Normal Serum Cytotoxicity for P^{32} -Labeled Smooth *Enterobacteriaceae*

II. Fate of Macromolecular and Lipid Phosphorus of Damaged Cells

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ABSTRACT

SPITZNAGEL, JOHN K. (University of North Carolina, Chapel Hill). Normal serum cytotoxicity for P^{32} -labeled smooth *Enterobacteriaceae*. II. Fate of macromolecular and lipid phosphorus of damaged cells. J. Bacteriol. 91:148–152. 1966.—Fresh, normal adult guinea pig serum released micro- and macromolecular forms of metabolically incorporated P^{32} from smooth *Escherichia coli*. A significant amount of P^{32} -labeled phospholipid was also released. The overall acid solubility distribution of the P^{32} label in bacterial suspensions was very little affected in fresh serum, although P^{32} -labeled cytoplasmic contents and cell walls of *E. coli* lost some P^{32} when they were suspended in fresh or heat-inactivated normal serum. These findings confirmed and extended earlier evidence that the peripheral wall and membrane structures of smooth *E. coli* are extensively damaged by factors in fresh normal serum.

Suspensions of P^{32} -labeled, smooth *Escherichia coli* in normal adult mammalian serum release their phosphorus label at rates 5 or 10 times faster than rates similarly measured by use of labeled *E. coli* suspended in salts-glucose solutions. Phosphorus release closely parallels serum bactericidal action (8). Experiments that were designed to evaluate and compare phosphorus compounds released under these two circumstances are the subject of this paper.

According to Rowley (7), serum phosphatases may degrade phosphorus compounds in certain enterobacterial endotoxin preparations. Cohn (2) suggests that bacterial nucleic acids are degraded in phagocytes by lysosomal nucleases. Jenkin's (5) experiments show, on the other hand, that events occurring during normal serum opsonization determine, independently of effects on phagocytic rate, the intracellular killing of *Enterobacteriaceae*. The possibility that autolytic enzymes of *Enterobacteriaceae* participate in in vivo enterobacterial degradation appears not to have been investigated.

Thirty per cent of the P^{32} -labeled compounds released in serum suspensions of *E. coli* show sedimentation characteristics that might be expected of large particles. That this is an indication

of severe damage to peripheral bacterial membranes with consequent loss of cytoplasm is attested by electron microscopic examination of the damaged cells (8). It is not clear, however, whether degradation of macromolecular phosphorus compounds to small molecular forms occurs in this situation.

Although it is evident that normal adult mammalian serum can participate in enterobacterial in vivo degradation, the degree of degradation attributable to primary serum effects is not known. It is, therefore, of interest to compare the acid solubilities of P^{32} compounds released from serum-suspended bacteria with those in untreated bacteria of the same labeled culture.

This paper shows that the acid solubilities and light-absorbing properties of the P^{32} compounds released from labeled *E. coli* in normal serum are consistent with those of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). An unexpected finding is that a considerable amount of phospholipid is released at the same time. The acid solubilities of bacterial phosphorus compounds are not altered during release from the bacteria.

MATERIALS AND METHODS

Cultures. *E. coli* O117:H27 was maintained and used as previously described (8).

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TABLE 1. Loss of phosphorous from P^{32} -labeled *Escherichia coli* O117:H27*

Suspending medium	Phosphorus measured as			
	P^{32}		Material absorbing light at 2,600 A (Increase in optical density of suspending medium/hr)	Total phosphorus (Per cent loss of bacterial sediment/hr)
	Per cent released into medium/hr	Per cent loss from bacterial sediment/hr		
50% serum-Gey's solution.....	70	73	0.40†	55
100% Gey's solution....	14	23	0.05†	16

* All determinations were made at 37 C.

† Increase in material extractable by hot perchloric acid from supernatant fluid (optical density).

Media. Growth media, solutions, and normal guinea pig serum were prepared as previously described. Serum was heat-inactivated at 56 C for 20 min where indicated.

Labeling of cells with P^{32} . Labeling methods have been described (8). Briefly, P^{32} as orthophosphate was added to a complex synthetic medium with limiting phosphate concentrations, thus assuring incorporation of a high proportion of label.

Preparation of P^{32} -labeled cell constituents. Cells were grown as described above and washed first in phosphate buffer ($\mu = 0.1$) at pH 7.0 followed by three washings in sterile water. After harvesting, 6 ml of cells [10 mg (dry weight) per ml] in water were broken by shaking for 40 min with 6 ml of 18-gauge Ballotini beads in a Mickle disintegrator and were then centrifuged at $10,000 \times g$ for 20 min. The supernatant fluid that remained after broken cells were removed by centrifugation at $10,000 \times g$ was taken as cell cytoplasm. The cell walls (crude sediment) were further purified by three washings in phosphate buffer ($\mu = 0.1$) at pH 7 and three washings in sterile water. These walls were then centrifuged through a sucrose gradient according to Roberson's (6) technique.

The specific activity of the labeled cell walls was $0.196 \mu\text{C}$ of P^{32} per mg (dry weight). Specific activity of the cytoplasm was $0.50 \mu\text{C}$ of P^{32} per mg (dry weight).

Light absorption at 2,600 A. Light absorption was determined in a Beckman model DB spectrophotometer with a 10-mm light path. Samples were prepared for absorption analysis by digestion with 2% perchloric acid (1).

Release of P^{32} . P^{32} release was measured by use of *E. coli* O117:H27 as previously described. The procedure was modified for measurement of P^{32} loss from labeled cell walls. Centrifugation was done at $10,000 \times g$ and 4 C for 10 min. Because measurement of P^{32} loss from labeled cytoplasm was meaningless as such, the procedure was modified to permit measurement of

increases in the cold acid or low molecular weight fraction. Each reaction mixture was treated with cold 2% perchloric acid at 4 C and the soluble P^{32} was measured. Results were recorded in terms of the per cent increase in cold acid-soluble P^{32} or 2,600 A absorbing material caused by addition of serum to the salts-glucose solution.

Radioisotope techniques. Isotopes were quantitated as previously described. Beta counting was done in a Packard Tri-carb liquid scintillation spectrometer (Packard Instrument Co., La Grange, Ill.). Results were corrected for quenching, volume, decay, and efficiency. Percentages of P^{32} activity were calculated on basis of total P^{32} introduced in the bacterial suspensions less any radioactivity found in the suspending medium at zero-time.

Total phosphorus determination. Bacteria treated by incubation in the various suspending media were sedimented and washed three times in distilled water. The residue was analyzed for total phosphorus by the Fiske-SubbaRow (4) procedure.

Fractionation of P^{32} compounds. Fractionation of P^{32} compounds was done by sequential extraction of supernatant fluids or sedimented cells by the method of Schmidt and Thannhauser as modified by Davidson and Smellie (3). Each fractionation step was repeated once or until significant counts of P^{32} were no longer obtained. Fractions recovered were interpreted as follows: cold trichloroacetic acid, small molecular intermediates or breakdown products; alcohol ether-soluble, phospholipids; potassium hydroxide, RNA; hot trichloroacetic acid, DNA.

RESULTS

Loss of P^{32} , total phosphorus, and ultraviolet-absorbing compounds from *E. coli*. These measure-

FIG. 1. Solubility distribution of metabolically incorporated P^{32} label lost from *Escherichia coli* in 50% serum-Gey's medium (□), 50% heat-inactivated serum-Gey's medium (▨), and 100% Gey's medium (■). The bacteria were sedimented after 1 hr at 37 C. The P^{32} activity in the supernatant media was fractionated by sequential extraction as indicated. The P^{32} activity in the fractions was determined and calculated as percentage by use of the total P^{32} activity of the bacterial inoculum as a basis. The solubility distribution of P^{32} label in a whole, untreated, unsedimented bacterial suspension (dot-filled bar) is included for comparison.

TABLE 2 Solubilities of P^{32} compounds* in *Escherichia coli* O117:H27 suspended in fresh serum, heat-inactivated serum, or 100% Gey's solution

Suspending medium	Hr at 37 C	Activity soluble in			
		Cold trichloroacetic acid (intermediates)	Alcohol-ether (lipids)	KOH (RNA)	Hot trichloroacetic acid (DNA)
50% fresh serum Gey's solution..	1	31†	14	43	10
50% heat-inactivated serum-Gey's solution.....	1	26	10	51	10
100% Gey's solution.....	1	40	14	38	7
100% Gey's solution.....	0	36	15	42	5

* Figures obtained by summation of P^{32} recovered from supernatant fluids with P^{32} recovered by corresponding procedures from bacterial residues sedimentable at $6,000 \times g$.

† Per cent total bacterial P^{32} .

ments of phosphorus loss were compared by use of *E. coli* suspended at 37 C for 1 hr in 50% serum-Gey's solution or in 100% Gey's solution. In fresh normal serum, 70% of bacterial P^{32} left the bacterial cells (Table 1); 14% left in Gey's solution. This loss from cells was balanced by corresponding gains in the suspending media.

Direct measurement of total phosphorus in bacteria centrifuged from serum-Gey's solution and plain Gey's solution suspensions before and after incubation showed losses in total phosphorus (Table 1) similar to those noted above. The increases in hot acid-soluble ultraviolet-absorbing compounds in the supernatant fluid were sufficient to account for this loss of total phosphorus. This conclusion was based on calculations made by assuming the phosphorus content of nucleic acids to be 8.13% and their absorbancy to be $E_{240}^{1\%} = 206$.

Fractionation of P^{32} -labeled compounds. The P^{32} compounds were released from *E. coli* [0.075 mg (dry weight) per ml] in 50% fresh serum-Gey's solution. Controls were suspended in heat-inactivated serum, in 50% Gey's solution, or in 100% Gey's solution. Sequential fractionation was done as described in Materials and Methods on the material not sedimentable at $6,000 \times g$ for 10 min. The use of heat-inactivated serum was introduced as an additional control because it was found that labeled *E. coli* lost even less P^{32} in such a suspension than they lost in 100% Gey's solution.

The results (Fig. 1) showed that *E. coli* O117:H27 labeled with P^{32} and suspended in fresh normal guinea pig serum lost macromolecular forms of P^{32} (RNA and DNA) as well as forms of low molecular weight that were soluble in trichloroacetic acid. The ratio between low molecular weight forms of P^{32} and nucleic acid P^{32} was greater in the material released into the suspending media than was the same ratio between cor-

responding fractions isolated from intact labeled cells. Experiments were done to determine whether this was due to degradation of RNA in fresh serum-treated bacteria or due to differences in diffusion of the low molecular weight materials and the RNA away from the cells.

The P^{32} in bacterial sediments and corresponding supernatant media was fractionated and measured, and the results for corresponding fractions were added and compared with analyses of the whole untreated bacterial suspensions. The results (Table 2) showed that in fact no marked change had occurred in the overall acid solubility distribution of P^{32} compounds but that, in fresh serum, compounds of low molecular weight had been lost into the suspending medium to a greater degree than compounds of high molecular weight. Also evident was the fact that P^{32} lost in 100% Gey's solution and in heat-inactivated serum came primarily from the low molecular weight fraction.

Continued metabolic activity. The RNA in the heat-inactivated serum suspensions continued to incorporate P^{32} at the expense of the low molecular weight fraction (Table 2). Other studies not shown revealed some bacterial multiplication under these conditions.

In 100% Gey's solution, the RNA fraction lost some P^{32} which apparently entered the low molecular weight pool. Plain Gey's solution with no nitrogen source was found to be bactericidal for *E. coli* (Spitznagel, unpublished data). An apparent tendency for P^{32} to be incorporated in DNA in serum, fresh or inactivated, was noted.

The loss of a significant amount of phospholipid (Fig. 1) into the suspending medium in a form not sedimentable at $6,000 \times g$ was unexpected. The same result was observed in several other experiments with *E. coli*.

Release of P^{32} from labeled cell walls. Cell walls of *E. coli* O117:H27 [0.075 mg (dry weight) per ml] labeled metabolically with P^{32} were suspended

TABLE 3. *Effect of guinea pig serum on cell walls of P³²-labeled Escherichia coli**

Suspending medium	Per cent sedimentable P ³² solubilized/hr
100% Gey's-solution.....	0.6
50% fresh serum-Gey's solution....	4.0
50% heat-inactivated serum-Gey's solution.....	4.9

* An amount of 0.075 mg (dry weight) of cell walls/ml (0.196 µc/mg) was used.

TABLE 4. *Effect of guinea pig serum on acid solubilities of P³² and 2,650 Å absorbing materials of labeled Escherichia coli cytoplasm**

Suspending medium	Per cent increase in cold acid-soluble P ³² fraction/hr	Per cent increase in cold acid-soluble 265 mµ absorbing material/hr
100% Gey's solution....	10	0.0
50% fresh serum-Gey's solution.....	20	17 (0.25) †
50% heat - inactivated serum-Gey's solution.....	20	19 (0.25) †

* An amount of 0.46 mg (dry weight) per ml was used.

† Optical density.

in 50% fresh guinea pig serum-Gey's solution, heat inactivated 50% guinea pig serum-Gey's solution, or 100% Gey's solution. Cell walls suspended in serum released 4 to 5% of their label per hour whether or not the serum was heat-inactivated (Table 3). Slightly more than one-tenth this amount of P³² was released by cell walls in 100% Gey's solution.

Conversion to acid solubility of P³² label in E. coli cytoplasm. *E. coli* O117:H27 cytoplasm which had been metabolically labeled with P³² was treated with fresh or heat-inactivated guinea pig serum in Gey's solution. A third sample was suspended in 100% Gey's solution. As shown in Table 4, there was an increase of 20% per hour of the cold acid-soluble P³² fraction in fresh or heat-inactivated serum and a 10% increase per hour in 100% Gey's solution. Measurement of the ultraviolet absorption of cold acid extracts of the treated cytoplasm showed that some of the 20% increase in the cold acid-soluble fraction in serum was probably due to degradation of nucleic acids. The 10% increase of P³² in the cold acid fraction that occurred in 100% Gey's solution was not accompanied by an increase in cold acid-soluble

ultraviolet-absorbing materials. Isotope exchange may have caused this.

DISCUSSION

The P³²-labeled compounds released from smooth *E. coli* in fresh normal serum included small molecular ones as well as RNA, DNA, and phospholipid. Small molecular compounds were released in relatively greater amounts than macromolecular ones. No evidence of substantial degradation of RNA or DNA to small molecular forms was obtained. Some evidence of phosphoesterase or nuclease activity was noted in the release of P³² from labeled, isolated *E. coli* cell walls and transfer of P³² from hot acid-soluble to cold acid-soluble fractions of *E. coli*. (It was not determined whether these hypothetical enzymes were from the serum or the bacterial protoplasm.) Evidence for isotope exchange between isolated cytoplasmic macromolecules and small molecular constituents of the media was obtained. These results confirm and extend physical and morphological studies on serum-treated *E. coli* that showed severe damage to peripheral cell membranes with loss of cytoplasm and nucleoplasm. The considerable escape of phospholipid correlates with previously demonstrated ultrastructural loss of cytoplasmic membranes (8).

Studies demonstrating release of P³² from labeled bacterial cell walls and cytoplasm suspended in serum confirmed Rowley's (7) observations. The possibility that enzymatic breakdown of nucleic acids and other P³² compounds occurs during the serum degradation of *E. coli*, therefore, exists, although it was not supported by our work done with intact bacteria. The source of these enzymes was not clear. They could have originated in serum. It is also possible that they originated in the bacteria and were somehow activated by systems present in the serum. These findings show that the role of the serum factors in degradation of *Enterobacteriaceae* includes damage to peripheral bacterial membranes, damage to permeability barriers, and release of cytoplasmic constituents in a macromolecular form apparently similar to that possessed by intact bacteria.

Jenkin (5) suggested that opsonization of *Enterobacteriaceae* by normal serum affects the intracellular fate of phagocytized organisms independently of opsonic effects on phagocytic rates. The present results suggest that, at least with smooth and rough *E. coli* (Amano and others have already shown that rough *E. coli* form osmotically sensitive spheroplasts in fresh serum), important degradative steps may be initiated due to interaction of the bacterial cell with serum prior to phagocytosis. The essential step is one that de-

stroys permeability barriers and possibly other protective actions of cytoplasmic membranes and cell walls. These findings suggest that the phosphorus compounds of the bacterial cytoplasm are not a major target in the 1st hr of antibacterial action of serum against intact bacteria.

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