned:

(*i*) To explain the increased toxicity of paraquat in spermidine-deficient cells, one might postulate that in wild-type cells spermidine competitively excludes paraquat from access to polyacidic components in the cell. It is well known, for example, that spermidine has a high affinity for such cellular components as ribosomes and DNA, and the binding of paraquat to DNA has recently been reported by Minchin (26). It is also possible that the damage resulting from the superoxide produced by the paraquat might depend on the cellular localization of paraquat. Imlay and Linn (27) have mentioned this possibility in their recent review on the relation of DNA damage and oxygen radical toxicity.

This explanation for the increased sensitivity of polyamine-deficient cells to paraquat—namely, an increase in the number of intracellular binding sites available for paraquat in polyamine-deficient cells—is consistent with the uptake data reported in Fig. 6.

Furthermore, in recent unpublished studies we have found that spermidine-deficient cells show only a small increase in sensitivity to sources of superoxide that do not contain a basic group in their structure, such as plumbagin and juglone. There is a larger increase, however, in the toxicity of these compounds in cells that lack both putrescine and spermidine. There is no increase in the toxicity of cobalt irradiation in either putrescine- or spermidine-deficient cells.

(*ii*) Polyamines might inhibit paraquat uptake from the medium. As mentioned in *Results*, our findings cannot be explained by a direct competition between exogenous spermidine and paraquat for an external transport system. However, we cannot exclude an effect of endogenous spermidine levels on a paraquat transport system.

In mammalian cells there have been some reports indicating that paraquat and polyamines might be taken up by the same transport system, but this has not been true of all cells (18–20). It is not clear, however, whether the effects observed were specifically on transport or on a change in the number of intracellular binding sites.

(*iii*) Spermidine might protect cellular components from damage by oxidation. For example, there have been reports in the literature on the effect of polyamines in reducing lipid peroxidation (21, 22) and on the radical scavenging properties of polyamines (28).

(*iv*) The amount of superoxide (or its dismutation products) generated by paraquat might be altered in amine deficiency, either by increased production of superoxide or by decreased degradation of superoxide or its dismutation products.

(*v*) In considering possible mechanisms for the degradation of superoxide, we first speculated that the novel spermidine derivative glutathionylspermidine (23, 24) might play a role in the degradation of superoxide, since glutathionylspermidine would be absent from spermidine-deficient cells. It is unlikely, however, that the absence of glutathionylspermidine is responsible for the increased paraquat toxicity in spermidine-deficient cells, since in preliminary experiments with a *gshA* mutant (JTG10) obtained from Bruce Demple (25) no increase in paraquat toxicity was observed (compared to the *gshA*$^+$ parent, AB1157). The *gshA* mutant lacks glutathione and hence would not have any glutathionylspermidine. These results are consistent with the reports of Greenberg and Demple (25) and of Imlay and Linn (27) that in *E. coli* intracellular glutathione does not protect the cells from lethal oxidative damage.

Finally, the studies reported in this paper will be of some practical value for investigators in the polyamine area. Previously, the marginal decrease observed in the growth rate of spermidine-deficient *E. coli* mutants precluded the use of growth rates to monitor the presence or absence of the genes for spermidine biosynthesis in transduction or transformation experiments. Now, the presence or absence of this pathway can be assayed by the effect of paraquat on the growth rate in liquid cultures or on agar plates with amine-free media.

The authors thank Hong T. Ngyuyen for technical assistance. This work was supported in part by U.S. Public Health Service Grant GM39933 to K.W.M.

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