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Effect of *cis*-Platinum(II)Diamminodichloride on Wild Type and Deoxyribonucleic Acid Repair-Deficient Mutants of *Escherichia coli*[1]

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The anti-tumor drug *cis*-platinum(II)diamminodichloride (PDD) induced extensive filamentation in wild-type *Escherichia coli* and in mutants lacking certain deoxyribonucleic acid (DNA) repair functions (*uvrA*, *recB*, *recC*, and *polA*); viability of repair-deficient mutants treated with PDD was significantly less than that of wild-type cells. PDD was highly toxic to *lex1*, *lex1 uvrA6* (where its effect was cummulative), and *recA13* mutants, all of which were killed without formation of filaments. ^{3}H-thymine incorporated into DNA of cells subsequently treated with PDD became trichloroacetic acid-soluble at rates similar to those observed after exposure to comparable doses of ultraviolet light (UV) or mitomycin C. PDD, like UV, induced extensive degradation of DNA in *recA* organisms. After a 30-min lag, PDD inhibited significantly the synthesis of DNA but not of ribonucleic acid or protein in *E. coli*. However, the relative differences between rates of DNA synthesis observed in PDD-treated and control cells decreased substantially when the duration of pulses (^{3}H-thymine) was prolonged from 2 to 5 min. These observations suggest that PDD-induced damage to DNA is reversible, possibly by defined mechanisms of excision and recombination repair.

cis-Platinum(II)diamminodichloride (PDD) causes significant regression of a variety of tumors in experimental animals (10, 13, 19, 22, 25, 26) and selectively blocks synthesis of deoxyribonucleic acid (DNA) in eukaryotic cells (6, 10, 11). PDD forms interstrand cross-links in nucleic acids (7, 16), but the frequency of cross-linkage is insufficient to account for lethality in bacteriophage (24). Similar to the effects produced by other DNA inhibitors, PDD induces temperate phage in lysogenic bacteria (15), causes filamentation of gram-negative organisms (18, 20, 21), and is exceptionally lethal to certain Exr$^-$, Hcr$^-$, and Fil$^-$ mutants of *Escherichia coli* (5).

In this paper, we report studies on PDD-induced filamentation and loss of colony-forming ability of a variety of *E. coli* strains which are blocked in various pathways of DNA repair. Also measured were the inhibition of DNA synthesis and the induction of DNA breakdown in the presence of PDD. The results are in accord with the hypothesis that PDD-induced lethality in bacteria is caused by the irreversible formation of interstrand cross-links in DNA.

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MATERIALS AND METHODS

Media. Minimal C medium (17), supplemented with glucose (0.01 M) and thymine (20 μg/ml), was used in most experiments. This medium contained unlabeled uracil (20 μg/ml) and cytosine (20 μg/ml) or L-isoleucine (33 μg/ml) and L-valine (29 μg/ml) when prepared for determination of rates of ribonucleic acid (RNA) and protein sythesis, respectively. Complex medium was tryptone-E broth (14). Difco nutrient agar was routinely used as a plating medium; diluent was 0.033 M potassium phosphate buffer, pH 7.0.

Bacteria. *E. coli* strain W 3350 *thy* was generally employed in experiments concerned with viability of filaments, macromolecular synthesis, and degradation of DNA. The origin of DNA repair mutants has been described (1); their properties are shown in Table 1.

Macromolecular synthesis. Rates of DNA synthesis were measured by pulse labeling. Samples of 1.0 ml of logarithmically growing cells were added to tubes (18 by 150 mm) containing 0.1 ml (10 μCi) of carrier-free ^{3}H-thymine (63 mCi/mmol). After aeration for 2 or 5 min in a model G76 gyratory water

TABLE 1. *Characterization of repair-deficient mutants of Escherichia coli strain K-12*

Strain	Relevant genotype	Remarks	Source and reference
W3350	*rec*$^+$		L. Snyder
AB1157	*rec*$^+$		P. Howard Flanders, 1
AB1886	*uvrA6*	Mutant from AB1157	P. Howard Flanders, 1
AB2463	*recA13*	Mutant from AB1157	P. Howard Flanders, 1
AB2494	*lex1*	Mating of AB2474 × AB2383	P. Howard Flanders, 1
NH4554	*lex1*	Selected from AB2494	P. Howard Flanders, 1
AB2474	*uvrA6 lex*	Mutant from AB1886	P. Howard Flanders, 1
JC5029	*rec*$^+$	*thy*$^+$ prototroph of JC5401	N. S. Willetts, 1
JC5412	*recB21 sbc8*	P1 transduction to JC5401	N. S. Willetts, 1
JC5426	*recC22*	P1 transduction to JC5401	N. S. Willetts, 1
JC4583	*rec*$^+$		S. Barbour, 2, 3
JC4584	*recB21 recC22*	P1 transduction	S. Barbour, 3
JC6722	*recB21*	P1 transduction of HF4733	A. J. Clark, 2
mr 2-41	*rec*$^+$	P1 transduction	M. Inouye, 12
mr 2-41	*recA*		M. Inouye, 12
W3110	*rec*$^+$		P. Delucia, 1
P3470	*polA*	Mutant from W3110	P. Delucia, 1

bath-shaker (new Brunswick Scientific Co., Inc., New Brunswick, N.J.), incorporation was stopped by addition of 2 ml of cold 5% trichloroacetic acid containing unlabeled thymine (40 μg/ml). After storage for 1 h at 0 C, the samples were centrifuged and the supernatant fluids were decanted. The precipitates were suspended in 0.2 ml of 2% NaOH, precipitated with 3 ml of 5% trichloroacetic acid containing unlabeled thymine, and then collected on a 0.45-μm diameter membrane filter (Millipore Corp., Bedford, Mass.). After being rinsed with 20 ml of cold 5% trichloroacetic acid containing unlabeled thymine, the membranes were dried and immersed in 10 ml of toluene base containing 0.4% 2,5-diphenyloxazole and 0.005% 1,4-di-2-(5-phenyloxazolyl)-benzene; radioactivity was determined in a Packard Tri-Carb scintillation counter.

Rates of RNA synthesis were measured similarly by addition of 1.0 ml of culture to tubes containing 0.1 ml (10 μCi) of ^{3}H-uracil (55 mCi/mmol). After pulsing for 5 min, incorporation was stopped by addition of 1 ml of cold 10% trichloroacetic acid containing 40 μg each of unlabeled uracil and cytosine per ml. After storage for 1 h at 0 C, the precipitates were collected on membranes, washed with 20 ml of cold 5% trichloroacetic acid containing uracil and cytosine, and dried, and radioactivity was determined as described previously. An identical procedure was used to determine rates of protein synthesis by using 0.1 ml (10 μCi) of ^{3}H-L-isoleucine (40 mCi/mmol). In this case the precipitates were washed with 20 ml of cold 10% trichloroacetic acid containing unlabeled L-isoleucine (66 μg/ml) and L-valine (58 μg/ml).

Degradation of DNA. Cells of an overnight culture were inoculated into fresh medium containing ^{3}H-thymine (2 μg/ml, 1.26 μCi/mmol) and allowed to grow for three to four generations. The cells were collected by centrifugation at 5 C (27,000 × *g* for 10 min), washed twice in cold 0.033 M potassium phosphate (pH 7.0), and then suspended in the same buffer at a concentration of about 5 × 10^7/ml. These suspensions were either irradiated with ultraviolet light (UV) or else directly innoculated, at a concentration of 5 × 10^6 cells per ml, into control medium or medium containing PDD or mitomycin C. The concentration of unlabeled thymine in these media was increased to 40 μg/ml.

During subsequent incubation at 37 C, duplicate samples of 1.0 ml were removed and precipitated with an equal volume of cold 10% trichloroacetic acid containing unlabeled thymine (40 μg/ml). After storage for 1 h at 0 C, each sample received 0.04 ml of a 0.5% solution of bovine serum albumin, in order to facilitate precipitation. After filtration and rinsing, the membranes were dried and radioactivity was determined as described previously.

UV irradiation. About 5 ml of tryptone-E broth containing repair mutants or their prototrophs, or phosphate buffer containing cells of strain W3350 *thy* (approximately 5 × 10^7 per ml), were placed in a standard glass petri dish. During irradiation, the dish was gently shaken beneath a 30-W General Electric germicidal lamp. Light intensity, as calibrated by comparison of inactivation kinetics with established values (9) was 13 ergs per mm^2 per s.

Miscellaneous. Increase of cell mass was determined spectrophotometrically at 620 nm. Extent of filamentation was monitored by direct observation with a microscope.

Reagents. Preparations of PDD were generously provided by Barnett Rosenberg. Mitomycin C and amino acids were purchased from Calbiochem (La Jolla, Calif.), and radioisotopes were obtained from New England Nuclear Corp. (Boston, Mass.).

RESULTS

Temperature and viability. Viability of PDD-treated cells decreased rapidly as a function of increased temperature of incubation (Fig. 1). For example, the times required to reduce viability of wild-type filaments by 37% in the presence of 35 μg of PDD per ml of medium were about 210 and 60 min at 25 and 40 C, respectively. The corresponding value obtained during incubation at 37 C was approximately 100 min.

DNA repair mutants. A series of mutants blocked in various steps of excision or recombination repair were tested for ability to form filaments and remain viable in the presence of PDD (Table 2). Isolates known to be especially sensitive to UV were rapidly killed by PDD and failed to undergo pronounced filamentation. This response was expressed in particular by *recA13* and *uvrA6 lex1* mutants, whereas single *uvrA6* or *lex1* isolates were significantly more resistant to PDD. Also of intermediate sensitivity were *polA*, *recB21*, *recC22*, and *recB21 recC22* isolates which were able to form extensive filaments in the presence of PDD. The UV suppressor *sbc8* in the *recB21* strain JC 5412 (23) was also able to provide protection against PDD.

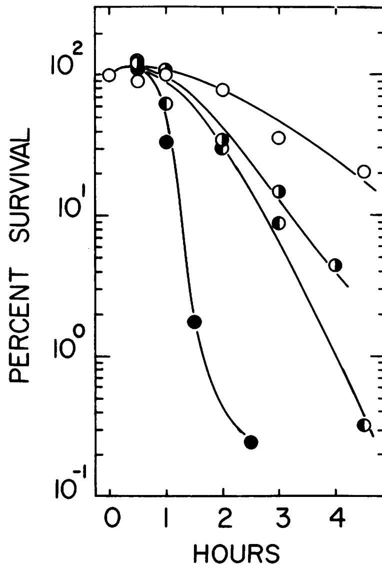

FIG. 1. *Survival (colony-forming ability) of filaments of Escherichia coli strain W3350 thy during incubation in C medium supplemented with thymine (20 μg/ml) at 25 C (○), 30 C (◑), 35 C (◐), and 40 C (●) in the presence of cis-platinam(II)diamminodichloride (35 μg/ml). The initial optical density was 0.1.*

TABLE 2. *Comparison of treatments with UV light and cis-platinum (II) diamminodichloride (PPD) on survival and filamentation of DNA mutants of Escherichia coli K-12*[a]

Strain		Time to reduce viability to e^{-1}			Ratio of cell mass (PDD to control)	Formation of Filaments[b]
		UV (s)	PDD (min)	Ratio (s)		
AB1157	*rec*⁺	144	>90		0.80	
AB1886	*uvrA6*	13	20	0.011	0.81	+
AB2463	*recA13*	2	5	0.007	0.58	0
AB2494	*lex1*	11	12	0.015	0.67	±
AB4554	*lex1*	14	12	0.019	0.53	±
AB2474	*uvrA6 lex1*	3	5	0.01	0.55	0
JC5029	*rec*⁺	155	>90		0.80	+
JC5412	*recB21 sbc8*	66	>90		0.86	+
JC6722	*recB21*	30	20	0.025	0.86	+
JC5426	*recC22*	21	45	0.008	0.89	+
JC4583	*rec*⁺	150	>90		0.91	+
JC4584	*recB21 recC22*	25	10	0.042	1.0	+
mr 2-41	*rec*⁺	90	>90		1.0	+
mr 2-41	*recA*	1	3	0.006	0.52	0
W3110	*pol A*⁺	204	>90		1.0	+
P3470	*polA*	24	30	0.013	0.94	+

[a] Cells growing logarithmically received, at an optical density of 0.1, either saline or PDD in saline to yield a final concentration of 35 μg/ml in tryptone-E medium containing added glucose (0.01 M) and thymine (20 μg/ml). Viability was monitored by plating, and the ratio of optical densities was determined after 3 h of incubation at 37 C.

[b] PPD-induced filamentation: (+), filaments 5 to 10 times longer than control cells: (±), cells about twice the length of control cells; and (0), cells equal in size or shorter than control cells.

Degradation of DNA. Washed wild-type cells which had previously been cultivated with ^{3}H-thymine were inoculated into fresh medium containing PDD; loss of trichloroacetic acid-insoluble radioactivity was monitored during further incubation. About 25% of the total radioactivity became soluble after incubation for 3 h in the presence of 50 μg of PDD per ml of medium (Fig. 2). Release of radioactivity was concentration dependent, as judged by a corresponding loss of about 15% after similar incubation with 25 μg PDD per ml. Wild-type cells were treated with approximately equivalent lethal doses of UV, mitomycin C, or PDD; the rate of PDD-induced release of radioactivity was intermediate between that promoted by UV and mitomycin C at the dosages tested (Fig. 3).

In view of the known ability of *recA* mutants to degrade their DNA extensively after irradiation with UV, a test of DNA stability was made in the presence of PDD. Addition of 50 μg of PDD per ml failed to induce significant breakdown of deoxyribonucleic acid in *recA*⁺ cells as

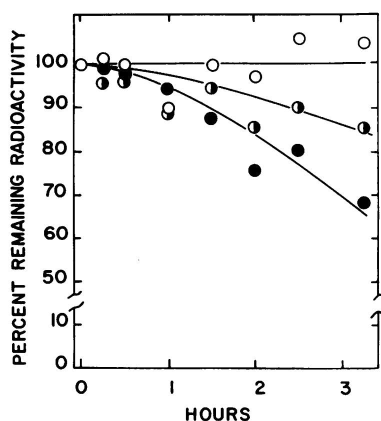

FIG. 2. *Loss of trichloroacetic acid-insoluble radioactivity from cells of Escherichia coli strain W3350 thy, previously grown with ³H-thymine, during incubation in C medium containing added unlabeled thymine (40 μg/ml) in the presence of 50 μg (●), 25 μg (◑), and no (○) cis-platinum(II)diamminodichloride per ml, respectively.*

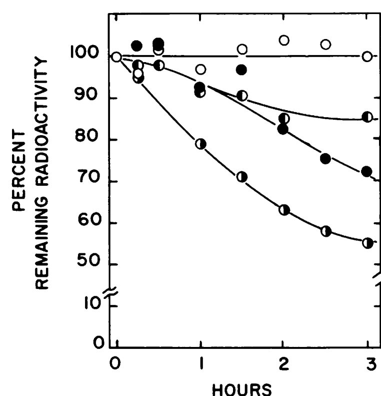

FIG. 3. *Loss of trichloroacetic acid-insoluble radioactivity from cells of Escherichia coli strain W3350 thy, previously grown with ³H-thymine, during incubation in C medium containing added unlabeled thymine (40 μg/ml) in the absence of treatment (○), after irradiation with 4,000 ergs of ultraviolet light per mm² (◑), and upon addition of 50 μg of cis-platinum(II)diamminodichloride (●) or 1 μg of mitomycin C (◐) per ml of medium.*

compared to that in a control culture lacking PDD. Slight breakdown occurred in a control culture of *recA* cells which was similarly enhanced by addition of 35 μg of PDD per ml or exposure to 1,000 ergs per mm² of UV light (Fig. 4).

Synthesis of DNA. The rate of DNA synthesis, as judged by pulse labeling with ³H-thymine, became significantly reduced in wild-type cells after 30 min of exposure to PDD. This compound failed to significantly influence the rate of protein synthesis, although that of RNA may have undergone slight reduction (Fig. 5). The extent of PDD-induced inhibition of DNA synthesis was inversely proportional to the duration of pulse with ³H-thymine. For example, an increase of pulse from 2 to 5 min in control cultures yielded an expected 2.5-fold increase in incorporation of isotope (Fig. 6). In contrast, a similar increase in cultures containing PDD resulted in a 3.5-fold increase in incorporation of ³H-thymine. The observation would be expected if PDD-induced inhibition of DNA synthesis was reversed with time or if PDD significantly increased the size of the deoxyribonucleotide pool.

DISCUSSION

Damaged nucleotides are not excised by *uvr* mutants which must, therefore, rely on processes of recombination to repair irradiated DNA (8, 28). Excision can occur in those repair mutants which are blocked in the process of recombination (8, 23, 28). Cells of both of these

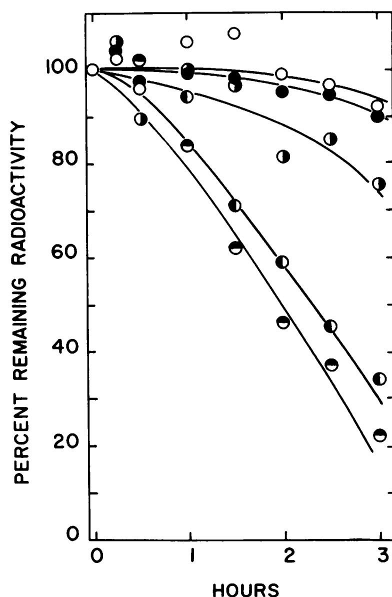

FIG. 4. *Loss of trichloroacetic acid-insoluble radioactivity from cells of Escherichia coli strain mr 2-41 previously grown with ³H-thymine, during incubation in tryptone-E medium containing added unlabeled thymine (40 μg/ml). cis-Platinum(II)diamminodichloride (PDD) in saline was added to a culture of recA⁺ cells (●) in this medium, and an equal volume of saline was added to a parallel control culture (○). A culture of recA cells was similarly divided into three subcultures, one of which was treated with 35 μg of PDD per ml (◐), another exposed to 1,000 ergs of ultraviolet light per mm² (⊖), and the third an untreated control culture (◑).*

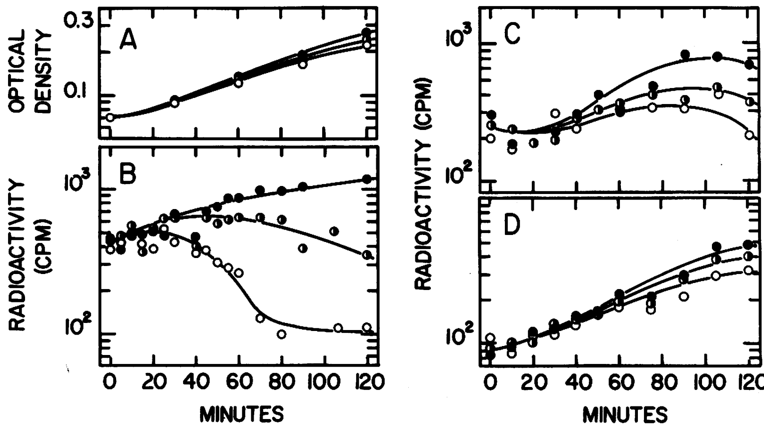

FIG. 5. *Increase in cell mass (A) and rates of deoxyribonucleic acid (B), ribonucleic acid (C), and protein (D) synthesis in control cells of Escherichia coli strain W3350 thy (●) and cells receiving 15 μg (◐) and 35 μg (○), respectively, of cis-platinum(II)diamminodichloride per ml of C medium. Procedures used for pulse labeling are described in the test.*

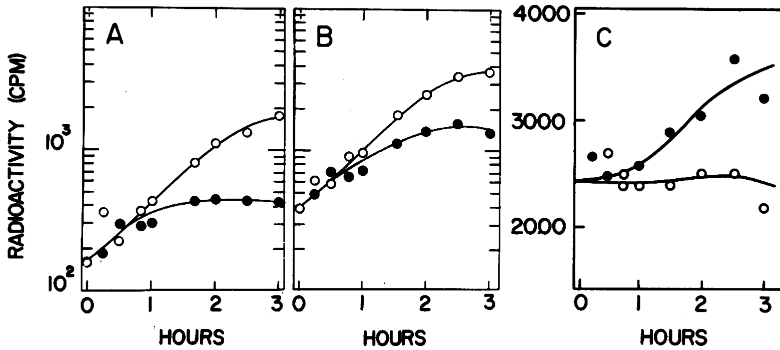

FIG. 6. *Rates of deoxyribonucleic acid synthesis in control cells of Escherichia coli strain W3350 thy (○) and cells treated at the initiation of the experiment with 35 μg of cis-platinum(II)diamminodichloride per ml of C medium (●); A, pulsed for 2 min with ^{3}H-thymine and B, pulsed for 5 min with ^{3}H-thymine. The difference between the values obtained during 2- and 5-min pulses is shown in C.*

mutant classes and an isolate lacking DNA polymerase I (*polA*) were killed by PDD at rates exceeding those of wild-type organisms. PDD was acutely toxic to those recombination mutants (*uvrA* and *lex*) which are known to undergo extensive autodegradation of DNA after treatment with UV. Similar degradation occurred in *recA* mutants after exposure to PDD. A double mutant blocked in both the excision and recombination pathways of DNA repair (*uvrA6 lex1*) was more sensitive to PDD than were single-excision (*uvrA6*) or recombination (*lex1*) mutants. Both of these pathways may be essential for the repair of interstrand cross-links (4).

PDD promoted the release of trichloroacetic acid-soluble fragments of DNA from growing cells. The nature of these fragments was not determined, but they may be analogous to the repair products which arise after treatment of bacteria with UV or certain radiomimetic agents (8, 28). In addition, PDD reversibly inhibited the systhesis of new DNA (Fig. 6), possibly by promoting the degradation of old DNA and thus increasing the size of the nonradioactive deoxyribonucleotide pool. These findings suggest that PDD can directly interact with bacterial DNA and that the resulting damage may be repaired by defined mechanisms.

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