THE FATE OF BACTERIA WITHIN PHAGOCYTIC CELLS

I. The Degradation of Isotopically Labeled Bacteria by Polymorphonuclear Leucocytes and Macrophages*

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Previous studies from this laboratory have indicated that the cytoplasmic granules of polymorphonuclear leucocytes are associated with a variety of hydrolytic enzymes and the bactericidal factor phagocytin (1). The composition and properties of these particles resemble in certain respects the rat liver lysosome initially described by De Duve (2). In both cases one of the suggested functions of these cytoplasmic elements is the breakdown of foreign materials which enter the cell. Considerable information is available concerning the viability of bacteria engulfed by leucocytes, a question of considerable interest to students of the host-parasite relationship. However, little is known regarding the ability of phagocytic cells to dispose of bacterial macromolecules (3). Studies on the conversion of C^{14}-labeled tubercle bacilli to $C^{14}O_2$ by guinea pig leucocytes have been published previously (4).

With the techniques available for the study of the interaction of bacteria and phagocytes, it seemed of interest to ascertain the potentialities of leucocytic enzymes for the destruction of bacterial constituents. This report will deal with the degradation of isotopically labeled bacteria following their phagocytosis by both polymorphonuclear leucocytes and macrophages.

Materials and Methods

Bacteria.—Strains of *Escherichia coli* K-12,28B2; *Bacillus subtilis*, *Micrococcus leisodykticus*, *Staphylococcus albus* "Air", *Staphylococcus aureus*, and *Salmonella typhimurium* RIA represented currently employed laboratory stocks. All strains were maintained on penassay agar slants and passed at least once a month.

Labeling and Growth.—The basic medium for the growth of all organisms contained: 0.2 per cent ammonium sulfate, 0.1 per cent ammonium citrate, 0.02 per cent magnesium sulfate-$7H_2O$, 1.4 per cent dipotassium phosphate, 0.6 per cent monopotassium phosphate, 0.1 per cent cassamino acids, and 0.05 per cent yeast extract dialysate. A small inoculum of bacteria was added to 10 ml of medium contained in a 50 ml flask and incubated at 37°C on a reciprocal shaker for 16 hours. The bacteria was then washed three times with large volumes of saline, collected by centrifugation at 9,000 *g* and resuspended to the appropriate concentration with saline.

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Bacteria to be labeled with P^{32} were grown in a medium in which phosphates had been replaced by 0.05 M tris (hydroxymethylaminomethane) buffer at pH 7.3. Phosphate was then added to a final concentration of 10^{-3} M. Carrier-free P^{32} orthophosphate was present at a concentration of 20 μc/ml.

Labeling of bacteria with D-glucose—C^{14}—uniformly labeled (Schwarz, Orangeburg, New York, specific activity 25 to 30 μc/mg) was accomplished in a modified medium in which the concentrations of casmino acids and glucose were reduced to 0.025 per cent and 0.01 per cent, respectively. The total volume of these cultures was maintained at 3.0 ml and contained 10 μc of C^{14}-glucose/ml. Incubation was carried out in tightly stoppered 25 ml flasks.

Heat-killed bacteria represented aliquots of viable washed cultures which were maintained for 30 to 60 minutes in a boiling water bath and washed twice with saline.

Leucocytes:

Rabbit Polymorphonuclear Leucocytes.—Homogeneous suspensions of cells were obtained from glycogen-induced peritoneal exudates by previously described methods (5).

Rabbit Macrophages.—Peritoneal macrophages were obtained from 3 to 4 kilo rabbits injected 4 days previously with 50 ml of sterile mineral oil (Nujol, extra heavy, Plough Inc.). The harvested cells were then washed twice with Hanks' solution and adjusted to the appropriate concentration. All preparations employed in this study contained at least 90 per cent macrophages.

Human Blood Leucocytes.—Heparinized blood was obtained from normal donors and the erythrocytes sedimented in the presence of dextran (6). The leucocytes were collected by low speed centrifugation and washed twice with Hanks' solution.

Horse Blood Leucocytes.—Heparinized blood was allowed to sediment spontaneously and the leucocytes were collected and processed as for human blood. All cells were adjusted to the appropriate concentration after duplicate cell counts had been performed. Differential counts were performed on both Wright-stained smears and wet mounts in the hemocytometer chamber.

Serum.—Normal rabbit, human, and horse blood was allowed to clot, stored at 4°C to allow clot retraction and centrifuged at 2,500 RPM. The supernatant serum was stored at −20°C for periods up to 30 days.

Reaction Mixtures.—Washed leucocytes obtained from different sources were suspended in a modified Hanks' medium (4) containing 150 mg per cent of glucose. Fresh frozen serum was present at a final concentration of 10 per cent for most studies, and at 3 to 5 per cent experiments employing *B. subtilis* and *M. leisodykticus*. The total volume of the final reaction mixture varied from 3 to 6 ml and contained 35 to 40 $\times$ 10^6 leucocytes/ml, 3 to 10 per cent serum and 3 to 8 $\times$ 10^7 labeled bacteria/ml. Phagocytosis took place in 12 $\times$ 100 mm siliconized glass tubes which were fitted with rubber stoppers and rotated end-over-end (rotator model ST, New Brunswick Scientific Co., New Brunswick, New Jersey) at 8 RPM in a constant temperature room at 37°C. All solutions were prewarmed to 37°C before mixing bacteria and, leucocytes. At intervals up to 180 minutes of incubation, aliquots of the suspension were removed for fractionation.

Fractionation of Leucocyte-Bacteria Suspensions.—The scheme employed for the fractionation of leucocyte-bacteria suspensions was patterned after that described by Roberts *et al.* (7). Aliquots of the incubation mixture were pipetted directly into 20 to 30 volumes of ice cold TCA (final concentration 5 per cent) and allowed to remain in an ice bath for 30 minutes. The precipitate was collected by high speed centrifugation and the supernatant fluid represented the *acid-soluble fraction*. The precipitate was extracted with 70 per cent alcohol-ether (1:1) at 45°C/15 minutes and again with ether alone. The combined alcohol-ether and ether supernatants were taken as the *alcohol-ether fraction*. Extraction of the residual precipitate with 5 per cent TCA at 95°C/15 minutes yielded the hot *TCA fraction*. The remaining solids

were then either dissolved in dilute alkali or resuspended in distilled water and constituted the *residue fraction.* All fractions were taken to constant volume and assayed for radioactivity. Preliminary observations revealed that the distribution of radioactivity in the fractions obtained from labeled bacteria alone and in the presence of serum-leucocytes was identical.

The nucleic acids of *E. coli* were separated by the Schmidt-Thannhauser method (8). Inorganic phosphorus in the RNA fraction, resulting from phosphoprotein hydrolysis, was removed by the technique of Delory (9).

The separation of inorganic and organic phosphorus in the acid-soluble fraction was performed as described in Umbreit *et al.* (10).

Experiments designed to measure the conversion of C^{14} to $C^{14}O_2$ were conducted in conventional Warburg respirometers. $C^{14}O_2$ was isolated from the center well and precipitated as the barium salt according to described procedures (11).

Determination of Radioactivity.—Known volumes of each of the fractions were placed on metal planchets and dried slowly under an infrared lamp. Radioactivity was then assayed with a micromil end-window gas flow counter. At least 4,000 counts above background were recorded for each sample. Fractions containing P^{32} were corrected for background. Samples containing C^{14} were corrected, when necessary, for self-absorption and expressed to infinite thinness. Self-absorption curves were prepared using leucocyte-serum mixtures and C^{14} glucose. The quantity of isotope contained in each of the fractions is expressed either as the per cent of total activity or as the per cent change in activity relative to the zero time sample. The sum total of radioactivity recovered in aliquots taken during the incubation period was always within 5 per cent of the zero time sample.

Chromatography and Radioautography.—The examination of the constituents of each of the fractions obtained from labeled bacteria alone, leucocyte-labeled bacteria mixtures, and degradation products was performed by the general methods outlined in Roberts *et al.* (7). The procedures employed for hydrolysis, solvent systems (two-dimensional descending chromatography) and characterization of isolated components were essentially as described for each of the fractions. The results obtained with *E. coli* K-12 corresponded to those of Roberts *et al.* (7) for *E. coli* B. Radioautograms were prepared with Kodak "no screen" x-ray film. Radioassay of chromatographically separated components was performed with a strip counter.

Determination of Phagocytosis and Intraleucocytic Killing.—Ingestion kinetics of viable bacteria-leucocyte mixtures was studied by previously described methods (4) in which the total, extracellular, and leucocyte-associated fractions were analyzed sequentially. In the case of heat-killed bacteria, aliquots of the reaction mixture were observed under the phase microscope as wet mounts and the proportion of extracellular and intracellular bacteria estimated.

RESULTS

Preliminary Observations.—Several control studies were performed which will be discussed in this section but will not be documented in detail. Repeated examinations revealed that with all of the organisms employed in this investigation the rates of phagocytosis and intracellular killing were rapid. Within 20 minutes after the addition of bacteria to leucocytes more than 95 per cent of the organisms had been ingested and could not be cultured on routine media. By 60 minutes more than 99 per cent of the bacteria had been phagocytosed and inactivated—the remaining fraction of 1 per cent was equally distributed between the extracellular fluid and leucocytes. It was therefore possible to achieve almost quantitative uptake of labeled bacteria so that a large labeled extracellular population did not confuse intracellular events.

Incubation of labeled bacteria in the complete medium, in the absence of

leucocytes, did not result in a significant change in the distribution of radioactivity for periods up to 120 minutes. Similarly, the interaction of labeled bacteria with leucocytes in the presence of heat-inactivated serum (56°C/30 minutes), conditions in which little or no phagocytosis occurred, was not associated with the degradation of bacterial acid-insoluble compounds. In addition, the lysis of sensitive strains, *e.g.* P^{32}-labeled *M. leisodykticus*, with egg white lysozyme, resulted in only a minor increase in acid-soluble radioactivity. From these and other studies it appeared that extensive degradation of acid-insoluble bacterial constituents was related to the intraleucocytic environment.

The influence of the number of bacteria ingested on the rate of degradation was examined in experiments in which different multiplicities of labeled bacteria/leucocyte were employed. Over a 100-fold range (0.1 to 10 bacteria/leucocyte) no alteration in the rate of formation of acid-soluble products was observed, whereas, at a ratio of 18 bacteria/leucocyte a marked reduction occurred. For all subsequent studies a multiplicity of 1 to 2 bacteria/leucocyte was employed.

Studies on the incorporation of P^{32} orthophosphate into the fractions of resting and phagocyting leucocytes (unlabeled bacteria) revealed striking differences. The most marked were in the acid-soluble and alcohol-ether fractions in which the activity of the phagocyting cell remained 2.5- to 3.0-fold higher than the control. The absolute incorporation was relatively low under both conditions and was considered not to be of significance in terms of the reincorporation of bacterial breakdown products. This point will be discussed in a later section.

The Fate of P^{32}-Labeled Bacteria within Rabbit Polymorphonuclear Leucocytes.— Fig. 1 illustrates the data obtained from three experiments in which each of five species of bacteria were engulfed and degraded by the rabbit granulocyte. The lower portion of the figure represents the conversion of acid-insoluble P^{32} into an acid-soluble form and is expressed in terms of total isotope. The upper portion shows the loss or gain of isotope in each of the three fractions as related to the initial distribution of isotope present in the bacteria at time zero. It can be seen that the rate of degradation (loss of isotope) of the acid-insoluble fractions differs for each organism but complements the rate of formation of acid-soluble products. Organisms such as *M. leisodykticus* and *B. subtilis* were broken down more rapidly than the other bacteria employed. This difference was not related to the rapidity of particle uptake by the phagocytes and is presumably a function of the susceptibility of these bacteria to leucocytic enzymes. *E. coli* appeared to be of intermediate susceptibility whereas both *S. albus* and *S. aureus* were less extensively degraded. It is clear from these results that the intraleucocytic death of the bacteria occurs much more rapidly than the extensive breakdown of bacterial protoplasm.

The fate of isotope in the alcohol-ether fraction deserves special comment. In most instances isotope was lost from this fraction and in terms of over-all

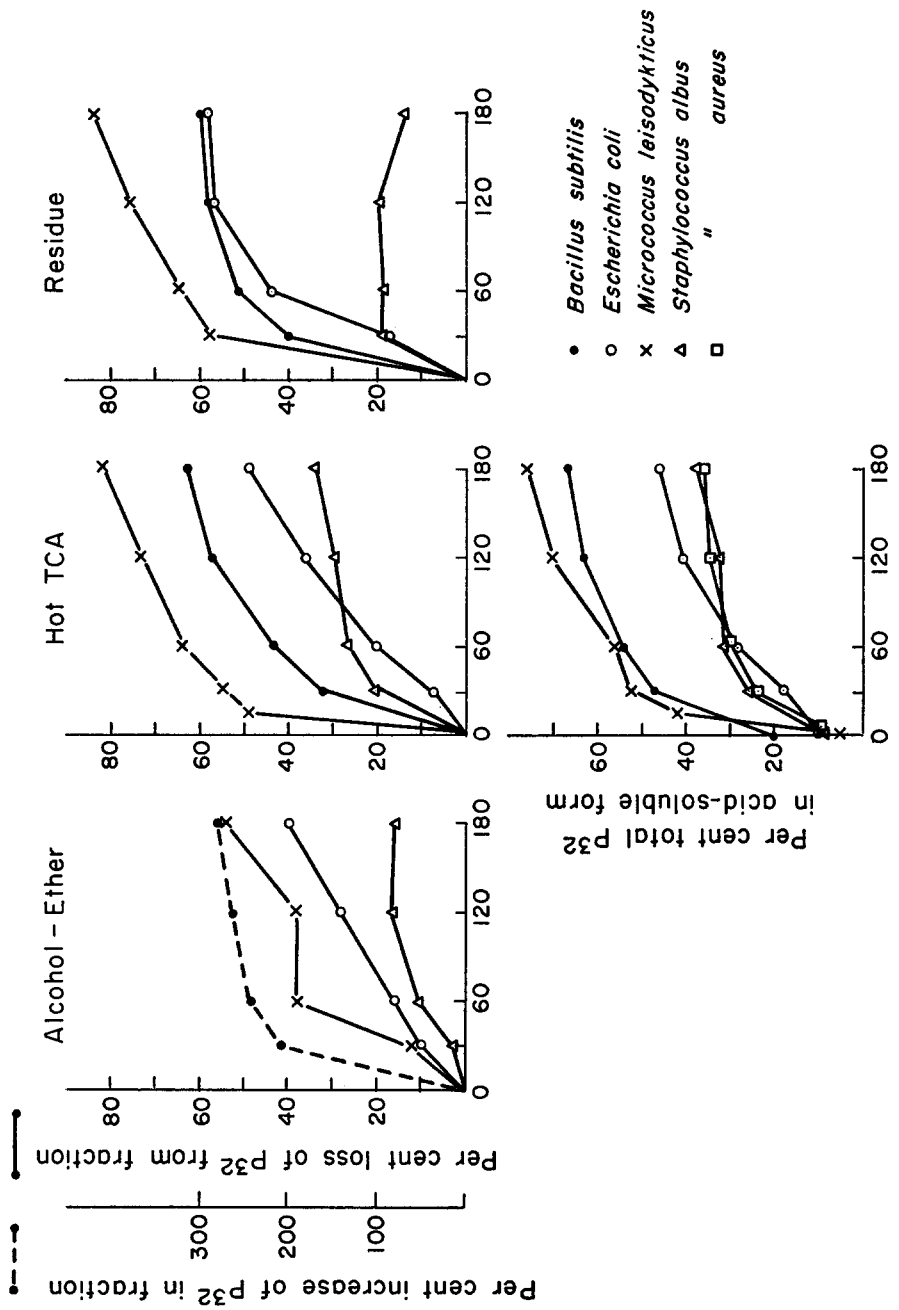

FIG. 1. The degradation of P^{32}-labeled bacteria by rabbit polymorphonuclear leucocytes.

recovery could be accounted for as acid-soluble material. This was not the case when *B. subtilis* was employed. The alcohol-ether fraction of *B. subtilis* alone, contained a lower percentage of P^{32} than the other bacteria, *i.e.* 3 to 4 per cent. Following exposure to leucocytes this fraction increased 200 to 300 per cent over the zero time sample. This was also the case when heat-killed bacteria were employed but did not occur when labeled bacteria were disrupted mechanically or by high concentrations of egg white lysozyme. These facts suggest that isotope may be incorporated into leucocyte lipids from *B. subtilis* degradation products. These findings would be in accord with the studies of Karnovsky in which phagocyting leucocytes incorporated considerably more isotope into their lipids than control cells (12). The other bacteria contained considerably higher levels of alcohol-ether–soluble P^{32} and therefore incorporation into leucocyte lipid may have been masked.

The hot TCA fraction originally contained the largest percentage of isotope and was the major contributor to the acid-soluble fraction after digestion. This fraction in the case of *E. coli* was composed primarily of nucleic acids whereas that from the Gram-positive organisms contained teichoic acids as well. The composition of the residue fraction of P^{32}-labeled organisms has not been completely resolved. In the case of *E. coli* 90 per cent of the isotope of this fraction could be solubilized with trypsin. This suggests the presence of labeled phosphoproteins, a finding recently reported by Rafter and Lane (13), the isotope of which is readily liberated during intraleucocytic residence.

Fig. 2 shows the fate of two of the previously studied organisms within rabbit macrophages. The rates of both ingestion and intraleucocytic inactivation were identical with those obtained with the polymorphonuclear leucocyte. Extensive degradation of both bacteria occurred and at the end of 180 minutes of incubation approximately 70 to 80 per cent of the total isotope was present in an acid-soluble form. The increase of P^{32} in the alcohol-ether fraction of *B. subtilis*-macrophage suspensions was less marked than in similar experiments with granulocytes. A comparison between the two types of cells revealed that the macrophages brought about a somewhat more rapid and extensive degradation of P^{32}-labeled acid insoluble compounds.

The efficacy of human and horse blood leucocytes, each containing mixtures of PMN's and monocytes is presented in Table I. Only the percentage of total P^{32} in an acid-soluble form is presented. It can be seen that cells from both sources were active in degrading bacteria and resembled the rabbit leucocyte in this respect. Although not shown, a similar increase in the P^{32} content of the alcohol-ether fraction occurred in suspensions of *B. subtilis* and human blood leucocytes, the majority of these cells being granulocytes.

The degradation of Viable and Heat-Killed Bacteria.—A question which arose in the interpretation of the preceding results regarded the role of bacterial autolytic enzymes. It seemed possible that following ingestion and killing these en-

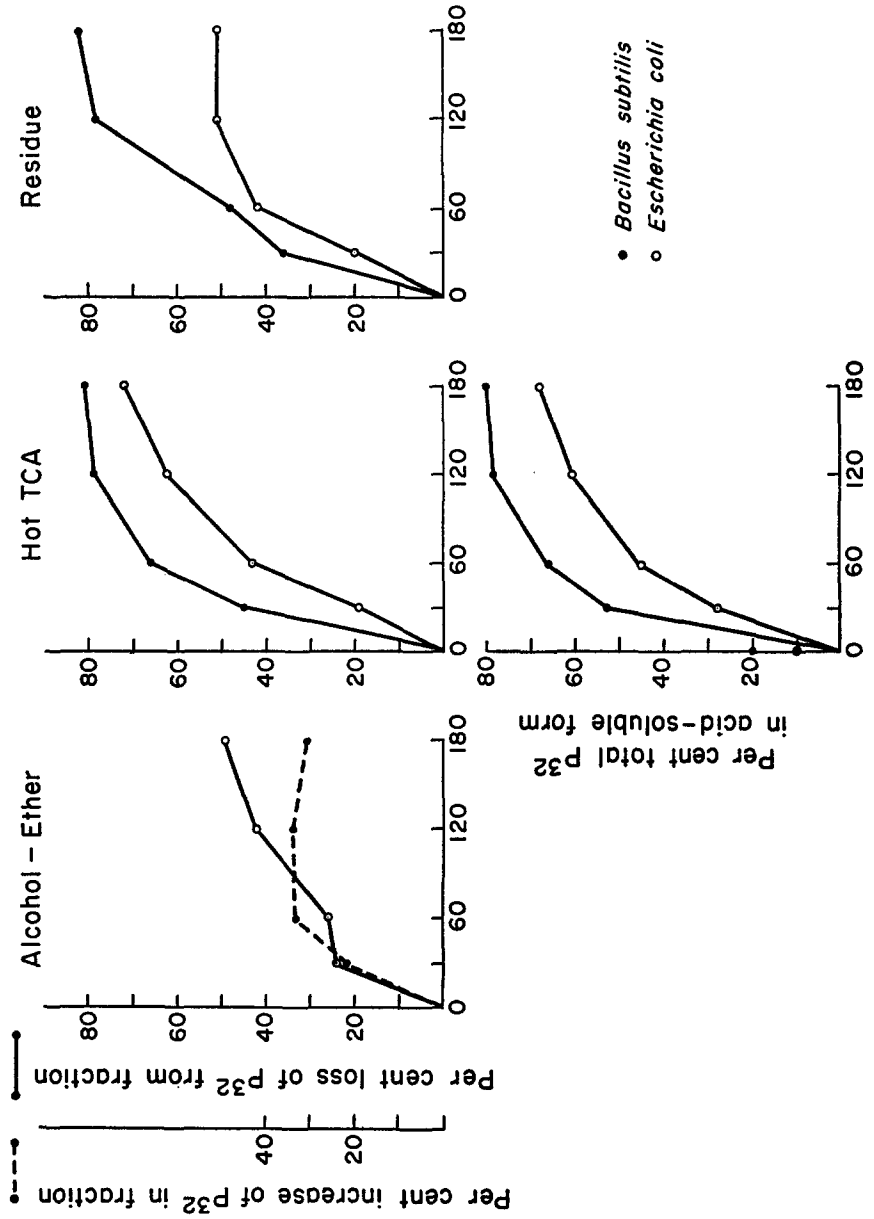

FIG. 2. The degradation of P^{32}-labeled bacteria by rabbit macrophages.

zymes might be activated and contribute to the breakdown of bacterial protoplasm. This point was examined by employing boiled bacteria in which the autolysins presumably had been inactivated. A comparison between viable and heat-killed bacteria is presented in Table II. In all cases the rate of formation

TABLE I

The Degradation of P^{32}-Labeled Bacteria by Human and Horse Blood Leucocytes

Bacteria	Leucocytes	PMN	Per cent of total P^{32} in acid-soluble form				
			0 min.	30 min.	60 min.	120 min.	180 min.
		per cent					
B. subtilis	Human	85	18	48	60	65	72
E. coli K-12	Human	83	11	46	52	58	67
E. coli K-12	Horse	78	11	23	30	46	57
E. coli 28 B2	Human	87	9	20	32	44	54
S. typhimurium RIA	Human	85	10	25	38	47	53
S. albus "Air"	Human	85	9	31	35	38	42

TABLE II

Comparison between the Degradation of Viable and Heat-Killed Bacteria by Rabbit Polymorphonuclear Leucocytes

Bacteria		Increase in per cent of total P^{32} in acid-soluble form				
		0 min.	30 min.	60 min.	120 min.	180 min.
B. Subtilis	Viable	—	20	26	38	43
	ΔK*	—	40	51	58	65
E. coli	Viable	—	7	18	30	38
	ΔK	—	34	42	48	53
S. albus	Viable	—	15	22	23	32
	ΔK	—	28	35	41	50

* 30 minutes in boiling water bath.

of acid-soluble isotope was increased when heat-killed organisms were presented to the cells. Although this data does not exclude the participation of autolytic enzymes in studies with viable organisms, it does suggest that leucocytic hydrolases are more effective when bacteria are altered by heat. Experiments with formalin-killed organisms gave similar results.

The Differential Breakdown of E. coli Nucleic Acids.—Previously presented experiments indicated that the hot TCA fraction was the major contributor to the acid-soluble materials accumulating in P^{32}-labeled *E. coli*–leucocyte mix-

tures. This fraction contained both RNA and DNA phosphorus. Table III presents 2 experiments in which RNA and DNA were separated and the isotope in each quantitated during intraleucocytic residence. It is apparent that RNA was degraded more rapidly and extensively than DNA. Only at 120 to 180 minutes was there significant loss of DNA·P^{32}. This relationship is also shown by the progressively decreasing ratio of RNA·P^{32}/DNA·P^{32}. It appears, therefore, that during the early phases of leucocytic action, RNA phosphorus contributes the largest amount of isotope to the acid-soluble pool.

The Nature of the Breakdown Products of P^{32}-Labeled Bacteria.—It was of some interest to investigate the nature of the products of leucocyte hydrolysis of high molecular weight bacterial compounds. Chromatography and radioautography of the acid-soluble fraction revealed a variety of labeled materials,

TABLE III

Degradation of E. coli Nucleic Acids in Rabbit Polymorphonuclear Leucocytes

Fraction	Per cent loss of P^{32}				
	0 min.	30 min.	60 min.	120 min.	180 min.
RNA	—	18	31	38	51
DNA	—	2	4	8	21
$\frac{\text{RNA}\cdot P^{32}}{\text{DNA}\cdot P^{32}}$	3.95	3.13	2.69	2.61	2.23

many of which have not been identified. Three areas on the chromatograms, which contained isotope, also absorbed ultraviolet light and upon elution in acid had ultraviolet absorption spectra characteristic of nucleotides. By far the major labeled product migrated just behind the solvent fronts and was identified as inorganic phosphate. Scanning with a strip counter revealed that at least 50 per cent of the P^{32} in this fraction was in the form of inorganic phosphate. Experiments in which the inorganic P^{32} of the acid-soluble fraction was estimated are presented in Fig. 3. Both viable and boiled organisms were employed and the results are expressed as per cent of the total P^{32} present in the aliquots. In both instances, as acid-soluble components accumulated there was a rapid increase in inorganic P^{32} as well. This was perhaps more extensive with heat-killed bacteria. Since the rabbit polymorphonuclear leucocyte contains both acid and alkaline phosphomonoesterases it is likely that these enzymes were operative in the digestive process.

The Fate of C^{14}-Labeled Bacteria.—The distribution of C^{14} in the four fractions of labeled bacteria was considerably different than with P^{32} and allowed the

evaluation of bacterial components other than those containing phosphorus. The majority of the isotope was present in the residue fraction and could be accounted for as labeled protein. Hydrolysis and chromatography of this fraction showed that at least 95 per cent of the isotope was present in the form of amino acids. The influence of various types of leucocytes on three species of C^{14}-labeled bacteria is shown in Table IV. It is apparent that extensive losses of label occurred in the residue fraction with a concomitant increase in acid-

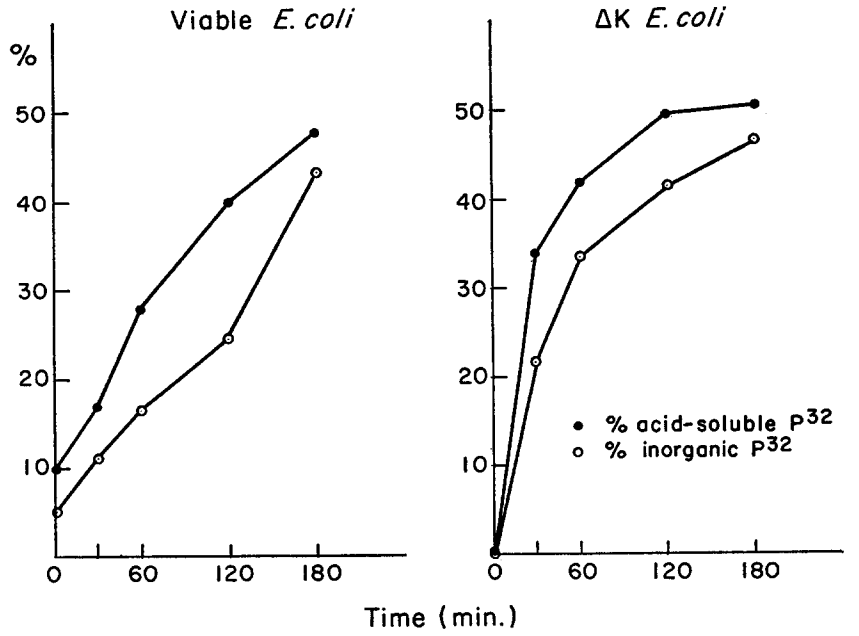

FIG. 3. The formation of inorganic phosphate from viable and heat-killed *E. coli* by polymorphonuclear leucocytes.

soluble radioactivity. This indicated that proteolysis was occurring with the accumulation of peptides and amino acids—a finding which was corroborated by the chromatography of the acid-soluble fraction after 180 minutes of incubation. Of considerable interest was the increase in radioactivity in the alcohol ether fraction—a finding confined to P^{32}-labeled *B. subtilis* in the former studies. At least 70 per cent of the isotope of this fraction was soluble in chloroform-methanol and presumably lipid. The lipid incorporation observed most likely represented uptake into leucocyte rather than bacterial lipid since heat-killed organisms were employed. The addition of acetate to the suspending medium reduced the increase of alcohol-ether C^{14} under these conditions. In general, the results with the other fractions of C^{14}-labeled bacteria gave results which were similar to P^{32}-labeled organisms.

The complete conversion of bacterial protoplasm to $C^{14}O_2$ is presented in Table V. Only a small percentage of the total isotope, added as labeled bacteria, was lost to the center well as CO_2. In a 2 hour incubation period less than 1 per

TABLE IV

The Degradation of C^{14}-Labeled, Heat-Killed Bacteria by Rabbit and Human leucocytes

Bacteria	Fraction	Rabbit PMN		Human blood		Rabbit macrophges	
		Per cent of total C^{14}		Per cent of total C^{14}		Per cent of total C^{14}	
		0 min.	120 min.	0 min.	120 min	0 min.	120 min.
E. coli K-12	Acid-soluble	1.0	51.6	1.0	47.8	1.3	45.0
	Alcohol-ether	21.4	15.5	23.0	15.6	15.5	24.2
	Hot TCA	8.6	2.9	11.5	4.6	13.5	3.2
	Residue	69.0	30.0	64.5	32.0	69.7	27.6
S. albus "Air"	Acid-soluble	0.3	25.2	0.3	20.3	0.4	27.0
	Alcohol-ether	6.5	10.1	2.1	6.4	4.5	7.1
	Hot TCA	34.4	24.1	21.2	20.0	23.0	17.0
	Residue	58.8	40.6	76.4	53.0	72.1	48.9
B. subtilis	Acid-soluble	14.	49.7	—	—	14.0	51.3
	Alcohol-ether	6.5	17.4	—	—	5.3	16.9
	Hot-TCA	25.0	12.3	—	—	26.1	12.8
	Residue	60.5	20.6	—	—	54.6	19.0

TABLE V

Oxidation of C^{14}-Labeled, Heat-Killed Bacteria to $C^{14}O_2$ by Rabbit Polymorphonuclear Leucocytes

Bacteria	No. of experiments	Per cent of total C^{14} recovered as $C^{14}O_2$/hr.*
B. subtilis	3	0.26
S. albus "Air"	3	0.21
E. coli K-12	4	0.16

* Experiment terminated after 4 hours' incubation at 37°C. Total CPM added as labeled bacteria varied between 7×10^4 and 2×10^5.

cent of the total radioactivity was converted to CO_2, even though large amounts of acid-soluble fragments were present.

Degradation Products in the Extracellular Medium and Leucocyte Fractions.— Further experiments determined the localization of degraded material both within and without the leucocyte. One such experiment, performed with *B. subtilis-P*[32] (Table VI) will illustrate the general results obtained with other

organisms; *e.g.*, *E. coli* and *S. albus*. At time 0, the distribution of radioactivity corresponded to that observed in the intact organism and was present in the medium prior to phagocytosis. After 15 minutes' incubation at 37°C the vast majority of bacteria were ingested, as evidenced by the large amounts of acid-insoluble P^{32} associated with the leucocytes. At this time almost all of the initially acid-soluble P^{32} was present in the medium. With prolonged incubation there was a progressive loss of leucocyte-associated acid-insoluble P^{32} and a concomitant increase in the acid-soluble radioactivity of the suspending medium. Throughout the experiment only small amounts of isotope were pres-

TABLE VI

The Interaction between P^{32}-labeled B. subtilis and Rabbit Polymorphonuclear Leucocytes. The Distribution of Acid-soluble and-Insoluble Radioactivity in Leucocyte Fractions and Medium

Fraction	Radioactivity	Per cent of total P^{32}				
		0 min.	15 min.	30 min.	60 min.	120 min.
Medium	Acid-soluble	18	22.7	34.9	52.1	62.7
	Acid-insoluble	82	3.1	1.1	1.0	1.2
10,000 *g* Cell supernate	Acid-soluble	—	2.0	2.6	2.2	3.6
	Acid-insoluble	—	0.8	0.9	0.9	0.9
10,000 *g* Cell sediment	Acid-soluble	—	2.8	4.5	3.7	4.7
	Acid-insoluble	—	68.6	56.0	40.1	26.9
Total acid-soluble		18	27.5	42.0	58.0	71.0
Total acid-insoluble		82	72.5	58.0	42.0	29.0

ent in the 10,000 *g* supernate from the disrupted leucocytes. It is likely that some portion of the acid-insoluble isotope present in the cell sediment represents radioactivity incorporated into leucocyte protoplasm.

It is concluded from this type of experiment that: (*a*) the initial intracellular event was the liberation of the bacteria's pool of small molecular metabolites, (*b*) the degradation of high molecular weight bacterial products within the leucocyte was followed rapidly by their transport to an extraleucocytic focus, and (*c*) no significant accumulation of small bacterial breakdown products occurred within the phagocyte.

The Influence of PMN Leucocyte Granule Extract on Labeled Bacteria.—In view of the fate of bacteria within intact leucocytes it was of some interest to compare these results with the effects of acidic extracts of PMN leucocyte granules which contain the bactericidal agent phagocytin.

The specific cytoplasmic granules from 9×10^8 rabbit PMN leucocytes were isolated by previously described procedures (1). The granules were washed once with 0.34 M sucrose, sedimented at 9,000 *g* and the entire pellet extracted with 0.095 M citric acid at 22°C for 4 hours. The extract was clarified by high speed centrifugation and employed as the stock phagocytin solution.

P^{32}-labeled *E. coli* were then added to buffer at pH 7.1 which contained either a 1:20 dilution of phagocytin or an equivalent amount of crystalline bovine serum albumin. At various times aliquots were removed for (*a*) determination of viable bacteria by plating techniques, (*b*) total acid-soluble and insoluble radioactivity, and (*c*) extrabacterial radioactivity following centrifugation at 20,000 *g* for 5 minutes.

The results of one such experiment are presented in Table VII. Within 1 minute after the addition of 2×10^8 bacteria/ml to the granule extract, the

TABLE VII

Influence of Granule Extract on the Viability, Degradation of Acid-Insoluble P^{32}, and Release of Acid-Soluble P^{32} from E. coli

Time	Viable count, bacteria/ml		Per cent of total P^{32} in Acid-soluble form		Per cent of total P^{32} released into medium	
	Extract	Control	Extract	Control	Extract	Control
min.						
Zero	2.7×10^8	2.5×10^8	10.4	10.2	<1.0	<1.0
1	3.0×10^6	—	—	—	—	—
5	$< 10^3$	—	12.6	—	12.0	—
30	$< 10^3$	2.6×10^8	15.6	11.5	14.7	3.2
60	$< 10^3$	—	16.4	12.1	17.8	—
120	$< 10^3$	3.0×10^8	18.5	13.3	19.1	5.0

viable count had fallen to 3.0×10^6, or a kill of 99 per cent. Within 5 minutes less than 10^3 viable organisms remained, whereas, the control tube was unchanged. During the 2 hours of incubation, although essentially all the organisms had been rendered non-viable, only a small increase occurred in acid-soluble radioactivity; *i.e.*, 8 per cent with granule extract and 3 per cent in the control. The most marked influence of granule extract was noted in the release of the bacteria's acid-soluble phase into the surrounding medium. Essentially all the acid-soluble radioactivity of the granule extract-treated bacteria was extracellular whereas this was not the case with the control. This suggested that subsequent to or concomitant with the loss of viability of the bacteria there was a release of acid-soluble P^{32}, perhaps on the basis of a change in the organism's permeability. In addition, the lack of extensive degradation of high molecular weight P^{32} was in contrast to the events within the cytoplasm of the intact leucocyte.

DISCUSSION

The current experiments employed a group of bacteria which under *in vitro* conditions, in the absence of immune serum, were ingested and killed by both granulocytes and macrophages. Under these conditions, extensive degradation of bacterial lipid, nucleic acids, and proteins occurred within both cell types. Temporally, the inactivation of the organism preceded any marked digestion of high molecular weight constituents. Thereafter, the rate of degradation depended primarily upon the nature of the particle rather than the source or type of leucocyte. It is likely that the composition of the bacterial surface plays an important role in the susceptibility of an organism to leucocyte enzymes. The availability of substrates within the bacteria is probably dependant to some extent on alterations in the cell wall or the integrity of other superficial structures. These factors, although explaining to some extent the over-all breakdown of a particular organism, do not offer insight into the differential destruction of particular classes of macromolecules. One example would be the delayed depolymerization of *E. coli* DNA within the polymorphonuclear leucocyte. Whether this depends upon steric factors or lack of sufficient hydrolytic enzymes is not clear at this time.

Degradation in terms of the present experiments, was equated with the formation of acid-soluble fragments. In most instances this implied extensive alterations in initially acid-insoluble components, although it is possible that relatively large fragments are also produced. In addition, the methodology employed did not allow one to distinguish between specific compounds of the bacteria, *e.g.* proteins etc., nor to account for immunologically active compounds such as the polysaccharides.

Electron microscopic studies (14) in collaboration with Dr. David J. Luck have revealed that bacteria ingested by polymorphonuclear leucocytes were always found within a membrane system. This structure in the past has been designated as the phagocytic vacuole. In the case of *B. subtilis* it was quite clear that within 30 minutes after phagocytosis, extensive fragmentation of the bacterial cell wall had occurred. This as well as other evidence (15, 16) strongly suggests that the phagocytic vacuole is the site for the death and destruction of particles. With the data available at this time, it appears that granule-associated components, including hydrolytic enzymes, are released into or in the vicinity of the phagocytic vacuole and play a role in the process of digestion. This sequence would allow intraleucocytic digestion to take place within an area removed from other cytoplasmic components as well as concentrating granule enzymes in apposition to the foreign particle. Under these conditions in which the leucocyte liberates preformed materials in response to a variety of particulates, it would appear difficult to influence the fate of an organism within the cell.

All of the experiments reported in this article were conducted in the presence

of fresh serum, which contained heat-labile opsonic factors and allowed rapid phagocytosis to take place. The nature of these opsonins has not been decided but it is considered likely that they combine with the bacterial surface in some manner. This interaction may influence the subsequent fate of the organism within the cytoplasm of the leucocyte, although evidence for or against this hypothesis is lacking. The influence of certain serum and cellular factors on the intracellular fate of bacteria will be developed in the following communication.

SUMMARY

The intraleucocytic fate of a variety of P^{32}- and C^{14}-labeled bacteria has been studied in both polymorphonuclear leucocytes and macrophages. Both cell types brought about extensive degradation of bacterial lipids, nucleic acids, and proteins. Intracellular breakdown was primarily dependant upon the composition of the ingested particle rather than on the type or source of the phagocyte. Evidence is presented for the reincorporation of bacterial constituents into leucocyte lipid. More than 50 per cent of the acid-soluble degradation products of P^{32}-labeled bacteria appear as inorganic phosphate. Bacterial RNA is degraded more readily than DNA.

Following phagocytosis, labeled bacteria lose their pool of small molecular weight intermediates. This is followed by the degradation of acid-insoluble constituents. The majority of bacterial breakdown products are then excreted by the leucocyte and appear in the medium.

Heat-killed bacteria were more readily broken down than viable organisms. Only small amounts of C^{14}-labeled bacteria were completely oxidized by leucocytic enzymes to $C^{14}O_2$.

Acid extracts of polymorphonuclear leucocyte granules, which were highly bactericidal, liberated the acid-soluble constituents of labeled bacteria but did not significantly degrade bacterial macromolecules.

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