RELEASE OF LIPOPOLYSACCHARIDE BY EDTA TREATMENT OF E. COLI

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Brief treatment of Escherichia coli with low concentrations of ethylenediaminetetraacetate (EDTA) results in a general increase in permeability, associated with little or no injury to viability, growth rate, or normal RNA and protein synthesis (Leive, 1965a,b). Since these results suggest a selective action of EDTA on the cell surface, they prompted experiments to determine the nature and extent of concomitant changes in cell wall structure and composition. It has now been found that brief exposure of E. coli to EDTA results in the rapid release of approximately half of the lipopolysaccharide cell wall layer.

Materials and Methods - E. coli 0111:B4 was used because its lipopolysaccharide contains colitose, which can easily be assayed in the presence of other components. It was grown with aeration at 37° on the previously described minimal medium buffered with Tris-Cl, with 0.5% glucose as the carbon source (Leive, 1965a) and was harvested at a density of 7×10^8 cells/ml.

(a) Preparation of supernatant fluids and cells to determine released components. Cells from 2 liters of medium were washed twice with 0.12 M Tris-Cl pH 8, and resuspended in the same buffer at a density of 10^{11} cells/ml. Permeable cells were prepared by adding EDTA, 5×10^{-3} M final concentration, and incubating 2 min. at 37°; the incubation was terminated by adding $MgCl_2$, 10^{-2} M final concentration. Control cells were washed and suspended in the same manner; solutions of $MgCl_2$ and EDTA were mixed in a separate vessel and then added to the cell suspension to give the same final concentrations as in the preparation of permeable cells. The suspension was then incubated 2 min. at 37°. Actino-

mycin sensitivity was tested as previously described (Leive, 1965b): aliquots of both permeable and control cells were diluted into medium containing actinomycin D (10 μg/ml) and compared with similar aliquots growing in actinomycin-free medium to determine their relative ability to incorporate ^{14}C-uracil into acid-precipitable material.

Control and permeable cells were chilled and centrifuged for 10 min. at 10,000 x g and both the cell pellets and supernatant fluids were lyophilized. The dry residue from each supernatant fluid was resuspended and separated into high molecular weight (estimated greater than 5000) and low molecular weight (estimated less than 5000) fractions on a Bio-Gel P-10 column. Lipopolysaccharide was isolated from the lyophilized cells by phenol extraction (Westphal et al 1952) followed by dialysis of the aqueous phase.

(b) Analytical methods. Colitose was determined by the thiobarbituric method of Cynkin and Ashwell (1960); color production was measured after periodate oxidation both at 55° and at room temperature. Standards of colitose and 2-keto-3-deoxy-gluconic acid were assayed in parallel with unknowns and the spectra of standards and unknown were measured between 500 and 590 mμ. On the basis of these analyses, the chromagen in all assays was presumptively identified as a 3, 6-dideoxyhexose*. Confirmation of this sugar as colitose was obtained by paper chromatography (see below). Not only was colitose the sole chromagen* in lipopolysaccharide, but whole cells also yielded only colitose by the above criteria. The following results substantiate this finding: (1) Hydrolysis and assay of purified E. coli K12 DNA indicated that less than 4% of the color yield could be attributed to DNA breakdown products (2) Hydrolysis and assay of whole cells of E. coli W, a strain which does not contain colitose, yielded 5% of the color obtained from O111:B4 under the same conditions.

Heptose was measured by the method of Dische (1953) as modified by Osborne

* 3-Deoxyoctulosonate (KDO), did not interfere with the assay, since only 10% as much KDO as colitose is present in the lipopolysaccharide of this organism, (Elbein and Heath, 1965) and the absorbance of KDO at 532 mμ, after oxidation at 55° and subsequent thiobarbituric acid assay, is less than 10% that of colitose; thus approximately 1% of the final absorbance was attributable to KDO.

(1963) using D-glycero-L-mannoheptose as a standard; glucose and galactose were measured with glucose oxidase and galactose oxidase (Worthington Biochemical Corp.) and hexosamine by the method of Boas (1953). Descending chromatography was performed using the solvent of Colombo et al (1960) and the sugars were detected with alkaline $AgNO_3$ (Anet and Reynolds, 1954). All assays were performed on samples without prior hydrolysis except as follows: For the determination of colitose, the samples were hydrolyzed at 95° for 15 min in 0.2 N H_2SO_4. Prior to chromatography and the enzymatic determination of glucose and galactose, samples were hydrolyzed at 95° for 2 hrs in 2N H_2SO_4 and neutralized with $Ba(OH)_2$. For the determination of hexosamine, the samples were hydrolyzed in 4 N HCl at 98° for 5 hrs.

Phosphorus was determined by the Fiske-SubbaRow method as modified by Ames and Dubin (1960), carbohydrate by the phenol-sulfuric acid method (Dubois et al, 1951) using glucose as a standard, and nucleic acids by their absorbance at 260 mμ, using a molar extinction of 13,000 for the low molecular weight fraction and 7,500 for the high molecular weight fraction. Protein was determined according to Lowry (1951), amino acids by ninhydrin (Troll and Cannon, 1953) using glycine as a standard, and fatty acid esters with hydroxylamine (Stern and Shapiro, 1953) using methyl stearate as a standard.

Results - E. coli 0111:B4, like other strains studied, is impermeable to actinomycin but becomes permeable when treated with EDTA, as indicated by the development of sensitivity to the drug (Table I). This increase in permeability is almost completely blocked by the presence of excess $MgCl_2$. Cells treated with a mixture of EDTA and $MgCl_2$ were therefore compared with permeable cells to determine whether they differed in the loss of various cells constituents. Table I shows that the low molecular weight fraction lost from such cells includes approximately as much amino acids and material absorbing at 260 mμ as the fraction lost from the control preparation. The high molecular weight material lost from permeable cells contained less nucleic acid but much more protein than the control.

TABLE I

CELL COMPONENTS RELEASED BY PERMEABLE AND CONTROL CELLS

Supernatant fluids from control and permeable cells were prepared and analyzed as described in Materials and Methods. Parentheses indicate that the amount present was too little to be assayed accurately; the figures within the parentheses indicate the amount which would have been detectable with the assay used. Values given are per 0.26 g dry weight of cells.

	Permeable Cells	Control Cells
Supernatant Material*		
Low Molecular Weight		
Nucleic Acid Bases	1.6	1.2
Amino Acids	2.6	2.3
Actinomycin Sensitivity	100%	5-10%

	Permeable Cells	Control Cells
Supernatant Material*		
High Molecular Weight		
Nucleic Acid Bases	(< 0.1)	0.3
Protein	1.0 mg	0.2 mg
Fatty Acid Ester	3.9	(0.4)
Carbohydrate†	8.0	1.0
Colitose	5.4	0.70
Glucose	4.4	<0.2
Galactose	2.6	(<0.4)
Heptose	3.0	(<0.2)
Hexosamine	14.0	0.8
Phosphorous	4.0	0.35

* Values in μmoles, unless otherwise noted.
† Expressed in glucose equivalents.

However, the protein lost even from permeable cells is negligible in amount, since it corresponds to less than 1% of the total cell protein.

A more striking difference was observed in the loss of two other high molecular weight components: 8 times as much carbohydrate and more than 10 times as much fatty acid ester was released from the permeable as from the control cells. In another experiment, when this material was extracted by shaking three times with an equal volume of $HCCl_3$, all of the fatty acid ester material remained in the aqueous phase, indicating that no free lipid was present. As shown in Table I, the stoichiometry of the various lipopolysaccharide components released was very similar to that reported by Elbein and Heath (1965) for purified lipopolysaccharide from this organism, except for hexosamine, which was higher than predicted. No attempt was made to quantitate released 3-deoxyoctulosonate. The corresponding supernatant fraction from control cells contained little or none of these components. To estimate what fraction of the total cell lipopolysaccharide had been lost, the permeable cells were extracted with phenol and the remaining polysaccharide assayed for colitose; by this criterion, approximately 30% of the

lipopolysaccharide had been released.

In the above experiment, an arbitrary 2 minute exposure to EDTA was used, solely because this procedure is sufficient to make cells permeable as determined by other criteria (Leive, 1965b). Since it appeared that only a fraction of the total lipopolysaccharide was released in this time period, the time course of its release, and its correlation with the permeability increase were measured in subsequent experiments.

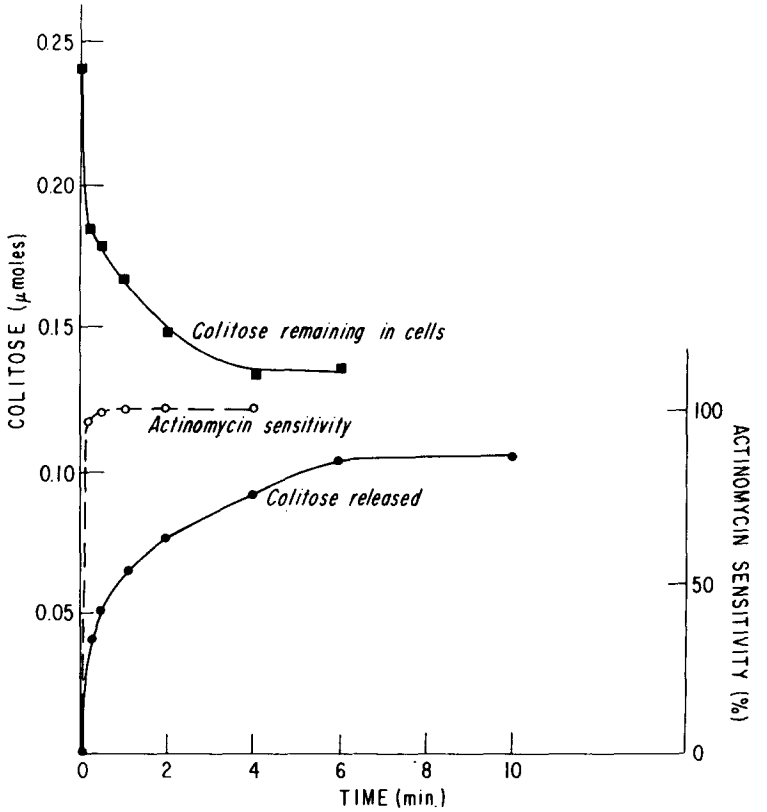

Fig. 1. Time course of release of colitose and production of actinomycin sensitivity. Cells were washed once with 0.12 M Tris-Cl pH 8, and resuspended in the same buffer at a density of 4 x 10^9 cells/ml. At 0 time, EDTA 5 x 10^{-4} M final concentration was added. Just before EDTA addition and at the indicated times thereafter, 1 ml aliquots were added to chilled tubes containing $MgCl_2$, 2 x 10^{-3} M final concentration. Portions of each sample were added to medium with or without actinomycin and tested for the percent inhibition by actinomycin of ^{14}C-uracil incorporation into acid-precipitable material. The remainder of each sample was centrifuged at 4°. Cell pellets and supernatant fluids were assayed for colitose after mild acid hydrolysis as described. Results are expressed as μmoles of colitose per cell pellet or supernatant from 1 ml of cell suspension.

Fig. 1 shows that treatment with EDTA caused release of approximately 45% of the cell colitose, and that nearly half of this loss occured within the first 15 seconds after EDTA addition. Sensitivity to actinomycin, which is one criterion of permeability, was 90% complete in 15 seconds. In other experiments, between 35 and 50% of the colitose was released.

Discussion. Treatment of E. coli with EDTA results in the release of approximately half of the lipopolysaccharide cell wall layer. This loss is very rapid, as is the permeability change caused by EDTA, and is also somewhat specific, since loss of several other cell components is apparently negligible. No correlation can be drawn between lipopolysaccharide release and permeability change. It should be noted, however, that actinomycin sensitivity is not a very precise measure of permeability, since if a cell with a given number of holes admits sufficient actinomycin to eradicate uracil incorporation, doubling this number can have no further observable effect.

This lipopolysaccharide release is not confined to E. coli 0111:B4, since other experiments indicate that it also occurs in other strains. Thus, treatment of whole cells with EDTA may be useful as a new means of isolating lipopolysaccharide. Current methods involve beginning with isolated cell walls (Osborn, et al, 1962) or require rather extensive manipulation to remove nucleic acid from the crude phenol extract (Westphal and Jann, 1965). Further work will be necessary to determine whether the material isolated by EDTA treatment is identical to lipopolysaccharide prepared by more conventional means. In addition, since only half of the lipopolysaccharide is released from the cell, it will be of interest to determine whether this limit is fortuitous or whether this fraction is in some way chemically or functionally distinct.

The rapidity and apparent specificity of the lipopolysaccharide loss suggests that it is a direct result of the action of EDTA on the cell surface. If a metal such as Mg^{++} participates in non-covalent bonds holding together the various layers of the cell wall, binding by EDTA might result in rapid and extensive changes in both function and composition of the cell surface, simultaneously causing both

altered permeability and loss of the lipopolysaccharide cell wall layer.*

Summary - The treatment of E. coli with ethylenediaminetetraacetate which causes an increase in permeability also results in the rapid release of 35 to 50% of the cell wall lipopolysaccharide. Since the same conditions produce negligible loss of several other cell components, it is possible that the release of lipopolysaccharide is a direct result of EDTA action on the cell surface. This treatment may be a useful tool in the isolation of chemically pure lipopolysaccharide.

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*After this manuscript had been completed, an article by Gray and Wilkinson appeared (J. Gen. Microbiol., 39, 385 (1965)) reporting that EDTA treatment of isolated cell walls of Pseudomonas aeruginosa and Alcaligenes faecalis solubilized material that contained phosphorous and carbohydrate and was therefore presumed to be lipopolysaccharide. However, in contrast to the results of the present paper, they found that cell walls of E. coli did not release this material. Since the conditions employed differed greatly from those reported here, it is impossible to comment on this discrepancy. Nevertheless, since it was previously postulated (Leive, 1965b) that EDTA alters a structure which is basic to the surfaces of gram-negative cells, it will be of great interest if lipopolysaccharide release proves to be a general consequence of its action.