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A NONSPECIFIC INCREASE IN PERMEABILITY IN ESCHERICHIA COLI PRODUCED BY EDTA

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E. coli is generally impermeable to ionized or charged molecules, with the exception of those for which it possesses specific transport systems. This permeability barrier is thought to be responsible for the resistance of *E. coli* to actinomycin, since the drug inhibits RNA synthesis in extracts of this organism but not *in vivo.*[1] However, sensitivity to actinomycin can be produced in this organism by treatment with ethylenediaminetetraacetate (EDTA).[2] It therefore appeared possible that sensitization results from increased permeability to the drug.

The present paper will show that treatment with EDTA does result in greater permeability to actinomycin, and that this increase in permeability is general, permitting entry of other, unrelated compounds. Moreover, by adjusting the conditions of EDTA treatment, it is possible to produce this permeability change without altering the growth rate and viability of the cells.

Materials and Methods.—*E. coli* AB 1105 (β-galactosidase-inducible, permease-negative; $i^+z^+y^-$) was grown at 37° on a minimal, Tris-Cl-based medium with required nutrients as described;[2] the carbon source was 0.5 per cent glucose unless stated otherwise.

The cells were harvested in exponential phase, at a density of 2–4 $\times$ 10^8/ml, washed once at room temperature with 0.12 *M* Tris-Cl, pH 8.0, and resuspended at a density of 2–10 $\times$ 10^9/ml in the same buffer. The suspension was incubated for 2

min at 37° with 2×10^{-4} *M* EDTA, and then 10 vol of growth medium were added to terminate the treatment. Controls were incubated without EDTA, and diluted in the same manner.

H^3-uracil, uracil-2-C^{14}, and C^{14}-*L*-leucine were purchased from the New England Nuclear Co., Boston. H^3-actinomycin D was the generous gift of Dr. H. Weissbach, and unlabeled actinomycin D was donated by Merck Sharp and Dohme, Rahway, N.J.

Incorporation of radioactive leucine and uracil into material precipitable at 4° by trichloroacetic acid (TCA) was measured by collecting the precipitates on Millipore (HA, 0.45 μ) filters, and counting them in scintillation solvent, as described previously.[2]

β-Galactosidase was assayed by measuring the hydrolysis of o-nitrophenylgalactoside; the assay and unit of enzyme activity have been described.[3] Ornithine transcarbamylase was assayed by measuring the formation of citrulline from added ornithine and carbamyl phosphate; the assay,[4] and unit of activity,[5] were as described previously.

Results.—For the present experiments it was desirable to have as nearly normal cells as was compatible with the EDTA effect. The previously described method of EDTA treatment[2] was modified (see *Materials and Methods*), primarily by lowering the EDTA concentration from 10^{-3} *M* to 2×10^{-4} *M*. As shown in Figure 1, cultures treated in this manner incorporated radioactive leucine and uracil at virtually the same rate as controls; nevertheless, they were fully sensitive to the action of actinomycin (Fig. 2). In other experiments, the rate of increase in optical density during several hours was the same in EDTA-treated and control cultures, and previous experiments[2] had shown that viability was not reduced. Thus, EDTA treatment can render a cell fully sensitive to actinomycin under conditions that do not impair viability or growth rate.

Actinomycin uptake: Actinomycin uptake was studied by suspending treated and untreated cells in H^3-actinomycin. At various times thereafter, aliquots were collected and washed by centrifugation at 4°, and the radioactivity of the cell pellets was determined. As shown in Figure 3, the EDTA-treated cells took up actinomycin to a final concentration of 0.6 μg/mg dry weight while none entered the control cells. Thus, the sensitivity of EDTA-treated *E. coli* to actinomycin is correlated with ability to take up the drug.

Somewhat unexpectedly, it was found that actinomycin uptake into EDTA-treated cells did not occur at 0°. Current views on the mode of action of actinomycin[6] do not predict that an enzymatic reaction should be necessary for its binding, and it is hardly likely that EDTA treatment has induced an active transport system. Perhaps the cell surface differs in permeability properties at 4° and 37°. Further experimentation is necessary to distinguish among these alternatives.

Permeability to enzyme substrates: Permeability to other molecules was studied by measuring the activity of enzymes whose substrates normally cannot enter *E. coli*. In many such cases, enzyme activity is not observed in whole cells incubated with substrate unless an agent such as toluene is added to destroy cell impermeability. If EDTA treatment results in breakdown of a permeability barrier, EDTA-treated preparations, unlike controls, might be expected to show enzyme activity even without toluene.

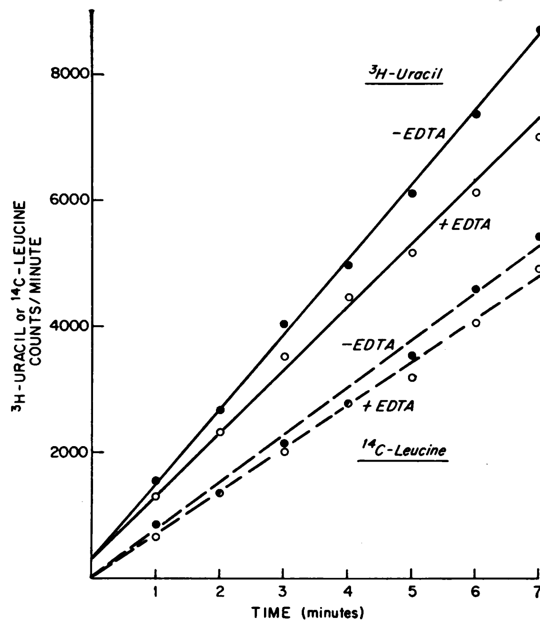

FIG. 1.—Incorporation of H³-uracil and C¹⁴-leucine into EDTA-treated and control cultures. EDTA-treated and control cell suspensions were prepared as described in *Materials and Methods*. The cell density of each after dilution with growth medium was 2×10^8/ml. After 4 min further incubation at 37°, C¹⁴-leucine (50 μc/μmole, 0.1 mM final concentration) and H³-uracil (1 mc/μmole, 20 μM final concentration) were added. Samples were assayed for TCA-precipitable radioactivity. Counts given are per 0.2 ml of culture. Solid lines, H³-uracil; dashed lines, C¹⁴-leucine.

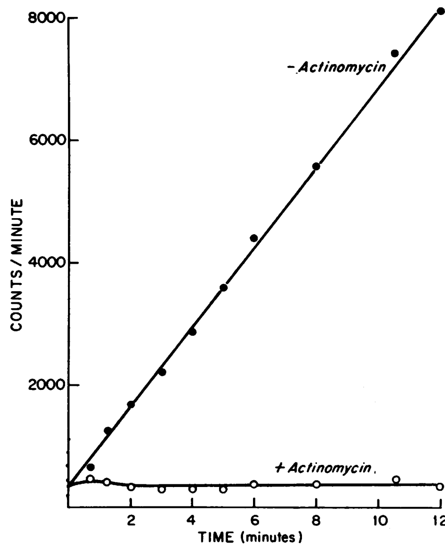

FIG. 2.—Incorporation of C¹⁴-uracil into EDTA-treated cells in the presence and absence of actinomycin. EDTA-treated cells were prepared as described in *Materials and Methods*. After dilution with growth medium to a density of 2×10^8/ml, the culture was incubated 4 min at 37° and then divided into two equal parts. At 0 time, actinomycin, 10 μg/ml, was added to one vessel, and C¹⁴-uracil (8 μc/μmole, 30 μM final concentration) to both. Incubation was continued, and samples were assayed for TCA-precipitable radioactivity. Counts given are per 0.2 ml of culture. In control cultures (not treated with EDTA) C¹⁴-uracil incorporation was not inhibited by actinomycin.

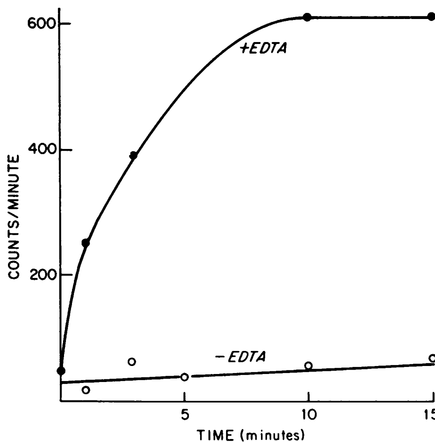

FIG. 3.—Uptake of H^3-actinomycin by EDTA-treated and control cultures. EDTA-treated and control cell suspensions were prepared as described in *Materials and Methods*. The cell density after dilution with growth medium was 1×10^9/ml. After 1 min further incubation at 37°, H^3-actinomycin (2670 cpm/μg, 5 μg/ml final concentration) was added to each. Aliquots were removed to chilled tubes, centrifuged, and washed at 4°, first with chilled medium and then with chilled H_2O. The precipitates were suspended in scintillation solvent and counted. Counts given are per 1.0 ml of culture.

(a) *β-Galactosidase:* The entry of o-nitrophenylgalactoside was assayed in a strain of *E. coli* possessing β-galactosidase but no galactoside permease.[7] As shown in Table 1, control cultures show no enzyme activity unless toluene is added, while EDTA-treated cultures yield 20 per cent as much activity in the absence of toluene as in its presence. When the cells were removed by filtration, no enzyme (less than 0.2%) remained in the supernatant fluid; thus, the activity demonstrable in EDTA-treated cells results from accessibility of the substrate to internal enzyme, and not to release of the enzyme to the medium.

(b) *Ornithine transcarbamylase:* The second enzyme used to assay cell permeability was ornithine transcarbamylase. Although *E. coli* possesses a transport system for one substrate, ornithine,[8] this enzyme cannot be assayed adequately in whole cells, presumably because the other substrate, carbamyl phosphate, cannot enter. However, as shown in Table 2, cells treated with EDTA, unlike controls, show full ornithine transcarbamylase activity in the absence of added toluene. As in the case of β-galactosidase, the enzyme is not released into the medium.

Discussion.—The above experiments indicate that brief treatment of *E. coli* with EDTA causes a generalized increase in permeability, which allows freer entry of compounds as diverse in structure as actinomycin, o-nitrophenylgalactoside, and carbamyl phosphate.[9] The size, shape, and charge of a molecule might influence its entry into an EDTA-treated cell, but such effects have not as yet been studied.[10]

At the present time, the nature of the surface change wrought by EDTA is completely unknown. It is reasonable to speculate that binding of some divalent cation(s) is involved, but it is noteworthy that the effect is not reversed by dilution of the cells into medium containing a large excess of all ions required for growth. Preliminary experiments indicate that 20 min of growth employing the conditions described above restores the permeability barrier in 10 per cent of the cells; extended growth results eventually in repair of the entire culture.

It appears likely that a barrier to the passage of ionized molecules would be helpful to a free-living cell, to prevent loss of internal co-factors and intermediates.[11] Although EDTA-treated cells admit molecules which normally cannot enter, the present experiments do not indicate whether or not any essential metabolites leave the cell. However, any such loss is presumably not injurious to the cells, since the growth rate is virtually unaltered.

TABLE 1

β-GALACTOSIDASE ACTIVITY IN EDTA-TREATED AND CONTROL CULTURES, WITH AND WITHOUT TOLUENE TREATMENT

	Units/mg Dry Weight		
	Treated with toluene	Not treated with toluene	Supernatant fluid
Treated with EDTA	1300	230	1–2
Not treated with EDTA	1140	11	—

E. coli AB 1105 was grown with 0.25% glycerol as the carbon source. One hour before harvesting, 5×10^{-4} *M* isopropylthiogalactoside was added. EDTA-treated and control cell suspensions were prepared as described in *Materials and Methods*. The suspensions were diluted with growth medium to a density of 3×10^8/ml and immediately chilled. Aliquots were agitated for 20 min at 37° with toluene (5% of the final volume). The supernatant fluid was obtained by filtering aliquots untreated with toluene on Millipore filters and collecting the filtrate. Assays for β-galactosidase were performed on aliquots that had or had not been treated with toluene, and on the supernatant fluid. The units for the supernatant fluid are for the supernatant from 1 mg dry weight of cells.

TABLE 2

ORNITHINE TRANSCARBAMYLASE ACTIVITY IN EDTA-TREATED AND CONTROL CULTURES, WITH AND WITHOUT TOLUENE TREATMENT

	Units/mg Dry Weight		
	Treated with toluene	Not treated with toluene	Supernatant fluid
Treated with EDTA	53.4	56.2	<0.5
Not treated with EDTA	54.4	4.2	<0.5

EDTA-treated and control cell suspensions were prepared as described in *Materials and Methods* except that the suspensions were diluted with H_2O instead of growth medium, to a density of 3×10^8/ml, and immediately chilled. Aliquots were agitated for 5 min at 37° with toluene (5% of the volume). The supernatant fluid was obtained by filtering aliquots on Millipore filters and collecting the filtrate. Assays for ornithine transcarbamylase were performed on aliquots that had or had not been treated with toluene, and on the supernatant fluid. The units for the supernatant fluid are for the supernatant from 1 mg dry weight of cells.

If the EDTA-treated cell surface is injured further, loss of internal substances does occur. Thus, EDTA treatment followed either by lysozyme or by osmotic shock has been found by Neu and Heppel to result in the release of several enzymes from *E. coli*,[12, 13] although these are not released by the use of EDTA alone (L. Heppel, personal communication).

Empirically, EDTA treatment has been used for some years to render *E. coli* sensitive to lysozyme.[14] The present experiments may help explain the efficacy of this treatment: presumably the alteration in permeability caused by EDTA permits this cell wall depolymerizing enzyme to approach its substrate.

Certain substances can enter *E. coli* despite the general permeability barrier since this organism possesses a number of active transport systems, each of which catalyzes the entry of one or more sterically related molecules.[15] Although no experiments have been performed to determine directly whether these transport systems are altered by EDTA treatment, indirect evidence indicates that they remain functional. For instance, *E. coli* treated with EDTA and lysozyme in a medium of high osmolarity forms spheroplasts; if the original culture possesses β-galactoside permease, the spheroplasts retain the ability to transport β-galactosides.[16] In the present experiments, the incorporation of radioactive amino acids into protein by EDTA-treated cultures proceeds at the same initial rate as in the control cultures, even when the amino acids are supplied at low external concentration (see Fig. 2), which would not be expected if the transport systems for these amino acids had been injured. It thus appears likely that EDTA treatment alters the permeability barrier of the cell without altering the function of specific active transport systems.

One of the most intriguing aspects of this problem is its possible bearing on the

basic differences between the cell walls and permeability properties of gram-positive and gram-negative organisms. Thus, gram-positive organisms are sensitive to lysozyme; gram-negative organisms are not—unless treated with EDTA. Gram-positive organisms are sensitive to actinomycin, gram-negative organisms are not—but treatment with EDTA makes *E. coli* sensitive. Gram-positive organisms can be genetically transformed by homologous DNA—it remains to be seen whether a molecule of this size can enter EDTA-treated *E. coli*. A further hint that these problems may all be interrelated is the finding[17, 18] that mutation to actinomycin resistance in *B. subtilis* results also in poor transformability. It appears likely that this mutation alters permeability, and it would be interesting to determine how general the alteration is, and whether EDTA treatment would make such mutants temporarily sensitive to actinomycin and restore their transformability. Such tests of cell physiology could profitably be combined with *in vitro* studies on the effect of EDTA on isolated gram-positive and gram-negative cell walls. The use of EDTA in this manner may well prove a helpful tool to dissect the complexities of the bacterial cell surface.

Summary.—*E. coli*, treated with EDTA under certain conditions, becomes permeable to several unrelated molecules to which it is otherwise impermeable. This increased permeability explains the sensitivity of such preparations to the antibiotic actinomycin, which is normally ineffective against *E. coli*. Despite this impairment of a permeability barrier, such cells grow at a normal rate.

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[10] Experiments in progress in the laboratory of Dr. A. Kornberg have now shown that nucleoside triphosphates can be used for DNA synthesis in *E. coli* cultures treated with EDTA (Buttin, G., L. Bertsch, and A. Kornberg, *Federation Proc.*, in press). Thus, the highly charged nucleoside triphosphates can be added to the list of compounds which can enter an EDTA-treated cell.

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