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Effects of the Rare Earth Cerium on *Escherichia coli*

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The rare earth cerium was found to bind rapidly to *Escherichia coli*. Cerium inhibited oxygen uptake in the presence of glucose as well as the endogenous respiration of glucose-grown cells. For a cell concentration of 4 mg per ml, maximal inhibition was obtained at 120 μg per ml. Greater concentrations did not increase the inhibitory effect. Cerium inhibited $^{14}CO_2$ evolution and ^{14}C uptake from uniformly labeled glucose. Marked changes in the distribution of ^{14}C incorporated into different chemical fractions of the cell were noted. The most striking changes occurred in the alcohol- and alcohol ether-soluble fractions, in which the ^{14}C activity was increased 5- to 20-fold in the presence of cerium.

The rare earth elements are not known to possess a functional role in living cells, but a clear physiological hazard from these elements exists. It has been established that various plants and animals are able to concentrate these elements from their environments (7, 9). Uptake of these elements by various organisms has resulted in drastic morphological changes in cells and tissues as well as the poisoning of some cellular systems (10, 13). Today, the possibility of exposure to rare-earth elements is great. Rare earths have numerous roles in industry, and, in addition, the rare earths are major radioactive components of fission products from nuclear explosions and reactor wastes (6).

The rare earths have long been known to be toxic for various organisms (1, 3), and more recent work has shown that the rare earths can inhibit respiration of fungi (Talburt and Johnson, Bacteriol. Proc., p. 171, 1967) and cause gross morphological aberrations (13). However, no data have been reported which would indicate the specific metabolic processes affected by rare earths.

This paper includes data concerning some physiological effects of a stable isotope of the rare earth cerium on cells of *Escherichia coli*.

MATERIALS AND METHODS

Organism and media. E. coli B was used in all experiments. The organism was maintained on Trypticase Soy Agar (BBL) slants and stored at 4 C. The defined medium used to grow cells for inocula and for all experiments had the following composition, in amounts per liter: glucose, 10.0 g; NH_4Cl, 2.0 g; K_2HPO_4, 1.22 g; KH_2PO_4, 0.78 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 36.7 mg. A trace element solution was added to give the following minimal concentrations (per liter): Fe as $FeSO_4 \cdot 7H_2O$, 1 mg; Mn as $MnSO_4 \cdot H_2O$, 10 μg; Mo as $NaMoO_4 \cdot 2H_2O$, 10 μg; Zn as $ZnSO_4 \cdot 7H_2O$, 10 μg; B as H_3BO_4, 1 μg; Co as $CoCl_2 \cdot 6H_2O$, 1 μg; Cu as $CuSO_4 \cdot 5H_2O$, 1 μg. Glucose and phosphate were autoclaved separately and added aseptically to the autoclaved medium. The final *p*H of the medium was 7.0. Distilled, deionized water was used in the preparation of all media, buffers, and cerium metal solutions. Cultures were grown in 200 to 300 ml of medium on a New Brunswick gyrotary shaker model "V" operated at 200 rev/min. The inoculum for each experiment consisted of cells grown in the defined medium at 35 C for 20 hr and adjusted to an optical density of 0.3 at 540 mμ. A 1-ml amount of the standardized inoculum was placed into each 100 ml of fresh medium. All cultures were grown at 35 C for 20 hr. For experiments with ^{14}C-labeled cells, the growth medium was supplemented with 2.0 μc of uniformly labeled glucose-^{14}C per 100 ml of medium.

Manometric studies. All manometric experiments were done at 36 C by use of conventional Warburg manometric techniques (14). Cells harvested from 20-hr cultures were washed twice in 0.06 M "HEPES" (*N* - 2 - hydroxyethylpiperazine - *N'* - 2 - ethanesulfonic acid) buffer (5), *p*H 6.6, containing 0.001 M $MgCl_2$. The *p*H was adjusted with sodium hydroxide, and the final buffer was 0.0028 M with respect to the sodium ion. After the final washing, the cells were resuspended in buffer of the same composition, and the cell suspension was adjusted so that a 1:10 dilution gave an 0.3 optical density at 540 mμ. A 2-ml amount of this cell suspension (approximately 10 mg, dry weight, of cells) was placed in the main compartment of each Warburg flask. Also added to the main compartment of each flask was 0.5 ml of an appropriate concentration of cerium as $Ce(NO_3)_3 \cdot$

$6H_2O$ in distilled water. For each exogenous system, 0.5 ml of 0.06 M HEPES buffer containing 5 μmoles of glucose were placed in the side arms. Center wells contained 0.2 ml of 20% (w/v) KOH. For labeling of cells for ^{14}C distribution studies, 50 to 200 nanocuries of glucose-U-^{14}C were combined with 5 μmoles of glucose in the side arm. The contents of the side arm were tipped into the main compartments of the Warburg vessels after the cells had been exposed to the cerium ions for 45 min. The final concentration of HEPES buffer in each flask, after tipping in the side arm contents, was 0.05 M. The $^{14}CO_2$, which was released from cells labeled with glucose-U-^{14}C by growth or by exposure to the labeled substrate, was trapped in KOH in the Warburg vessels. The center well contents were transferred along with washings to combustion tubes (Pyrex stoppers, Corning no. 7665) for analysis for radioactivity. Supernatant fractions, recovered after separation from labeled cells by centrifugation, were also saved for subsequent measurement of radioactivity.

Chemical fractionation and analysis of labeled cells. At intervals after the tipping-in of labeled glucose from the side arms, Warburg flasks were removed from the manometer arms and chilled in ice water to retard further uptake of substrate. The cells from 2.0 ml of the contents of the main compartment were harvested by centrifugation, the supernatant fluid was removed, and the cells were washed once in cold HEPES buffer to remove residual supernatant radioactivity. The resulting cell pellet was fractionated by the procedure described by Clifton and Sobek (2). This yielded a cold trichloroacetic acid-soluble fraction, an acidic alcohol-soluble fraction, an alcohol ether-soluble fraction, a hot trichloroacetic acid-soluble fraction, and an insoluble residue fraction.

Radioactivity measurements were made on dried cells and chemical fractions by combustion to $^{14}CO_2$ and measurement in a Dynacon (Nuclear-Chicago Corp., DesPlaines, Ill.) electrometer system. Trapped respiratory $^{14}CO_2$ was released into the ion chambers by neutralization of the KOH and acidification of the sample with 25% (v/v) H_2SO_4.

Adsorption of cerium. The rate of adsorption of cerium to the bacterial cells was determined at different *p*H values. Cells were suspended in HEPES buffer at twice the concentration used in the Warburg experiments and exposed to cerium at a concentration of 240 μg/ml in Erlenmeyer flasks shaken on a gyrotary shaker at 35 C. This was twice the concentration of cerium used in the Warburg experiments in which 300 μg of cerium was added to each flask. At selected time intervals, samples were removed and centrifuged to remove the cells. The residual cerium in the supernatant fraction was determined by the alizarin red S colorimetric determination (11).

Chemicals and reagents. HEPES buffer was obtained from Calbiochem (Los Angeles, Calif.); glucose-U-^{14}C was obtained from New England Nuclear Corp. (Boston, Mass.); and cerium nitrate [$Ce(NO_3)_3 \cdot 6H_2O$], 99.99% purity, was obtained from G. Frederich Smith Chemical Co. (Cleveland, Ohio).

RESULTS

Adsorption of cerium. Figure 1 shows the rate of disappearance from the supernatant fraction of 240 μg/ml of cerium at different *p*H values by adsorption to washed suspensions of *E. coli* suspended in HEPES buffer. One of the major problems in working with the rare earth elements is that they rapidly form virtually insoluble phosphate and hydroxide compounds at slightly acidic, neutral, and alkaline *p*H values and precipitate from solution. Cerium will also react with protein and a variety of other organic compounds. This problem may be obviated with the fungi by allowing adsorption to take place in distilled water with an acidic *p*H (D. E. Talburt, Ph.D. Thesis, Univ. of Arkansas, Fayetteville, 1965). Such a solution is not feasible with bacteria because of the adverse effects of low *p*H on the cells and the desire to carry out all operations in a buffered system. A buffer system was needed which would buffer near the neutral *p*H range and not react with, nor chelate, the rare earth metal. The HEPES buffer described by Good et al. (5) offered promise of meeting these requirements. No precipitate could be observed for 8 to 24 hr when 0.06 M HEPES buffer, *p*H 6.6, containing 0.001 M $MgCl_2$ was incubated at 37 C with various concentrations of cerium, some in considerable excess of those used in the experimental protocols. The rates of disappearance of cerium from the supernatant fraction by adsorption to cells suspended in buffer at *p*H values of 6.2 and 6.6 were considerably slower than at *p*H 7.0, but in all three cases, adsorption was complete within approximately 30 min, and the data indicate that similar amounts were bound (Fig. 1).

Inhibition of endogenous and exogenous respiration. The effects of various concentrations of cerium on the endogenous and exogenous respiration of *E. coli* are shown in Fig. 2 and 3. In order to allow for complete adsorption of cerium before exposure to the substrate, the contents of the side arms in exogenous Warburg vessels were not tipped into the main compartments until the cells had been exposed to the metal for 45 min. Measurement of endogenous oxygen uptake was also begun 45 min after exposure of the cells to cerium. Little effect on the endogenous or exogenous oxygen uptake rates had been noted in earlier experiments until 45 min after exposure to cerium. Concentrations of 0 to 150 μg of cerium per Warburg flask had little effect on the rate or extent of the exogenous oxygen uptake over a 2-hr period. Inhibition could be noted at a concentration of 200 μg per flask. The inhibition

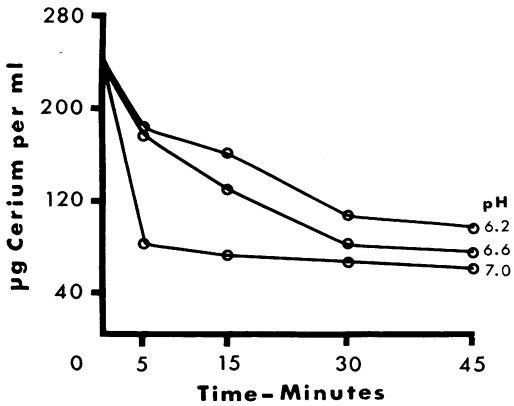

FIG. 1. *Residual supernatant cerium, during adsorption to Escherichia coli at different pH values.*

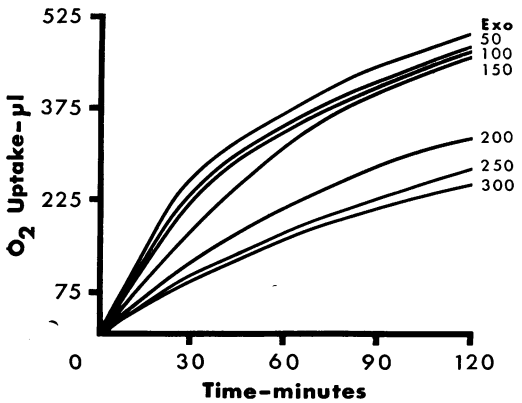

FIG. 2. *Effect of cerium on the oxidation of glucose by Escherichia coli. Figures to the right of the curves represent μg of cerium per Warburg flask. "Exo" refers to the exogenous control flask containing no cerium.*

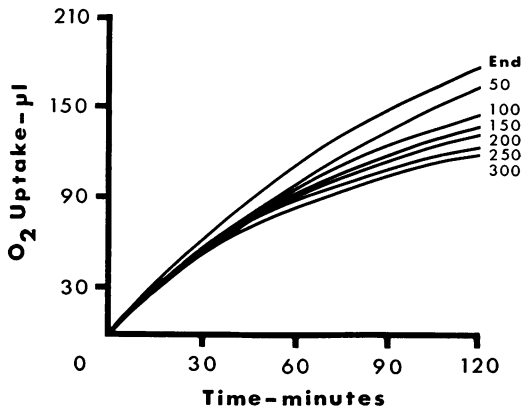

FIG. 3. *Effect of cerium on the endogenous oxygen uptake of Escherichia coli. Figures to the right of the curves represent μg of cerium per Warburg flask. "End" refers to the endogenous control flask containing no cerium. Measurements started after 45-min exposure to cerium.*

became more apparent when 250 and 300 μg per flask were used. In contrast, the endogenous respiration was affected by the lowest concentration of metal used (50 μg per flask) and showed progressively increasing inhibition up to 300 μg per flask. Correction of the exogenous oxygen uptake curves for their respective cerium-treated endogenous curves did not change the pattern of inhibition noted in the uncorrected curves, except to decrease slightly the differences between the total oxygen uptake observed with the 0 to 150 μg per flask concentrations of cerium. Concentrations of cerium from 400 to 800 μg per flask did not suppress the endogenous or exogenous oxygen uptake below that observed with 300-μg amounts. At 600 and 800 μg per flask, the exogenous oxygen uptake was slightly greater than that observed with the 300-μg concentration. The 300-μg concentration gave a 40 to 50% inhibition of total exogenous oxygen uptake, and 50 to 60% inhibition of the total endogenous oxygen uptake by the end of 2 hr. In some experiments, marked clumping and settling of the cells could be noted, particularly in flasks containing concentrations of cerium above 200 μg per flask. This phenomenon has been observed by earlier workers (4, 12). Stained smears showed cells with typical Gram reaction but with marked clumping, in contrast to the predominantly unclumped appearance of untreated cells.

Effect of cerium on the integrity of the cell. Cells labeled by growth on medium containing glucose-U-^{14}C were allowed to respire in Warburg flasks in the presence and absence of 300 μg of cerium per flask. Radioactivity measurements made on the supernatant fractions revealed no differences between the metal-treated versus control cell flasks, over a 3-hr period. The activity in the supernatant fraction at time 0 was 0.69 nc (nanocuries) in both sets of flasks, and at the end of 180 min the activity of the supernatant fractions was identical, 0.51 nc. The initial activity of the cells was 23.9 nc per flask. This indicated that, while the respiration of the cells was inhibited, the cell wall or the membrane, or both, remained intact during the course of an experiment at this concentration of cerium. Electron micrographs of whole cells exposed to 600 μg of cerium showed knob-like protrusions not observable in untreated cells (*unpublished data*). At 300 μg of cerium per flask, fewer cells displayed these lesions. These protrusions were

similar to those observed in rare earth-treated fungi (13). The presence of unlabeled glucose in the flasks did not significantly affect the amount of radioactivity in the supernatant fraction. The supernatant activity of untreated cells in the presence of glucose was 0.72 nc at the end of 135 min, and 0.36 nc in the control flask. Such small differences are probably not significant.

Effect of cerium on the ^{14}C distribution in cells. The decrease in $^{14}CO_2$ evolution from oxidation of uniformly labeled glucose by metal-treated cells, when compared to control flasks, began with the 100-μg per flask concentration and reached a maximal inhibition of approximately 30% at the 300-μg concentration. Concentrations above 300 μg showed less inhibition of $^{14}CO_2$ evolution. The decrease in whole-cell activity also began at the 100-μg per flask level and reached a maximum at 300 μg per flask. The per cent inhibition of whole-cell radioactivity observed in the 300-μg flask was variable, averaging 40 to 50%.

Table 1 shows the distribution of ^{14}C from uniformly labeled glucose in various fractions of untreated and cerium-treated *E. coli*. In most experiments, samples were removed for chemical fractionation and analysis at 40 or 80 min after addition of the labeled substrate. The changes in the intracellular distribution of ^{14}C radioactivity followed the same pattern in all experiments. Significant changes in the amount of radioactivity occurred in all fractions, except the hot trichloroacetic acid-soluble fraction, which showed only a slight depression in activity. The most striking differences occurred in the acidic alcohol- and alcohol ether-soluble fractions, which comprise the alcohol-soluble "protein" and lipids of the cell. The alcohol-soluble fraction amounted to only 5 to 8% of the total cell activity in control cells. The activity of this fraction showed a three- to fivefold increase in cerium-treated cells. Similarly, the activity of the alcohol ether-soluble fraction was increased five- to 20-fold in cerium-treated cells. In control cells, this fraction amounted to only about 1 to 2% of the total activity. The percentage of radioactivity in the cold trichloroacetic acid and residue fractions was decreased in all experiments. The results of fractionation experiments were similar when cells were removed from reaction vessels after 40, 80, or 105 min of exposure to labeled glucose; i.e., the percentage of distribution in fractions of control or cerium-treated cells did not change significantly with time. Table 1 also shows the effects on *E. coli* of nitrate (as $NaNO_3$) alone at a concentration equivalent to that used in the 300-μg cerium flasks, to which the rare earth was added as the nitrate salt. The percentage of the activity in the hot trichloroacetic acid and residue fraction was higher than that observed in the control flasks, but the activity of the cold trichloroacetic acid and alcohol fractions was slightly less. The activity in the alcohol-ether fraction remained essentially the same. Sodium nitrate, at the same concentration (0.004 M) as that used during adsorption in the 300-μg cerium flask, had no effect on the oxygen uptake in the presence of glucose and no effect on the endogenous respiration. In addition, viable plate counts on the cells from the control flasks and nitrate-treated cells were essentially the same at the end of the Warburg experiment (i.e., 3.29×10^{10} viable cells per ml in the control flask and 3.20×10^{10} viable cells per ml for the nitrate-treated cells). One might expect a shift, during assimilation, in the direction of synthesis of higher molecular weight nitrogenous compounds (i.e., the hot trichloroacetic acid-soluble and residue fractions) in the presence of both a carbon and nitrogen source, when compared to a carbon source alone. This, however, does not obscure the effects of the cerium, which causes an accumulation of alcohol and alcohol ether-soluble material.

DISCUSSION

The results show that cerium is rapidly adsorbed by bacterial cells, and that the rate of disappearance of cerium from the supernatant

TABLE 1. *Effect of cerium and nitrate on the intracellular distribution of radioactivity from glucose-U-^{14}C*[a]

Fraction	Control cells		Cerium-treated cells[b]		Nitrate-treated cells	
	nc	%	*nc*	%	*nc*	%
Whole cells	34.8	—	28.4	—	16.9	—
Cold trichloroacetic acid-soluble	18.00	47.0	5.18	18.7	3.35	22.1
Alcohol-soluble	2.09	5.5	9.15	33.8	0.36	2.4
Alcohol ether-soluble	0.50	1.3	5.70	21.0	0.31	2.0
Hot trichloroacetic acid-soluble	2.76	7.2	1.26	4.6	3.43	22.7
Residue	14.9	39.0	5.79	21.9	7.67	50.8

[a] Activity is expressed as nanocuries (nc). Percentages are the percentages of the total activity in all fractions.

[b] In amounts of 300 μg per flask.

fraction is somewhat *p*H-dependent. At a *p*H of 7.0, cerium uptake by *E. coli* was essentially complete within 15 min. At *p*H values of 6.2 and 6.6, the rate of disappearance of cerium from the supernatant fraction was slower, but complete within 30 min. Johnson and Kyker (8), however, report that cerium uptake by *Saccharomyces cerevisiae* is optimum at *p*H 2.5 to 4.0. The difference in chemical composition of the bacterial and fungal surfaces may account for the difference. The observed decrease in the rate of cerium disappearance, as the *p*H is lowered, could reflect a decrease in the number of negatively charged groups on the cell surface resulting in a decreased attraction for the cerium cations.

In appropriate concentrations, cerium will inhibit the oxidation and assimilation of glucose. The endogenous respiration of the cells is also inhibited. Increasing the concentration of metal beyond a certain level does not increase the extent of inhibition of either the endogenous or exogenous oxygen uptake of the cells. This suggests that there may be a fixed maximal binding capacity for a given concentration of cells. The maximal inhibition of both endogenous and exogenous respiration is reached at 300 μg per flask (120 μg/ml) for a cell concentration of 4 mg (dry weight) per ml. In fungi, the rare earths affect growth, and gross changes in morphology have been observed suggesting breakdown of the cell wall or the cell membrane, or both (13). In *E. coli*, similar cell-surface damage has been observed and, in addition, the alterations in the distribution of radioactivity in almost all fractions of the cell suggest penetration of the cerium into the cytoplasm of the cell and interference with several metabolic functions.

The most striking changes in the distribution of radioactivity in the cerium-treated cells occurred in the alcohol- and alcohol ether-soluble fractions of the cell (Table 1). The material accumulating in these two fractions is unknown but may be lipid or low-molecular-weight material. The rare earth elements combine readily with a variety of compounds, particularly with protein or phosphate compounds. Therefore, the observed effects could be due to nonspecific binding to proteins, such as enzymes, or, possibly, a binding to and inactivation of phosphate compounds in the cell. If some of the nucleotides were inactivated, this could lead to derangement of the cellular synthetic reactions in the direction of compounds requiring less energy for synthesis or to a "pooling" of precursor material. Wurm (15) has suggested that failure of *Streptococcus faecalis* to grow in the presence of lanthanum was due to the depletion of phosphate from the medium by the metal. Wurm also suggested that relatively high concentrations of lanthanum could deplete the intracellular phosphate and inhibit cellular metabolism.

Studies are currently underway to determine the cellular adsorption sites of rare earths, the morphological changes accompanying rare earth adsorption, and the precise metabolic events which are affected by the rare earth elements.

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