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Tracer Studies to Locate the Site of Platinum Ions Within Filamentous and Inhibited Cells of *Escherichia coli*

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The distribution of platinum ions within *Escherichia coli* after the induction of filaments with *cis*-$Pt(NH_3)_2Cl_4$, and after growth inhibition by $PtCl_6^{2-}$, has been determined with radioactive metal compounds (^{191}Pt, with a half-life of approximately 3 days) by the simple chemical procedure of Roberts et al. In the filamentous cells, the platinum metal is associated with metabolic intermediates, nucleic acids, and cytoplasmic proteins; whereas, in inhibited cells, the platinum is combined only with the cytoplasmic protein. Similar experiments with gram-positive cells of *Bacillus cereus* and *Staphylococcus aureus*, which show no filamentous growth in the presence of *cis*-$Pt(NH_3)_2Cl_4$, reveal that the metal complex does penetrate the cell wall and subsequently becomes bound predominantly by metabolic intermediates.

It has been observed in this laboratory (4) that *Escherichia coli* cells form long nonseptate, multinucleated filaments, up to 300 times the length of a normal cell, when certain group VIIIb transition metal complexes of platinum, rhodium, and ruthenium are added to the culture medium. Subsequent investigations, carried out primarily with platinum salts, showed that hexachloroplatinate [$PtCl_6^{2-}$] is bactericidal to all the microorganisms tested, whereas *cis*-di-ammino-tetrachloro-platinate [*cis*-$Pt(NH_3)_2Cl_4$] is effective in forcing filamentous growth in gram-negative bacilli without markedly affecting cellular growth (3, 5). Gram-positive bacilli and cocci show little effect in the presence of the latter compound. Cytokinesis (cross-septation) can be initiated in these filamentous forms of *E. coli* only by removal of the metal compound from the medium. This contrasts with experiments in which cross-septation of filamentous cells can be induced by pantoyl lactone, by divalent cations, or by an elevated temperature (3).

We performed experiments with radioactive platinum (^{191}Pt) designed, first, to locate the site of the metal within the bacterium and, second, to investigate differences in the mode of action of the various ionic species of platinum. Since the tracer available at the time of these experiments has a half-life of 3 days, we carried out only simple and quick chemical fractionations (2). This procedure reveals the distribution of a tracer among various broad classes of compounds.

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MATERIALS AND METHODS

Cultivation. Stock cultures of the various bacteria were maintained on nutrient agar slants and subcultured at monthly intervals. Nutrient broth, Brain Heart Infusion, and the synthetic mineral "C" medium of Roberts and co-workers (2) containing 0.2% glucose as the energy source, were used. Early log-phase subcultures were used to inoculate 1-liter amounts of the media, giving initial cell counts of approximately 10^8 cells/ml. Two-liter flasks were equipped with fritted-glass bubblers through which compressed air was passed at a constant rate. For each experiment, the particular radioactive platinum compound was added to give a final concentration of 10 to 50 μg/ml of platinum. Samples (25 ml) were removed at intervals. The absorbance of the culture was measured at 850 mμ in a model B spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), and the total number of cells was determined with a Petroff-Hausser counting chamber and a phase-contrast microscope.

Preparation of the radioactive tracer. The tracer experiments were performed with the radioisotope ^{191}Pt, since this isotope could be generated from naturally occurring iridium metal by bombardment with protons. The latter service was available to us through the courtesy of the staff of Michigan State University Cyclotron laboratory. With a half-life of 3 days, ^{191}Pt decays via electron capture, emitting γ rays. The spectrum of energies has recently been

published by Blichert-Toft et al. (1). The short life of this species frustrated attempts to extract the platinum isotope and to synthesize it into *cis*-diaminotetrachloroplatinate, $Pt(NH_3)_2Cl_4$, which is most effective in forcing filamentous growth of *E. coli*. Hence, the platinum was extracted from iridium and immediately converted to a solution of $(NH_4)_2PtCl_6$. The latter was diluted with an equal volume of "C" medium and irradiated for 45 min under a low-pressure mercury lamp. The resulting solution forced filamentous growth, and, as was shown in earlier work (5), contains the diamminotetrachloroplatinate complexes, possibly both the *cis*- and *trans*-isomers.

The iridium foil, supplied by Engelhard Industries, Inc., Newark, N.J., has a minimal purity of 99.8%; the major impurities are the platinum group metals. Irradiation of five pieces of foil, $1 \times 1 \times 0.0038$ cm^3 (approximate weight, 70 mg), with 32 Mev protons on three separate occasions, for 45 min, generated activities between 100 and 500 Mrad/hr. The γ-ray spectrum, kindly measured by W. Kelley, showed the radioactive product to be predominantly ^{191}Pt. Small quantities of osmium and gold were also present. Studies on the half-life of the purified radioactive platinum indicated that ^{197}Pt was also generated.

The extraction procedure developed is a modification of that of Thompson and Rasmussen (6).

The foil of iridium was shredded and added to a melt of alkali (50% KOH-50% KNO_3). The alkali was heated strongly for 10 to 15 min until dark brown. After cooling, the mass was extracted with concentrated HCl, giving a blue supernatant solution and a heavy deposit of KCl. The solution was centrifuged to remove the solids (the latter were re-extracted with further quantities of acid to increase the yield). The major part of the activity was now found in the solution phase. Platinum and gold carriers were added at this stage; one small crystal of $HAuCl_4$ and 0.1 g of $(NH_4)_2PtCl_6$ dissolved in dilute HCl.

One extraction with an equal volume of butyl acetate gave a colorless organic layer with little radioactivity. This step removes any gold produced from platinum impurities in iridium foil. A little stannous chloride solution in HCl was added to the aqueous phase. The murky brown color cleared and a deep orange color formed, signifying the reduction of platinum from oxidation state +4 to +2. The color is extractable into butyl acetate (two portions of 50 ml each). The radioactivity is predominantly in the butyl acetate layer. After separating and washing the organic layer with 3 N HCl to remove the stannic chloride, it was flamed in an evaporating dish, yielding a grayish metallic deposit.

The metal deposit was dissolved in the minimal quantity of hot aqua regia. A few crystals of sodium chloride were added and the solution was evaporated to near dryness. The residue was covered with HCl and again evaporated to near dryness to destroy nitrocomplexes. After drying for 1 hr, the residue was covered with water and evaporated to dryness on a steam bath. Boiling water was finally added and the *p*H was adjusted to alkalinity with hot sodium bicarbonate solution. The flocculent white precipitate produced was centrifuged away, and a clear, pale yellow supernatant fluid was obtained. Addition of the ammonium ion as NH_4Cl and reduction of volume on a steam bath gave a solution of $PtCl_6^{2-}$, as shown by the electronic absorption spectrum.

The platinum concentration was determined by the tin chloride test. Total recovery of platinum was never greater than 50%.

This solution of $PtCl_6^{2-}$ was diluted with "C" medium to provide a stock medium containing the required concentration of platinum. This contains the doubly negative-charged species. The neutral species active in forcing filamentous growth was generated by irradiation of the diluted solution with a low-pressure mercury lamp.

Fractionation of cells. Various samples of the culture were placed in 50-ml plastic centrifuge tubes and immediately cooled in an ice bath. After centrifugation in the cold, the cells were washed three times in a 0.9% sodium chloride solution to remove extracellular radioactivity. Resuspension and extraction with 5% trichloroacetic acid for 30 to 60 min at 0 C produced the cold trichloroacetic acid-soluble fraction. After further centrifugation, the cells were resuspended in 70% ethyl alcohol for 30 min at 40 to 50 C, cooled, and centrifuged. The supernatant fluid was the alcohol-soluble fraction. The alcohol-ether soluble fraction was then produced with a 50-50 mixture of 75% ethyl alcohol and ether after heating the resuspended cells for 30 min at 40 to 50 C, cooling, and centrifuging. Finally, the sedimented cells were added to 5% trichloroacetic acid and heated for 30 min in a boiling-water bath to obtain the hot trichloroacetic acid-soluble fraction. The precipitate so produced was washed twice in 75% ethyl alcohol to remove the residual trichloroacetic acid (the acidified-alcohol wash). The precipitate, or crude cytoplasmic protein-cell wall fraction, was suspended in 10 ml of 0.02 N NH_4HCO_3 and adjusted to *p*H 8.0 with NH_4OH. Trypsin was added to a concentration of 0.5 mg/ml and allowed to stand overnight in a 37 C water bath to digest the cytoplasmic proteins and liberate the purified cell walls. This suspension was then passed through a 0.45 filter (Millipore Corp., Bedford, Mass.) and the filter was washed several times with distilled water.

Samples (0.1 to 0.2 ml) from each fractionation step, and from the Millipore-filtered suspension, were placed on planchets and dried under an infrared lamp. Radioactivity was measured with a gas-flow Geiger counter (Nuclear Chicago Corp., Des Plaines, Ill.); the total activity of each fraction is expressed as counts per minute per volume. This enabled the percentage of radioactivity for each fraction to be calculated.

RESULTS AND DISCUSSION

To locate the platinum within the elongated bacterial cells, *E. coli* strain B was grown for 3 hr in the synthetic mineral-salts medium containing 10 μg/ml of the radioactive neutral species of platinum, *cis*-$Pt(NH_3)_2Cl_4$. Elongation of the cells up to 50 times the length of normal cells, was observed. Table 1 shows the distribution of the

radioactive platinum among the various fractions. Using the chromatographic data of Roberts et al. (2), which identified the components of each fraction, the average distribution (from two or more experiments) for the platinum among the four classes of cellular compounds was calculated (Table 2).

At intervals after the inoculation of platinum tracer, the distribution of radioactive platinum was determined in this manner. Figure 1 shows the time course for the incorporation of radioactive platinum by different classes of cellular compounds. The organism was grown in the synthetic medium containing 11 μg/ml of platinum, the neutral species. Filamentous forms were detected within 0.5 hr, and, after 3 hr, over 90% of the cells were filaments 10 to 20 times their normal length. Also plotted in the Fig. 1 is the total uptake of platinum per cell. This reveals a continuous uptake of platinum as the cell elongates.

It is evident that the neutral platinum complex is associated predominantly with protein fragments, although significant quantities are found

TABLE 1. *Distribution of ^{191}Pt (neutral ionic species from ammonium hexachloroplatinate) among the various fractions of filamentous Escherichia coli*

Fraction	Percentage of radioactive platinum	Percentage of ^{35}S reported by Roberts et al. (2)
Cold trichloroacetic acid soluble	20	25
Alcohol soluble	3	12.5
Alcohol-ether soluble	1	0
Hot trichloroacetic acid soluble	34	0
Acidified alcohol wash	1	
Trypsin digest	40	62.5
Purified cell walls	1	

TABLE 2. *Average distribution of ^{191}Pt (neutral species from ammonium hexachloroplatinate) among the various classes of cellular compounds in three bacteria*

Fraction	Percentage of radioactivity		
	E. coli	*B. cereus*	*S. aureus*
Metabolic intermediates	19	74	60
Lipids	6	2	1
Nucleic acids	30	19	20
Cytoplasmic protein	45	5	19

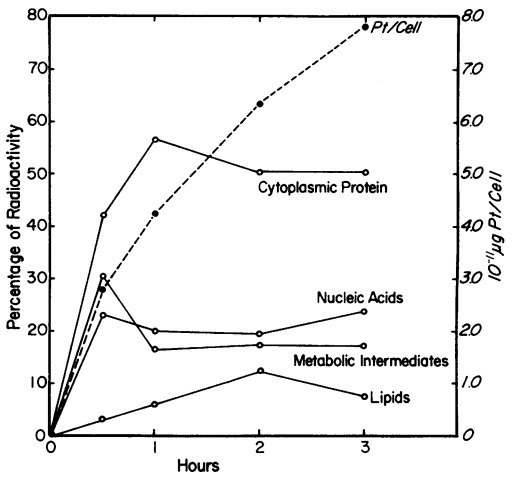

FIG. 1. *Incorporation of ^{191}Pt, as cis-$Pt(NH_3)_2Cl_4$, by various classes of cellular compounds during the growth of filamentous Escherichia coli B, and the total uptake of radioactive platinum per filamentous cell.*

with the nucleic acids and the metabolic intermediates. The plot with time reveals a rapid accumulation of the platinum complex by the metabolic intermediates and a subsequent attachment to the cytoplasmic protein. The chemical properties of platinum would suggest a considerable affinity of the metal for sulfur ligands. For comparison, the distribution of sulfur in the fractions of *E. coli* as determined by Roberts et al. (2) is shown in column 2 of Table 1. Evidently, such a correlation cannot account for all the bound metal, the occurrence of the platinum with nucleic acids being noteworthy. Incubation of *E. coli* in the presence of the neutral platinum species and glutathione, both reduced and oxidized forms does not diminish the efficacy of the platinum salt in forcing filamentous growth (L. Van Camp, *unpublished data*).

Since the development of filamentous growth in the presence of the neutral platinum complex occurs only with gram-negative bacilli, all gram-positive bacilli and cocci tested showed no observable effect (3). A comparison has been made of the distribution of the radioactive platinum within *E. coli* and two species of gram-positive bacteria, *B. cereus* and *S. aureus*. *E. coli* was grown in the synthetic medium containing 10 to 12 μg/ml of platinum and the gram-positive cells were grown in nutrient media containing 30 to 60 μg/ml of platinum. Higher concentrations of platinum were employed in the nutrient medium, because previous studies (3) indicated that twice as much platinum is needed to produce an effect comparable to the synthetic medium. We have

ascribed this to a probable chelation of the platinum by the nutrient polypeptides. Table 2 shows the results of the fractionation procedures with the three species. The two species of gram-positive cells examined took up approximately the same weight of radioactive platinum per cell as the *E. coli* from a medium of nutrient broth containing 26 μg/ml of neutral platinum species. This result was given by a comparison of the total count of radioactive platinum from equal weights of washed and dried cells of the three species.

It is evident that the neutral platinum species can permeate the walls of all three species tested. The predominance of the radioactive metal in the metabolic intermediates of the gram-positive species suggests that the platinum is retained in the pool, possibly by metabolities complexing with platinum.

When *E. coli* is incubated in the dark with fresh ammonium hexachloroplatinate, $(NH_4)_2[PtCl_6]$, the bacteria are immediately killed, the efficacy being dependent upon the concentration of platinum (5). *E. coli* was incubated with 10 to 13 μg/ml of the double negative hexachloroplatinate and the distribution of radioactive platinum was determined (Table 3). The platinum metal is associated predominantly with cytoplasmic protein, leading to cellular death.

Since the radioactive metal is associated with the protein fraction of the filamentous cells, an attempt was made to separate the cellular proteins of *E. coli*. In this way, any specificity of the platinum complex might be detected. Filamentous cells were disrupted with a Blackstone ultrasonic probe and the supernatant fluid, containing over 85% of the radioactivity, was separated into crude protein fractions both by column chromatography on Sephadex G-200 and by electrophoresis on cellulose acetate and starch gel. In no case was it possible to separate an unlabeled band or a disproportionately labeled fraction.

TABLE 3. *Average distribution of ^{191}Pt (double negative and neural species of ammonium hexachloroplatinate) among the various classes of cellular compounds of Escherichia coli*

Fraction	Percentage of radioactivity	
	Double negative species	Neutral species
Metabolic intermediates	1	19
Lipids	3	6
Nucleic acids	1	30
Cytoplasmic protein	96	45

We have considered the possibility that, during the fractionation procedures, the chemical reagents used to leach the cells may dislodge or solubilize the radioactive metal. The reproducibility of our results with a given species of cell, and the drastic difference in the distribution of the tracer within three different species of bacteria, suggest that the tracer distribution found is a function of the biological material and is independent of the fractionation procedure employed. We are currently using a long-life platinum isotope (^{193}Pt, with a half-life of 500 years, from New England Nuclear Corp., Boston, Mass.) and the techniques of ultracentrifugation to examine the distribution of metal throughout the organelles of *E. coli*.

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