JOURNAL OF BACTERIOLOGY, Mar. 1978, p. 1150-1155
0021-9193/78/0133-1150$02.00/0
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Vol. 133, No. 3
Printed in U.S.A.

Role of Glutathione in Reversing the Deleterious Effects of a Thiol-Oxidizing Agent in *Escherichia coli*†

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Received for publication 27 September 1977

Diamide was found to be much less specific for the oxidation of glutathione in vivo in *Escherichia coli* than had been previously assumed. In vivo, only a slight alteration of the ratio of reduced to oxidized glutathione was found, whereas a significant amount of glutathione was found in the form of mixed disulfide with proteins. This latter occurrence was postulated as being responsible for the bacteriostatic effect of diamide.

To assess the in vivo role of reduced glutathione, Kosower et al. (5) utilized the specific thiol-oxidizing agent diamide [diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)]. They concluded that diamide at concentrations ranging from 1×10^{-4} to 50×10^{-4} M did not react with protein sulfhydryls but exerted an effect on cellular metabolism solely via oxidation of low-molecular-weight thiols. In non-metabolizing erythrocytes, the oxidation of glutathione was accompanied by a stoichiometric reduction of diamide. Wax et al. (9) found that diamide temporarily inhibited growth of an exponential culture of *Escherichia coli*. Growth resumed after a lag period, the length of which was porportional to the initial diamide concentration. These studies led to the conclusion that reduced glutathione, which is the major low-molecular-weight thiol in *E. coli* (8), is essential for cellular metabolism, but did not exclude the possibility that oxidized glutathione could inhibit growth by stopping protein synthesis as reported for an in vitro protein synthesis system (4).

The recent isolation and partial characterization of *E. coli* mutants unable to synthesize γ-glutamyl cysteine (1) or glutathione (1, 3) indicated that *E. coli* can grow in the absence of glutathione. To further the understanding of the role of glutathione in vivo, we have investigated the effects of diamide on wild-type *E. coli* and on an *E. coli* mutant unable to synthesize glutathione.

MATERIALS AND METHODS

Glutathione and *N*-ethyl maleimide (NEM) were obtained from Sigma Chemical Co. Diazenedicarboxylic acid bis(*N*,*N*-dimethylamide), hereafter referred to as diamide, was obtained from Calbiochem. Carrier-free [^{35}S]sulfate was obtained from Amersham/Searle.

Bacterial strains. The *E. coli* K-12 F^- strains used in this study were derived from X-88 and its parent, KK1004, previously described (3). To eliminate the requirement for sulfur-containing amino acids, methionine prototrophic derivatives of KK1004 and X-88 were obtained by P1-mediated transduction to yield KK1007 and KK1009, respectively. A second P1-mediated transduction was then used to obtain a cysteine-prototrophic derivative of KK1009 designated KK1010. KK1010 was found to contain no detectable glutathione, but to accumulate γ-glutamyl cysteine, as does *E. coli* X-88.

Growth of bacteria. Cultures were grown in minimal medium (2) supplemented with 20 μg of thymidine per ml. In experiments involving labeling with [^{35}S]sulfate, the sulfate concentration of the medium was reduced to 10^{-4} M.

Extraction of acid-soluble compounds from cells. Cultures were quickly chilled in ice and centrifuged at 15,000 × *g* for 10 min. The pellets were suspended in a volume of 5% trichloroacetic acid equivalent to 1 to 5% of the original volume of culture and allowed to stand overnight at 0 to 4°C. These suspensions were centrifuged at 15,000 × *g* for 10 min, and the supernatant was extracted with 1.5 ml of diethyl ether to remove the trichloroacetic acid. Ethylenediaminetetraacetic acid (EDTA) was added to bring the concentration to 20 mM to prevent oxidation of thiols. Two further ether extractions completed the removal of trichloroacetic acid, and the aqueous phases were used to quantitate total sulfhydryl groups, glutathione, and other low-molecular-weight sulfur-containing compounds. In experiments involving chromatographic separation of low-molecular-weight sulfur-containing compounds, 1 mg of NEM was added to a chilled culture to derivatize thiol groups just before addition of trichloroacetic acid. In all experiments, the trichloroacetic acid precipitate was dissolved in 1 ml of 0.1 N NaOH to determine protein content (6) or in some cases to determine ^{35}S incorporation into protein.

Quantitation of sulfhydryl groups. The concentration of sulfhydryl groups was determined spectrophotometrically by the reduction of 5,5′-dithiobis-(2-nitrobenzoic acid). To 0.1 ml of sample was added 0.9 ml of an assay mixture containing 0.09 M potassium

† Scientific paper no. 10,042 of the Minnesota Agricultural Experiment Station.

phosphate buffer (pH 7.0), 0.04 M ethylenediaminetetraacetic acid, and 0.004% 5,5'-dithiobis-(2-nitrobenzoic acid). The increase in absorbance at 412 nm was followed at room temperature with a Sargent SRL recorder attached to a Beckman DB spectrophotometer until no further increase occurred (about 2 min).

Analysis of intracellular pools of sulfur-containing compounds. To determine the composition of the pool of acid-soluble sulfur-containing compounds, cultures labeled for two generations with [^{35}S]sulfate (1 to 2 μCi/ml) were treated with NEM and trichloroacetic acid as described above. Portions of the acid-soluble fractions were spotted on Whatman no. 1 chromatography paper along with standards (NEM-glutathione, NEM-γ-glutamyl cysteine, NEM-cysteine, oxidized glutathione, diglutamyl cysteine, and cystine) and chromatographed with a solvent containing isopropanol, formic acid, and water (195:3:102). Strips were scanned in a Nuclear-Chicago strip scanner to locate radioactive spots, and the standards were located visually after being sprayed with ninhydrin.

Quantitation of diamide. The oxidized form of diamide was experimentally determined to have a molar extinction coefficient of 1.3×10^3 at 340 nm, whereas the reduced form had little or no detectable absorbance at this wavelength. The reduction of diamide in a culture was followed by measuring the absorbance of a culture at 340 nm after removing cells by centrifuging for 2 min in a Brinkmann microcentrifuge.

RESULTS

Attempts to investigate the effect of diamide in non-metabolizing *E. coli* cells indicated that diamide did not alter sulfhydryl content when growth was stopped by either low temperature or glucose limitation (data not shown). Thus, it was necessary to investigate the effects of diamide on growing *E. coli* cells. Diamide is bacteriostatic for *E. coli* KK1007 and KK1010, but growth resumes after a lag, the length of which is dependent upon the initial concentration of the diamide (Fig. 1). The results with strain KK1007 were similar to those reported by Wax et al. (9), whereas KK1010 exhibited a considerably longer lag before growth began. Since KK1010 cannot synthesize glutathione but accumulates γ-glutamyl cysteine, these results indicate that the bacteriostatic effect of diamide is not due to accumulation of oxidized glutathione (4). The simplest explanation for these results would be that diamide inhibits growth, but that growth resumes when diamide is reduced and this reduction is more rapid in the presence of glutathione.

The rates of reduction of diamide in cultures of KK1007 and KK1010 were 330 and 350 nmol/min per mg of protein, respectively (Fig. 2), indicating that the rate of diamide reduction is independent of the presence of glutathione in the cell. In both strains, growth resumed only after diamide reduction was essentially complete. It is not possible to compare the growth lag from this experiment with those of experiments shown in Fig. 1, because the initial cell concentration in this experiment was fivefold higher, and this experiment was terminated before exponential growth began. Nevertheless, it would appear that the longer lag before growth of KK1010 resumed after diamide treatment compared with that of KK1007 was due to some cellular alteration that glutathione could either prevent or repair in KK1007, rather than due to a slower rate of diamide reduction.

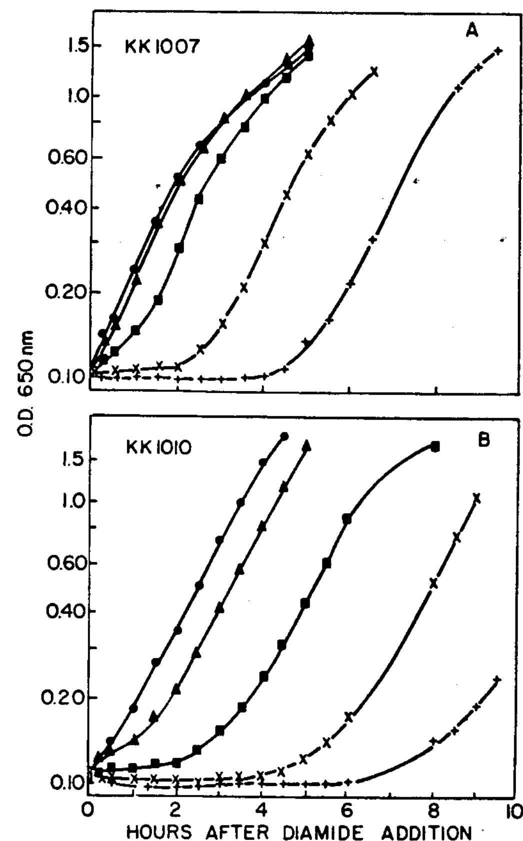

FIG. 1. *Growth of E. coli after diamide addition. Diamide was added to exponentially growing cultures of E. coli KK1007 (A) and KK1010 (B) (initial optical density at 650 nm [O.D. 650 nm] of 0.1) at 37°C to give the following concentrations: no diamide (●), 1 × 10^{-4} M (▲), 3 × 10^{-4} M (■), 6 × 10^{-4} M (×), and 1 × 10^{-3} M (+). Growth was followed by measuring the optical density at 650 nm at various times after diamide addition.*

To distinguish the lag in exponential growth due to the time required to reduce diamide from the lag caused by diamide-induced cellular alterations, cultures of KK1007 and KK1010 were treated with excess diamide for various lengths of time before diamide was removed. Both

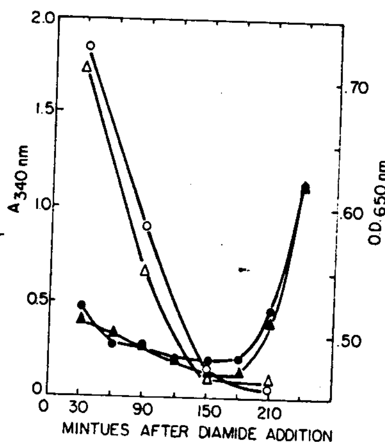

FIG. 2. *Diamide reduction by E. coli cultures. Diamide was added to cultures of E. coli at 37°C to give an initial concentration of 2×10^{-3} M; the initial optical densities at 650 nm (O.D.$_{650\,nm}$) of the cultures were 0.52 and 0.51 for strains KK1007 and KK1010, respectively. The reduction of diamide by KK1007 (○) and KK1010 (△) was followed by measuring the absorbance at 340 nm (A$_{340\,nm}$) of the cultures. Growth of KK1007 (●) and KK1010 (▲) was followed by measuring the optical densities (at 650 nm) of the cultures.*

strains exhibited a lag before growth resumed, indicating that diamide induced cellular alterations (Table 1). The presence of glutathione either decreased the amount of damage or speeded up the recovery from diamide, since the lag was shorter with KK1007. Thus, whereas the rate of diamide reduction was glutathione independent, the resumption of growth after exposure to diamide was glutathione dependent.

To explore the effects of diamide on the metabolism of all low-molecular-weight sulfur compounds in *E. coli*, cultures of KK1007 and KK1010 were grown in [^{35}S]sulfate medium for two generations to label these compounds, and then diamide was added, together with unlabeled sulfate, to prevent further incorporation of [^{35}S]sulfate into these pools. The low-molecular-weight sulfur-containing compounds were extracted from the cells by treatment with 5% trichloroacetic acid after the cells had been treated with excess NEM to derivatize all sulfhydryl groups. The supernatant after centrifugation was subjected to paper chromatography (3). After diamide addition, the amount of acid-soluble sulfhydryl compounds of strain KK1010 (essentially only the NEM derivative of γ-glutamyl cysteine) dropped rapidly, whereas the oxidized ^{35}S compounds rapidly increased (Fig. 3). In contrast, in strain KK1007 glutathione was only slowly oxidized to the disulfide. By 1 min after addition of diamide to KK1007, the ratio of reduced glutathione to total acid-soluble glutathione dropped from 0.92 to 0.85 (Table 2). Under identical conditions, the ratio of reduced γ-glutamyl cysteine to total acid-soluble γ-glutamyl cysteine in KK1010 dropped from 0.60 to 0.05.

The possibility that the reduced glutathione pool in strain KK1007 can be maintained by turnover (excretion of oxidized glutathione and de novo synthesis of reduced glutathione) can be ruled out by the results shown in Fig. 4. Diamide drastically reduced incorporation of [^{35}S]sulfate into both protein and the acid-soluble fraction. The difference between the ratio of thiol to disulfide in KK1007 and KK1010 after diamide treatment in Fig. 3 can be explained if the rate of oxidation of glutathione by diamide in vivo is the same order of magnitude as the reduction of oxidized glutathione by glutathione reductase. Since oxidized γ-glutamyl cysteine is not a substrate for glutathione reductase (3), the pool of reduced γ-glutamyl cysteine can only be

TABLE 1. *Growth lag after diamide treatment*

Exposure to 3×10^{-3} M diamide (min)	Lag[a] (min)	
	KK1007	KK1010
30	35	70
60	75	95
120	100	120
180	110	125

[a] Time before growth resumed after diamide was removed.

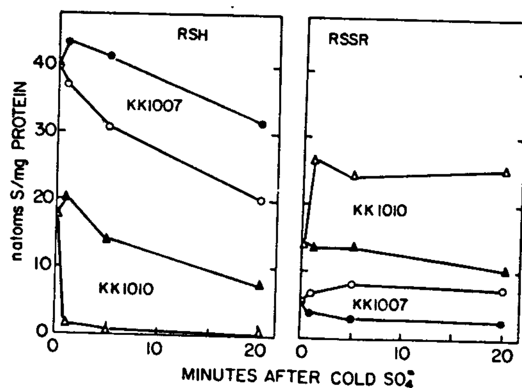

FIG. 3. *Effect of diamide on acid-soluble thiols and disulfides. Diamide (3×10^{-3} M) and ammonium sulfate (1×10^{-2} M) were added to cultures labeled at 30°C for two generations with [^{35}S]sulfate (10 µCi/ml). Incubation was continued, and at various times samples were removed and treated with NEM and trichloroacetic acid. The acid-soluble extracts were analyzed by paper chromatography. The amounts of thiols (RSH) and disulfides (RSSR) in the absence (●, ▲) and presence (○, △) of diamide were determined for strains KK1007 and KK1010.*

maintained by de novo synthesis, which was severely depressed by diamide (Fig. 4).

The decrease in trichloroacetic acid-soluble thiol compounds in the presence of diamide was greater than the observed increase in acid-soluble disulfides (Fig. 3), suggesting that these compounds were being lost from the acid-soluble pool. One possible explanation for this discrepancy is that disulfides were being formed between low-molecular-weight thiols and proteins. This could occur either directly during diamide treatment or via disulfide exchange between low-molecular-weight disulfides and protein sulfhydryls. Such disulfides would no longer be acid soluble. To determine whether mixed disulfides with proteins were being formed by glutathione or γ-glutamyl cysteine during diamide treatment, an experiment similar to the previous one was conducted, except that the acid-insoluble material was collected, washed, and treated with excess dithiothreitol to reduce all disulfide bonds. Excess NEM was added to derivatize all sulfhydryls, and the proteins were again precipitated with trichloroacetic acid. The acid-soluble fraction was chromatographed to permit identification of the acid-soluble compounds present. Within 1 min of diamide treatment, there was a 5- to 10-fold increase in the number of mixed disulfides between proteins and low-molecular-weight thiols (Fig. 5). Some of the mixed disulfides formed with proteins would be expected to inactivate sulfhydryls essential for activity of key enzymes needed for growth and could therefore account for the bacteriostatic effect of diamide.

After removal of diamide from strain KK1007, the mixed disulfides containing glutathione could be reduced either directly by glutathione reductase or indirectly via reduction of oxidized glutathione and thiol exchange. In strain KK1010, disulfides containing γ-glutamyl cysteine could not be reduced by glutathione reductase. If a significant amount of the glutathione or γ-glutamyl cysteine were in the form of protein mixed disulfides after diamide treatment, one might expect that newly synthesized low-molecular-weight thiols made after diamide treatment may be used in thiol-exchange reactions generating protein thiols and low-molecular-weight disulfides, particularly in KK1010. To investigate this possibility, strains KK1007 and KK1010 were treated with diamide for 30 min, diamide was removed, [^{35}S]sulfate medium was added, and low-molecular-weight thiols and disulfides were analyzed as before. The accumulation of newly synthesized thiols and disulfides

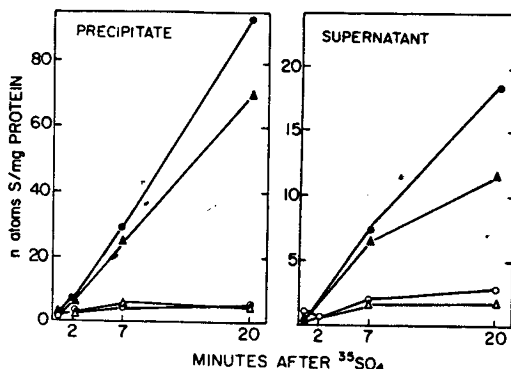

FIG. 4. *Effect of diamide on sulfate incorporation. Diamide (3 × 10^{-3} M) and [^{35}S]sulfate (10 µCi/ml) were added to exponentially growing cultures at 30°C, and at various times samples were removed and treated with NEM and trichloroacetic acid. The amount of [^{35}S]sulfate incorporated into the acid-soluble and acid-insoluble fractions in strain KK1007 in the absence (●) and presence (○) of diamide and in strain KK1010 in the absence (▲) and presence (△) of diamide was determined.*

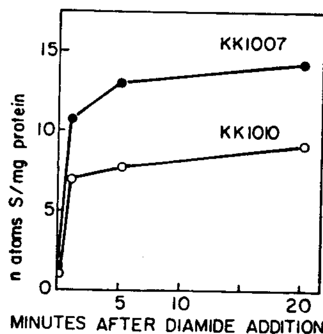

FIG. 5. *Effect of diamide on disulfides involving proteins. Diamide (3 × 10^{-3} M) was added to cultures labeled at 30°C for two generations with [^{35}S]sulfate (15 µCi/ml). Incubation was continued, and at various times samples were removed, treated with NEM, and centrifuged. The pellet was treated with trichloroacetic acid and dithiothreitol, and the amount of ^{35}S released by dithiothreitol from the acid-insoluble fraction of strain KK1007 (●) and KK1010 (○) was determined.*

TABLE 2. *Effect of diamide on acid-soluble thiols and disulfides*[a]

Strain	Diamide concn (M × 10^{-3})	[^{35}S] in thiols (%)	Thiol/disulfide (cpm)
KK1007		92	23
	3	85	11
KK1010		60	3
	3	5	0.1

[a] Data are calculated from the data shown in Fig. 3 corresponding to 1 min after addition of diamide. Thiol/disulfide is equal to counts per minute of ^{35}S in thiol/0.5 cpm of ^{35}S in disulfides.

in strain KK1010 was delayed compared with that in KK1007, presumably reflecting the longer lag before growth resumption in KK1010 (Fig. 6A and C). Acid-soluble disulfides accumulated very significantly in both KK1007 (Fig. 6B) and KK1010 (Fig. 6D), suggesting that thiol-exchange reactions between newly synthesized thiols and glutathione and γ-glutamyl cysteine covalently bound to proteins via disulfide bonds were occurring. The extent of accumulation of oxidized glutathione in KK1007 was surprising since untreated cells maintained 10-fold-more reduced glutathione than oxidized glutathione, whereas, even 60 min after diamide treatment when strain KK1007 was in exponential growth, the disulfide was accumulating at a rate equal to the thiol. These results again indicate that neither the greatly elevated level of oxidized glutathione nor the altered thiol disulfide ratio can prevent bacterial growth.

DISCUSSION

The results presented in this paper clearly do not support many assumptions previously made concerning the action of diamide in vivo (5, 7). The similar rates of diamide reduction in vivo in an *E. coli* cell with a functioning glutathione

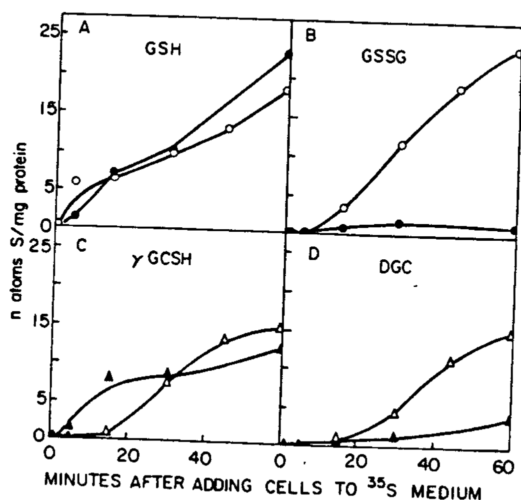

FIG. 6. *Effect of pretreatment with diamide on [^{35}S]sulfate incorporation into acid-soluble thiols and disulfides. Cells were treated for 30 min at 30°C with an excess of diamide (2×10^{-3} M), removed by centrifugation, and suspended in medium containing 1×10^{-4} M [^{35}S]sulfate. The amount of [^{35}S]sulfate incorporated into glutathione and γ-glutamyl cysteine in untreated strains KK1007 (●) and KK1010 (▲) and in strains KK1007 (○) and KK1010 (△) pretreated with diamide was determined. Abbreviations: γ-GCSH, γ-glutamyl cysteine; DGC, di-γ-glytamyl cysteine; GSH, glutathione; GSSG, oxidized glutathione.*

reductase system compared with one lacking glutathione indicate that an alternate system for reduction of diamide exists and is predominant, or else both pathways share some component that is rate limiting. One such alternate pathway known in *E. coli* is the thioredoxin reductase system (7). Thioredoxin has two thiols that can be rapidly oxidized to an intramolecular disulfide. This system, as well as glutathione reductase, utilizes reduced nicotinamide adenine dinucleotide phosphate as a reductant. If reduced nicotinamide adenine dinucleotide phosphate generation is rate limiting in the reduction of diamide, loss of either pathway would not alter the rate of reduction of diamide.

The observation that oxidized glutathione does not accumulate during diamide treatment but that protein-glutathione mixed disulfides do suggests that glutathione reductase may reduce oxidized glutathione more efficiently than mixed disulfides of glutathione.

The glutathione-deficient mutant accumulated not only mixed disulfides, but also diglutamyl cystine, probably because glutathione reductase could not reduce this disulfide. When diamide was removed from a treated culture, we found that newly synthesized glutathione or γ-glutamyl cysteine rapidly appeared as an oxidized compound. It would seem that the oxidized glutathione and γ-glutamyl cysteine were being formed via exchange of protein-glutathione mixed disulfides that accumulated during diamide treatment. If this was the case, it would indicate that diamide was doing substantial damage to the cell. This agrees with our finding that exposure of cells to 3 mM diamide and removal of the diamide before sufficient time has elapsed for the cells to reduce excess diamide caused a growth lag proportional to the time of exposure. This is in contrast to an earlier conclusion that the lag is due to time needed to reduce excess diamide (9).

Although it would be very difficult to prove that the bacteriostatic effect of diamide is due to either the mixed disulfides formed between low-molecular-weight sulfhydryl and protein or those formed between sulfhydryls of proteins, it would fit well with the data presented in this paper. Fuchs and Warner showed previously that at least one protein sensitive to oxidation of sulfhydryls, the B1 subunit of ribonucleotide reductase, was only partially active in a *gshB* mutant, and that this defect could be directly attributed to the *gshB* mutation (3). This paper indicates that the ratio of thiol to disulfide in trichloroacetic acid-soluble compounds in the parental strain of the *gshB* mutant is 23, whereas in the *gshB* strain it is 3. In the case of ribonucleotide reductase, the cell appears to compen-

sate for the loss of activity of the B1 subunit of ribonucleotide reductase by increased enzyme synthesis (3). It thus appears that the absence of glutathione and the resulting change in the ratio of thiol to disulfide can be tolerated because of the ability of *E. coli* to regulate enzyme synthesis or, in some cases, to use alternate pathways.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM 20884 (J.A.F.) and grants GM 21014 and GM 21464 (H.R.W.) from the National Institute of General Medical Sciences. P.B.B. and K.A.H. were supported by Public Health Service training grant GM 00345 to the Department of Biochemistry. H.R.W. is a recipient of Public Health Service Research Career Development Award GM 45729 from the National Institute of General Medical Sciences.

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