Comparison of the Antigenicity of Phenol and Ethylenediaminetetraacetate Complexes Isolated from Cell Walls of *Salmonella enteritidis*¹

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Cell walls of *Salmonella enteritidis* were extracted with phenol and with ethylenediaminetetraacetate (EDTA). Phenol extracts were consistently more antigenic in chickens than EDTA extracts. Differences in the action of EDTA and phenol upon bacterial cell walls were demonstrated by the use of radioisotopes.

Ethylenediaminetetraacetate (EDTA) has been reported by several workers to remove portions of bacterial cell envelopes containing lipopolysaccharide (3, 4, 6). Prior to the use of EDTA, phenol was employed to extract this material (9). The antigenicity of phenol and EDTA extracts from *Escherichia coli* were compared, and the EDTA extract was more antigenic in mice (4). The present report will show that, in White Leghorn chickens, material isolated by phenol extraction of cell walls of *Salmonella enteritidis* is more antigenic than that isolated by EDTA. Through the use of radioisotopes, it is shown that differences in antigenicity are possibly related to differences in the modes of action of EDTA and phenol upon bacterial cell walls.

Cells of *S. enteritidis* were inoculated in Trypticase soy broth and incubated for 24 hr at 37 C. They were then harvested by centrifugation and washed twice with physiological saline and distilled water.

Two-gram (wet weight) samples of washed cells were suspended in 50 ml of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.0, and sonically treated (Branson sonifier) for 3 min at 5 C. Sonic extracts were centrifuged at 3,640 × g for 20 min at 2 C to separate whole cells. Supernatant solutions were decanted and recentrifuged at 27,000 × g for 15 min, and the liquid was discarded.

Cell wall pellets were suspended in 50 mg each of deoxyribonuclease and ribonuclease, incubated overnight at 10 C, and then centrifuged. This procedure is a modification of one described previously (2). The absence of significant amounts of cytoplasmic contamination was established by the absence of electron-dense material in electron micrographs (1) and the failure to detect ribose in acid hydrolysates of cell walls (3).

Cell walls (500 mg dry weight) were resuspended in 50 ml of 1.0 mM EDTA (tetrasodium dihydrate) at 22 C for 15 min. The suspension was then centrifuged, and the supernatant solution was decanted, dialyzed against several changes of distilled water at 4 C for 3 days, and lyophilized. An identical procedure was used to prepare the phenol extract, except that cell walls were resuspended in 50 ml of 90% (v/v) phenol at 65 C for 15 min (9).

The isolated extracts were each dissolved in physiological saline (10 mg/ml). Two milliliters of the phenol extract was injected intravenously into each of six adult female White Leghorn chickens. A different group of six chickens was inoculated in an identical manner with the EDTA extract. Procedures for collection of blood and measurement of antibody titer by tube agglutination have been outlined previously (8). Antigen concentrations used in titrations were 200 µg (dry weight) of either the EDTA or phenol extract per ml of distilled water.

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Basically, the procedure for titration was as follows. Antigen (0.5 ml) was added to tubes containing 0.5 ml of physiological saline plus serial dilutions of serum. The contents of each tube were thoroughly mixed and incubated for 24 hr at 37 C. Positive reactions were indicated by a granular-type agglutination.

Zinc is one of the mineral constituents (1) in gram-negative bacteria, and aspartic acid is one of the major amino acids (7) in cell walls of Salmonella. Extracting isolated cell walls labeled with radioisotopic markers of these components should provide insight into the modes of action of EDTA and phenol and help to explain observed differences in the antigenicity of these extracts. Accordingly, cells were inoculated; 4 hr later, 3 ml of either 65ZnCl2 or aspartic-1-14C acid (150 μCi/ml, New England Nuclear Corp.) was added to each 250 ml of broth, and the cultures were incubated. The cells were then harvested, and labeled cell walls were isolated and extracted with either phenol or EDTA. Radioactivity was measured for duplicate 1-min intervals, and the percentage lost was calculated by subtraction from controls.

Table 1 indicates that in chickens the phenol extract produced a consistently higher antibody titer than did the EDTA extract. The differences in titer remained significant throughout the 8-week period of testing. Similar results have been reported in a previous study (5). When cell walls of Pseudomonas aeruginosa were treated with EDTA, the antigenicity of the isolated lipopolysaccharide was lowered when injected in mice.

Perhaps the basis for differences in antigenicity of phenol and EDTA extracts lies in their modes of action upon bacterial cell walls. Approximately two-thirds of the 65Zn2+ and 14C-aspartic acid was removed from labeled cell walls by extraction with EDTA (Table 2). However, less than 10% of the radioactivity was removed by phenol extraction. In both extractions, the radioactivity was nondialyzable. In a previous study (4), differences were found in the composition of lipopolysaccharide fractions isolated by phenol and EDTA from cells of E. coli. It was reported in that study that EDTA extracts were more antigenic and at least as lethal in mice.

In the present study, it appears that in chickens differences in antigenicity of phenol and EDTA extracts may be related to the mode of action of these compounds upon bacterial cell walls, as demonstrated by differences in their ability to extract radioactive components from the walls.

### Table 1. Response of chickens to phenol and EDTA extracts

| Time post-injection (weeks) | Range of agglutination titers (reciprocals) |
|-----------------------------|---------------------------------------------|
|                             | Phenol extract* | EDTA extract* |
| 0                           | <10            | <10           |
| 1                           | 5,120-10,240   | 1,280-2,560   |
| 2                           | 5,120-10,240   | 640-1,280     |
| 3                           | 2,560-5,120    | 640-1,280     |
| 4                           | 2,560-5,120    | 160-640      |
| 5                           | 1,280-2,560    | 80-320        |
| 6                           | 640-1,280      | 40-160        |
| 7                           | 160-640        | 20-80         |
| 8                           | 40-160         | 10-40         |

* Twenty milligrams injected.

### Table 2. Comparison of the removal of 65Zn2+ and 14C-aspartic acid from cell walls of Salmonella enteritidis by phenol and EDTA extractions

| Treatment               | Radio-isotope | Radioactivity in pellet* (%) | Radioactivity in supernatant fluid* (%) |
|-------------------------|---------------|------------------------------|----------------------------------------|
| Washed pellet*          | 65Zn2+        | 100 ± 1                      | 0 ± 1                                  |
| Extracted with water    | 100 ± 5       | 5 ± 1                        |                                        |
| Extracted with EDTA     | 39 ± 3        | 61 ± 4                       |                                        |
| Washed pellet*          | 14C           | 100 ± 2                      | 0 ± 1                                  |
| Extracted with water    | 100 ± 2       | 0 ± 1                        |                                        |
| Extracted with EDTA     | 90 ± 1        | 9 ± 2                        |                                        |

* Five hundred milligrams dry weight.

**LITERATURE CITED**

1. Eagon, R. G., and K. J. Carson. 1965. Lysis of cell walls and intact cells of Pseudomonas aeruginosa by ethylenediaminetetraacetic acid and by lysozyme. Can. J. Microbiol. 11:191-201.
2. Gray, G. W., and S. G. Wilkinson. 1965. The action of EDTA on Pseudomonas aeruginosa. J. Appl. Bacteriol. 28:153-164.
3. Gray, G. W., and S. G. Wilkinson. 1966. The effect of ethylenediaminetetraacetic acid on the cell walls of some gram-negative bacteria. J. Gen. Microbiol. 39:385-399.
4. Leive, L., V. K. Shovlin, and S. E. Mergenhagen. 1968. Physical, chemical, and immunological properties of lipopolysaccharide released from Escherichia coli by ethylenediaminetetraacetate. J. Biol. Chem. 243:6384-6391.
5. Michaels, G. B., and R. G. Eagon. 1966. The effect of ethylenediaminetetraacetate and of lysozyme on isolated lipopolysaccharide from Pseudomonas aeruginosa. Proc. Soc. Exp. Biol. Med. 122:666-668.
6. Rogers, S. W., H. E. Gilleland, Jr., and R. G. Eagon. 1969. Characterization of a protein-lipopolysaccharide complex released from cell walls of Pseudomonas aeruginosa by ethylenediaminetetraacetic acid. Can. J. Microbiol. 15:743-748.
7. Salton, M. R. J. 1964. The bacterial cell wall. Elsevier Publishing Co., New York.
8. United States Department of Agriculture, Agricultural Research Service. 1967. The national poultry and turkey improvement plans and auxiliary provisions. Miscellaneous Publication no. 739. Washington, D. C.
9. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol—water and further applications of the procedure, p. 83-91. In R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press Inc., New York.