to sour (room temperature, 4 days) and was then worked up as sample #4. As no increase in $DMSO_2$ was noted, it is probable that the $DMSO_2$ isolated from cows' milk is not a product of micro-organisms in the milk but rather arises from metabolic processes in the intact animal.

Summary. Dimethyl sulfone has been detected and isolated from pasteurized cows' milk. The source of the sulfone is not yet known. Dimethyl sulfoxide was not detected.

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The Effect of Ethylenediaminetetraacetate and of Lysozyme on Isolated Lipopolysaccharide from *Pseudomonas aeruginosa.** (31273)

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During studies on the endotoxic properties of lipopolysaccharide of *Pseudomonas aeruginosa*, it was noted that its lethal toxicity for mice was reduced or destroyed by incubation with ethylenediaminetetraacetate (EDTA) and with lysozyme. Previous reports have indicated that cells of *P. aeruginosa* died rapidly in the presence of EDTA (6,9). It was suggested by both groups of workers that EDTA acted through its chelation of metal cations and that these metal cations were essential to the structural integrity of *P. aeruginosa*. More recently Eagon, Simmons and Carson(7) showed that Ca^{2+}, Mg^{2+} and Zn^{2+} were components of the cell wall of *P. aeruginosa*.

Lysozyme, on the other hand, did not induce lysis of cells of *P. aeruginosa*. This reagent, however, was not completely without effect since electron microscopy of cells incubated with lysoyzme revealed alterations of the cellular surfaces(6). Furthermore, electron micrographs of thin sections of cells incubated with lysozyme also indicated extensive alterations of the cell walls(4). These observations were interpreted to indicate: (1) that the mucopeptide component of the unaltered cell walls of *P. aeruginosa* is sensitive to lysozyme; and, (2) that the mucopeptide component is not solely responsible for the rigidity of the cell wall of this microorganism.

The investigations described here, therefore, were undertaken to provide further information on the structure of the lipopolysaccharide and of the cell wall of *P. aeruginosa*.

Materials and methods. P. aeruginosa was cultivated for 12-14 hours at 37°C on a rotary shaker in an enriched medium. After collection and washing, cells were killed by suspending in 2% phenol for 1 hour at room temperature with shaking. These cells were then washed twice with sterile 0.85% saline and lyophilized. This material was designated as killed whole cells.

Cell walls were prepared according to the technique of Eagon and Carson(6). Cell membranes were prepared as described by Carson and Eagon(3). Protoplasm was prepared by disruption of a saline suspension of cells at 10,000 psi in the French Pressure Cell followed by centrifugation at 27,000 × *g* for 1 hour to remove walls, membranes, large particles and unruptured cells.

Lipopolysaccharide was prepared by the aqueous ether-ethanol method as described by Foster and Ribi(8). Whole cell lysate was prepared by lysing cells with EDTA plus lysozyme as described by Eagon and Carson

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TABLE I. Lethality of Cellular Fractions and of Treated and Untreated Lipopolysaccharide as Determined by the Mouse Toxicity Test.

Preparation	LD_{50}
Killed whole cells	2.5 mg
Cell walls	1.25 "
Lipopolysaccharide	1.25 "
Cell membranes	negative*
Protoplasm	4.0 mg†
Whole cell lysate	negative*
Lysozyme-treated lipopolysaccharide (Tris buffer suspension)	" *
Lysozyme-treated lipopolysaccharide (phosphate buffer suspension)	" *
EDTA-treated lipopolysaccharide	" *

* Quantities higher than 5 mg were not used. Thus, the experimental results were considered to be negative if 5 mg were not lethal.

† Estimated value calculated from results obtained with 2.5 mg and 5 mg doses.

(6). Lysozyme-treated lipopolysaccharide was prepared by incubating lipopolysaccharide with lysozyme (2 mg lysozyme per 200 mg lipopolysaccharide in 12 ml of 0.1 M tris-(hydroxymethyl)aminomethane (Tris) buffer, pH 7.6) for 30 min at room temperature after which the lipopolysaccharide was collected by centrifugation, washed 3 times with Tris buffer and relyophilized. EDTA-treated lipopolysaccharide was prepared in a similar fashion by the substitution of 33 μmoles of EDTA for lysozyme.

For these experiments, assays for the presence and activity of lipopolysaccharide were determined by the mouse toxicity test by intraperitoneal injection into male HA/ICR mice according to the technique described by Ribi *et al*(12). Groups of 8 mice were used for each dilution of material tested.

Experimental results. Table I shows LD_{50} values in mice as calculated 7 days after intraperitoneal injection. At this time complete recovery of all survivors had occurred. Most deaths due to toxicity of lipopolysaccharide, moreover, occurred within 24 hours and all within 48 hours.

The results shown in Table I clearly confirm that the lipopolysaccharide has endotoxic activity and that it is located in the cell wall. Lethal endotoxic activity was not detected in the whole cell lysate, indicating that either lysozyme or EDTA or both reagents altered the lipopolysaccharide. When these reagents were used singly on isolated lipopolysaccharide, lethal endotoxic activity was destroyed. In order to rule out that Tris buffer may have altered the lipopolysaccharide, this material was incubated with lysozyme in phosphate buffer. As shown in Table I, these results confirmed that lysozyme and not Tris buffer was the active agent.

Discussion. Hypotheses to explain the sensitivity of the lipopolysaccharide to lysozyme and to EDTA may be tentatively advanced as follows:

(1) Since lysozyme is considered to be a $\beta(1\rightarrow4)$N-acetylglucosaminidase, these results indicate the probable presence of $\beta(1\rightarrow4)$glycosidic linkages between adjacent N-acetylglucosamine units in the lipopolysaccharide. Recently, Clark, Gray and Reaverly (5) presented evidence that the 'lipid A' component of the lipopolysaccharide of *P. aeruginosa* contains glycosidically-linked D-glucosamine units. Thus, whether 'lipid A' or another component of the lipopolysaccharide is the site of attack by lysozyme cannot be discerned from these experiments. Similarly, whether this same site is also attacked by lysozyme in the intact cell is unknown. If it is, it is not essential for cellular integrity since lysozyme alone does not provoke lysis of intact cells.

(2) The effect of EDTA implicates divalent cations as components of the lipopolysaccharide of *P. aeruginosa*. Burton and Carter (2) reported that Ca^{2+} and Mg^{2+} were present in lipid A from the lipopolysaccharide from *Escherichia coli*. Similarly, Eagon, Simmons and Carson(7) demonstrated the presence of these substances in the cell wall of *P. aeruginosa*. Chelation of these metal cations by EDTA, moreover, is considered to result in lysis of *P. aeruginosa*(6,9). Moreover, the release of material with characteristics of lipopolysaccharide by treatment of isolated cell walls of *P. aeruginosa* with EDTA has been reported(10). Finally, multivalent cations have been shown to be involved in the organization, structure and assembly of the cell wall of *P. aeruginosa* (1). Thus, the evidence indicates that divalent metal cations may be essential for the structural integrity of the lipopolysaccharide

and, consequently, the cell wall *per se* of *P. aeruginosa*.

The results of our experiments reported herein, moreover, indicate that the treatment of cell walls of *P. aeruginosa* by EDTA cannot be used as a tool in the isolation of chemically pure lipopolysaccharide as suggested by Leive(11) for *E. coli* since we have shown that lipopolysaccharide thus treated was altered in its biological activity.

Summary. Subcellular fractions and extracted lipopolysaccharide of *P. aeruginosa* were assayed for toxic activity by the mouse lethality technique. Toxic activity was demonstrated to be associated with the cell wall fraction. The isolated lipopolysaccharide was shown to be altered by treatment with lysozyme and with EDTA as evidenced by its loss of lethality for mice.

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A Whole Blood Microculture Technique for Cytological Examination Of Neonatal Rabbits.* (31274)

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Several techniques describing methods for culturing small quantities of human, cattle, sheep, deermouse, and mink whole blood for the subsequent study of chromosomes have been developed(1-4). These procedures (primarily for human whole blood) have not always been directly applicable to laboratory animals. Although the quantitative values associated with these techniques vary, the literature indicates that whole blood cultures are in general composed of chemically defined culture media, blood serum, phytohemagglutinin, heparinized whole blood, and colchicine or its synthetic analog, colcemide. A variety of methods for the hypotonic treatment of the cultured cell, cell fixation, slide preparation, and staining also exists and alterations in these steps are apparently based on individual preference.

Since our study of spontaneous and induced malformations in newborn rabbits would be aided by the cytological examination of such malformed neonates, it was necessary that a repeatable technique be found which would insure good chromosome spreads with limited effort. The whole blood microculture seemed most applicable, since leucocyte separation procedures(5,6) and the bone marrow biopsy technique(7) would be impractical because of the neonatal rabbit's size. Extension of a working microtechnique to older rabbits would also be advantageous because of the small quantities of blood required. The following technique has been developed for efficient chromosomal assay of the neonatal rabbit.

Materials and methods. Erlenmeyer flasks (30 ml) and rubber serum caps were prepared for sterilization by soaking and washing in

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