ANTIGENIC AND RELATED BIOCHEMICAL PROPERTIES OF *LISTERIA MONOCYTOGENES*

I. Preparation and Composition of Cell Wall Material[1]

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Received for publication April 11, 1960

Recently *Listeria monocytogenes* has assumed considerable importance as a pathogen. This has stimulated a number of studies directed toward a more complete characterization of the bacterium. It is striking that in spite of the astonishing variety of susceptible hosts, almost 50 different species of mammals and birds, its bacteriological and immunological characteristics are quite constant. Renewed studies on its antigenic structure, employing several techniques, revealed new groups which differ only slightly from the four described by Paterson (1939, 1940). Although some lesions produced by *L. monocytogenes* suggest the action of a toxin, attempts to confirm this assumption have led to inconclusive and confusing results. With the exception of Stanley (1949), who partially purified the monocyte-producing fraction of the cell, there is a paucity of information on the localization and nature of its various immunological components.

Antigens residing in the cell wall of an organism can be appreciably purified by preparation of cell wall material free of protoplasm before chemical or other physical purification steps are attempted. A knowledge of the chemical composition is ultimately sought for many antigens and therefore the chemical composition of the parent structure (cell wall, cytoplasmic membrane, etc., where applicable) is of importance.

The preparation and chemical characterization of cell wall and membrane material from many organisms has been practiced by investigators for some time, as indicated in a recent review by Mitchell (1959). Such work has been prompted by many reasons including interest in the cytological location of various antigens (Munoz, Ribi, and Larson, 1959; Ribi, Milner, and Perrine, 1959), of enzymatic activities (Marr and Cota-Robles, 1957; Mitchell, 1959), and of metals (Keeler, Carr, and Varner, 1958). Other reasons have included interest in the transport of materials into and out of the cell (Mitchell and Moyle, 1956; Mitchell, 1959), in the mode of action of antibiotics (Strominger, Park and Thompson, 1959), and also inherent interest in the chemical and structural nature of cell wall and membrane material.

It is possible to prepare bacterial cell wall or membrane material by a number of techniques, including lysis or protoplast formation by agents such as lysozyme (Repaske, 1956; Weibull, 1956), by osmotic rupture of whole cells or protoplasts (Robrish and Marr, 1957), by shaking with glass beads (Mickle, 1948; Nossal, 1953), and by sonic oscillation (Marr and Cota-Robles, 1957; Cota-Robles, Marr, and Nilson, 1958).

Several investigators prepared extracts of *L. monocytogenes* by various methods. Most of these studies were directed toward the isolation of a toxic or monocyte-producing fraction (Patocka et al., 1959*a*,*b*; Stanley, 1949; Uher and Uher, 1956), or a fraction which, when given together with the bacterium, might enhance its pathogenicity (Patocka et al., 1959*a*,*b*), or an antigen that might be employed as a skin test for listeric infection (Sachse and Potel, 1957; Zipplies, 1957). Roots (1958*b*) was the first to employ mechanical means for obtaining cell wall preparations of *L. monocytogenes*. He used ether extraction followed by 4 hr agitation with glass beads and digestion with trypsin, ribonuclease, and deoxyribonuclease for the preparation of cell wall material. In subsequent studies with flagella preparations Roots (1958*a*) found that the flagella of *L. monocytogenes* contained only H

[1] Contribution from the Montana Veterinary Research Laboratory (Montana Experiment Station and Livestock Sanitary Board cooperating), Montana State College, Agricultural Experiment Station, paper no. 489, journal series. Supported in part by research grant no. E-2571, National Institute of Allergy and Infectious Diseases, U. S. Public Health Service.

antigens, but no further mention was made of the antigenic components in the cell wall-membrane complex.

We have sought to obtain a suitable method of breakage of *L. monocytogenes* which would allow the recovery of cell wall material and have attempted a partial chemical characterization of this fraction. These experiments, reported below, were performed as a requisite to the study of the antigens of this organism.

METHODS

Growth and harvesting of cells. L. monocytogenes strain VRL 10275, serological type 4b, isolated in 1958 from a sheep brain by Dr. I. C. Blore, (University of Nebraska), was grown in shake cultures of approximately 1 liter at 37 C in tryptose phosphate broth (Difco). The cells were harvested after about 18 hr growth while still in the log phase. They were removed by centrifugation at 2,000 × *g* for 10 min and washed two times in 0.05 M phosphate buffer, pH 7.0, prior to resuspension in that buffer for cell breakage.

Purity of the cultures was checked by plating on tryptose agar. After 18 hr incubation at 37 C the plates were examined by means of a binocular scanning microscope using the illumination suggested by Henry (1933) and Gray (1957). Viable cells were counted by plating.

Where required, whole cells or fractions were suspended in 1 per cent agar and photographed on Contrast Process Pan film.

Cell breakage and preparation of fractions. Cells were broken using sonic oscillation (Raytheon 10 kc). Next, 40 ml of the cell suspension containing about 100 mg of cells were subjected to oscillation at an audio frequency current of about 1.1 to 1.2 amp with the temperature of the cell suspension maintained below 5 C.

In kinetic experiments 1-ml samples were removed at various time intervals during the course of the oscillation treatment. Generally only about seven or fewer 1-ml samples were removed and, for this reason, no buffer was added to replace that removed. In experiments in which it was desirable to fractionate the broken cells, a 16-min oscillation time was used. Fractionation of the disrupted cell preparation was accomplished by an initial centrifugation at 2,000 × *g* for 10 min which removed whole cells. Some broken cell wall material was also lost in this residue. The supernatant from this centrifugation contained cell wall and protoplasmic constituents. Centrifugation of the supernatant at 10,000 × *g* for 30 min sedimented cell wall material which had not been comminuted to extremely small particles. This wall material was then repeatedly washed.

Chemical analysis. Visible and ultraviolet spectral curves were run on the wall and protoplasmic fractions using a Beckman model DU spectrophotometer. Turbidity was determined by spectrophotometric assay at 660 mμ. Kjeldahl nitrogen was determined by the method of Johnson (1941). Protein was also determined by the Folin method (Lowry et al., 1951). Hexose, hexosamine content (Winzler, 1955), and dry weight were also determined on the fractions.

Sugar chromatography was run on both acid and resin hydrolyzates of the fractions (3 N HCl autoclaved for 4 hr at 120 C, and acid regenerated Permutit Q at the same temperature). The hexosamine assays were run on the acid hydrolyzates, as were the amino acid chromatograms. The sugar chromatograms were run by triple ascension in *n*-propanol:ethyl acetate:water (7:1:2). The location of the sugars was disclosed by dipping in benzidine-acetate (Aronoff, 1956). The two-dimensional amino acid chromatograms were run in *sec*-butanol:formic acid:water (7:1:2), and in phenol:water:ammonia (18:2:0.6). Amino acid spots were developed by dipping in ninhydrin-acetone (Toennies and Kalb, 1951).

The data reported are averages of at least three determinations.

Radioisotope methodology. Either Ca^{45} as Ca^{++} ion, P^{32} as $PO_4^{=}$, or Fe^{59} as Fe^{+++} ion was added to the liter cell cultures, in which calcium, phosphorus, or iron distribution was to be determined. The isotopes were added shortly after the cultures had entered the log phase to allow the cells to become completely equilibrated with the isotopes. Assay of the activity was accomplished using an end window GM tube with a window thickness of 1.4 mg/cm. A standard scaling circuit was used. Differences in the weights of the fractions were small enough that a counting error of less than 5 per cent could be attributed to self-absorption differences, and for this reason self-absorption corrections were disregarded. Counting time was of sufficient duration to yield a standard error of 1.6 per cent or less.

Terminology. The terms Folin protein and

Kjeldahl protein are used to describe the Folin and Kjeldahl positive material in the fractions including the cell wall fraction, even though it is recognized on the basis of the results reported here that the amino acids of the cell wall could hardly be described as typical protein(s) since only a few amino acids are present. The 10,000 × *g* residue is called the wall fraction throughout, although the results presented here suggest that it may be contaminated with some cytoplasmic membrane material.

Assay for monocyte-producing agent. Groups of five mice were inoculated intravenously with 0.02 ml of either whole cells, cell wall, or protoplasmic material. Since it was possible that both the wall and protoplasmic fractions might contain some viable cells, portions of these were treated with terramycin (oxytetracycline hydrochloride) at a concentration of 0.2 mg per 0.02 ml of inoculum for 2½ hr before exposure. Two groups of five mice which received either wall or protoplasmic fractions were given 0.2 mg of terramycin intraperitoneally at the time of exposure. Groups of five mice which received whole cells were treated with terramycin at the time of exposure or received terramycin-treated cells. Blood smears were made from tail vein blood at time of exposure and at 3-day intervals thereafter for a period of 10 days. Smears were stained with Wright's stain and 100 leucocytes per film were counted. All mice which died or were killed were necropsied and the liver and spleen cultured, using the maceration and refrigeration technique of Gray et al. (1948); Gray (1957).

RESULTS AND DISCUSSION

Preparation of wall material and recovery. Sonic oscillation of *L. monocytogenes* as with Azotobacter (Marr and Cota-Robles, 1957) results in the disruption and killing of the cell and comminution of the cell and intracellular components. Figure 1 shows a plot of both total viable cells and optical density versus time of oscillation. The rate of decrease of optical density is less than the rate of decrease of viable cells. This suggests that cell wall fragments accumulate during oscillation (Marr and Cota-Robles, 1957). Phase microscopical observations verified the accumulation of microscopic size particles. Prior to oscillation only whole cells are seen. Shortly after oscillation begins, whole cells and large fragments from the cells which appear to be about ½ the

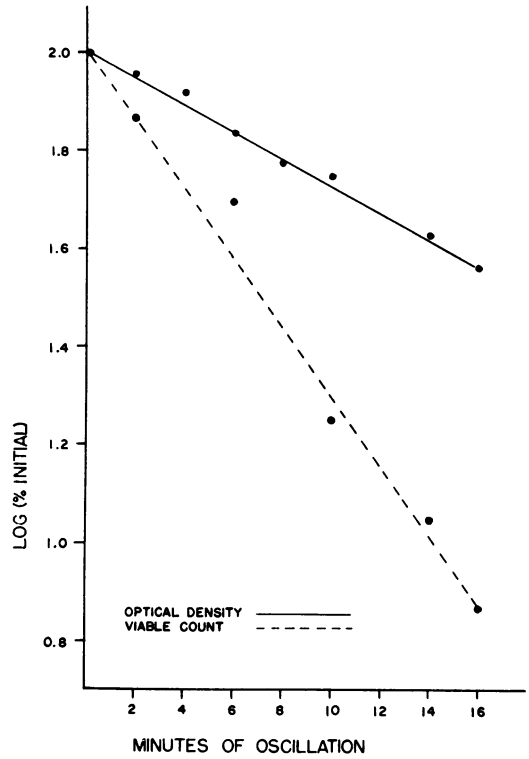

Figure 1. A plot of the logarithm of the percentage remaining of both total viable cells of *Listeria monocytogenes*, and optical density of the cell suspension at 660 mμ versus the length of time of oscillation.

size of whole cells are visible. A few fragments of an apparent size range of $\frac{1}{10}$ to $\frac{1}{15}$ the size of whole cells are also seen. With longer oscillation times, the proportion of whole cells and large fragments decreases in comparison to those fragments of the size range $\frac{1}{10}$ to $\frac{1}{15}$ whole cell size. Figure 2 presents photographs of whole cells, an oscillated preparation, and also purified wall material. Oscillation for 16 min allows the greatest recovery of wall material.

On the basis of dry weight measurements, an average of 9 per cent of the total dry weight of the cell was found in the wall fraction. A rough approximation can be made of the yield of wall material prepared in this way if a value of 20 to 30 per cent is assumed for the actual dry weight of the wall, as is the case for most gram-positive organisms (Mitchell, 1959). This would mean that our recoveries were from 30 to 50 per cent of the total wall material.

Attempts to prepare wall material by proto-

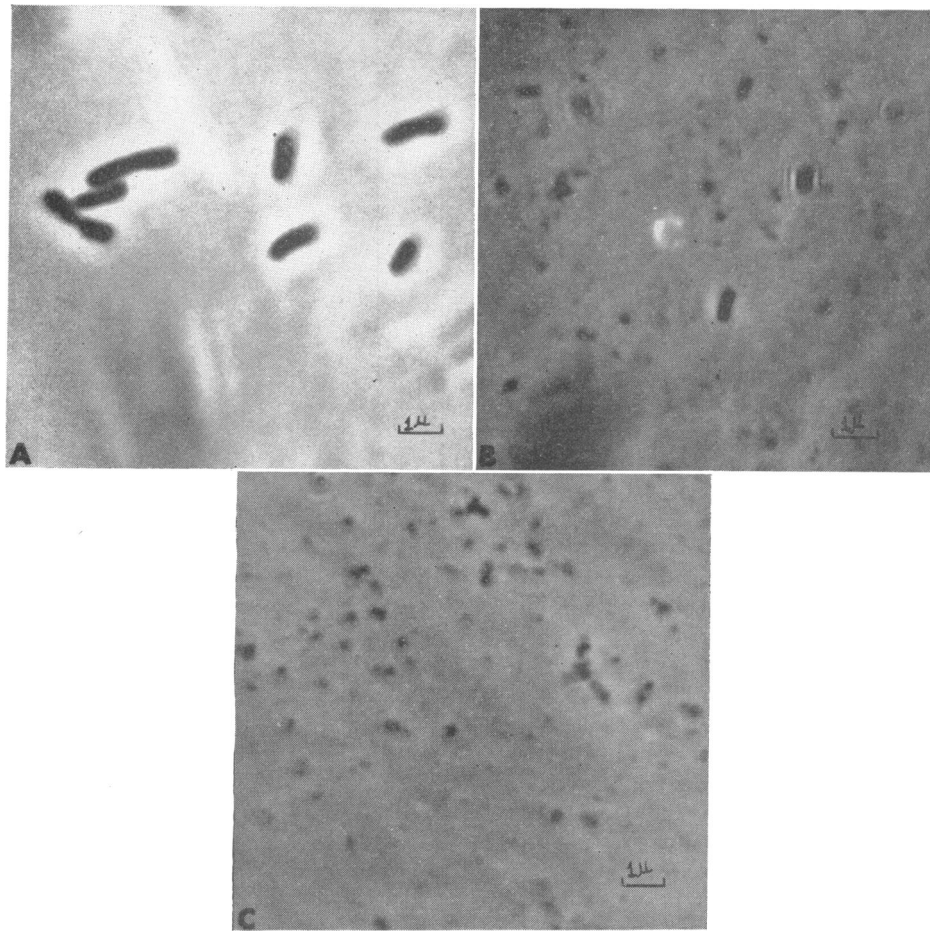

Figure 2. Phase photomicrographs of preparations of *Listeria monocytogenes*. (*A*) Whole cells. (*B*) A preparation oscillated for 16 min. (*C*) A purified wall preparation.

plast formation using Weibull's (1956) lysozyme method for gram-positive cells or modifications did not prove successful. Furthermore, direct osmotic lysis was also unsuccessful. Cells were incubated for various lengths of time in 0.5 molal solutions of glycerol, ethanol, propylene glycol, sucrose, or glucose and then subjected to rapid dilution with distilled water, but no cell lysis occurred, as judged by the lack of optical density change at 660 mμ. Thus, it has not been possible to check the yield of wall material obtained by oscillation by using a more accurate value for actual wall dry weight obtained by protoplast formation or direct lysis techniques.

Composition of wall material. The ultraviolet spectra of the protoplasmic, the unwashed wall, and the washed wall fractions are shown in figure 3. In contrast to the protoplasmic and unwashed wall preparations, the washed wall material shows no apparent absorption at 260 mμ. This suggests the absence of nucleic acid material in the purified wall preparation.

It was of interest to us to determine whether cytochromes were present in the wall fraction, since it has been shown that cytochromes are a part of the cytoplasmic membrane of certain cells (Mitchell, 1959; Marr and Cota-Robles, 1957). It would be valuable in subsequent antigenic studies to know whether membranes were a contaminant of wall material prepared by our method. The visible spectra of both wall and protoplasmic fractions was determined. There was no evidence of a peak in the Soret region in either the wall or protoplasmic material, suggest-

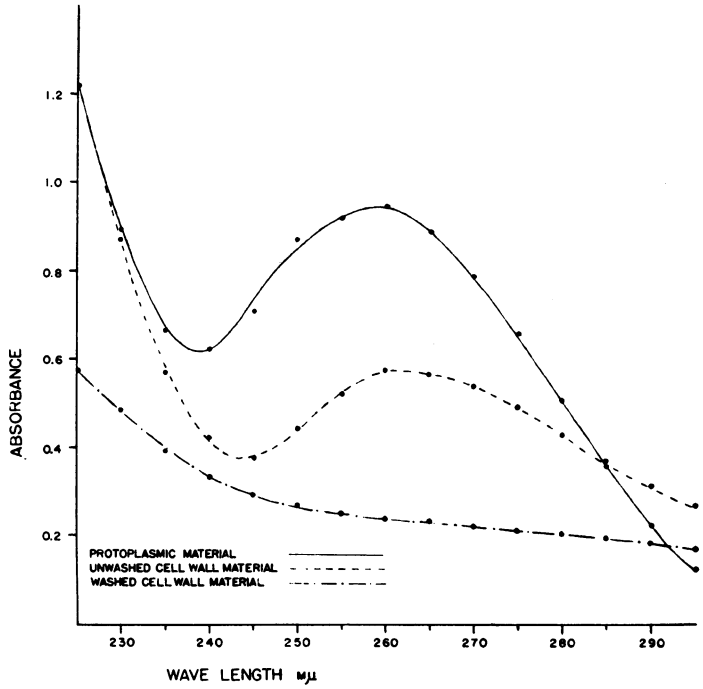

Figure 3. The ultraviolet absorption spectra of protoplasmic, unwashed wall, and washed wall preparations of *Listeria monocytogenes*.

ing a very low cytochrome level in these microaerophilic cells. A more sensitive technique was obviously needed for these cytochrome-poor cells. Localization of iron can be used as a fairly accurate method for determining the location of cytochromes (Keeler et al., 1958) since a relatively large proportion of the iron of bacterial cells may be cytochrome bound. Preliminary experiments with Fe^{59} indicated that about 15 percent of the iron taken up by the cell could be recovered in the wall preparation. Assuming a recovery of only ½ of the wall material, one could conclude that at least 30 per cent of the iron was present in this wall fraction. It has been shown by Keeler et al. (1958) that membrane material from Azotobacter prepared by lysozyme lysis and osmotic rupture, contains ⅔ of the total iron taken up by that organism. The cytochromes have been reported to reside in the cytoplasmic membrane of that organism (Marr and Cota-Robles, 1957). Thus these data with *L. monocytogenes* indicated the possibility that some of the cytoplasmic membrane was adhering to the wall during preparation by sonic oscillation.

The wall preparation has been found to contain hexoses, hexosamine, and nitrogen (table 1). It gives a positive Folin protein reaction although the concentration of protein by this method is only ⅓ to ½ that value for protein given by Kjeldahl nitrogen. This discrepancy suggested an absence or low concentration of aromatic amino acids in the wall preparation. It has been reported by Salton (1953), Mitchell (1959), and others that most gram-positive organisms examined contain no aromatic amino acids and only a limited number of nonaromatic amino acids in the wall material.

Amino acid paper chromatography of acid hydrolyzates of the wall preparations revealed the presence of only the five amino acids shown in table 2 at the relative concentrations indicated. All are nonaromatic. The protoplasmic material, on the other hand, contained a full complement of 17 to 20 detectable amino acids.

The hexose of the wall material has been found by paper chromatography to be composed solely of glucose and galactose. Protoplasmic material contains at least seven sugars of about the same concentration as those in the wall material. Three of the sugars of protoplasmic material were assumed to be glucose, galactose, and ribose from R_F values while the rest were unidentified, al-

TABLE 1

Cell wall material composition of Listeria monocytogenes

Component Present	Percentage of the Total Dry Weight of the Cell Wall
Hexose	17–20
Hexosamine	2–5
Protein	
Folin	18–21
Kjeldahl	45–50

TABLE 2

Amino acid composition of cell wall fractions of Listeria monocytogenes

Amino Acid	Relative Concn
Alanine	+++
Glutamic acid	++
α,ϵ-Diaminopimelic acid	++
Aspartic acid	+
Leucine	+

though their R_F values were found not to coincide with mannose, rhamnose, fucose, arabinose, fructose, or xylose.

Distribution of components in wall and protoplasmic material. Table 3 shows the minimal distribution of various components in unwashed and washed wall preparations as a percentage of the total for that component in the cell. These data suggest that a rather large proportion of the total hexosamine, calcium, iron, and hexose of the cell are to be found in the wall fraction, considering the weight of this structure.

A comparison of radioisotope distribution in the wall and protoplasmic fractions was made in table 4 by utilizing specific activity calculations (counts:min:g dry weight). The relative specific activity of P^{32} was the same in both fractions, while that of Fe^{59} was only slightly greater in the wall fraction. With Ca^{45}, however, the specific activity was 10-fold greater in the wall than in the protoplasmic fraction.

The chemical form or role of calcium in *L. monocytogenes* wall material is unknown. The role of the small proportion of phosphorus is likewise undetermined. It may be a component of phospholipid (Marr and Cota-Robles, 1957) since lipid is reported to be a component of the cell wall-membrane complex of some organisms (Salton, 1953; Mitchell, 1959). Another possibility is ribitol phosphate, reported present in cell walls of certain organisms (Mitchell, 1959).

The proportion of the total incorporated Fe^{59} and P^{32} which remains in solution after sedimentation at 10,000 $\times$ *g* for 30 min was determined for specific time increments during the course of oscillation. The data were plotted by the kinetic method of Marr and Cota-Robles (1957) (figure 4). Values for P^{32} described a curve about midway between that for optical density and that for viable count. The curve was not exponential, but rather approached the viable count curve early in the oscillation period, and then as time progressed, shifted towards the optical density curve. This suggests, according to the reasoning of Marr and Cota-Robles, that this component was derived from both the wall and protoplasm. The Fe^{59} values very closely paralleled the optical

TABLE 3

Minimum amounts of various components in cell wall preparations of Listeria monocytogenes as a percentage of total in cell

Component	Percentage in Unwashed Wall	Percentage in Washed Wall	Percentage Loss by Washing
Hexose	16.7	13.8	17
Hexosamine	37.0		
Dry weight	9.2		
P^{32}	8.7	5.4	38
Ca^{45}	37.2	35.8	4
Fe^{59}	18.2	14.9	18
Kjeldahl protein		7.5	
Folin protein	7.9	5.4	22

TABLE 4

Specific activity of radioisotopes in cell wall and protoplasmic fractions of Listeria monocytogenes

Radio isotopes	Fraction	Total Weight	Total Activity	Specific Activity
		g	*counts/min*	*counts:min:g*
P^{32}	Wall	0.0083	1,080	13.0×10^4
	Protoplasm	0.0795	10,340	13.0×10^4
Fe^{59}	Wall	0.0078	5,300	68.0×10^4
	Protoplasm	0.0585	23,800	41.0×10^4
Ca^{45}	Wall	0.0040	73	1.8×10^4
	Protoplasm	0.0690	123	0.18×10^4

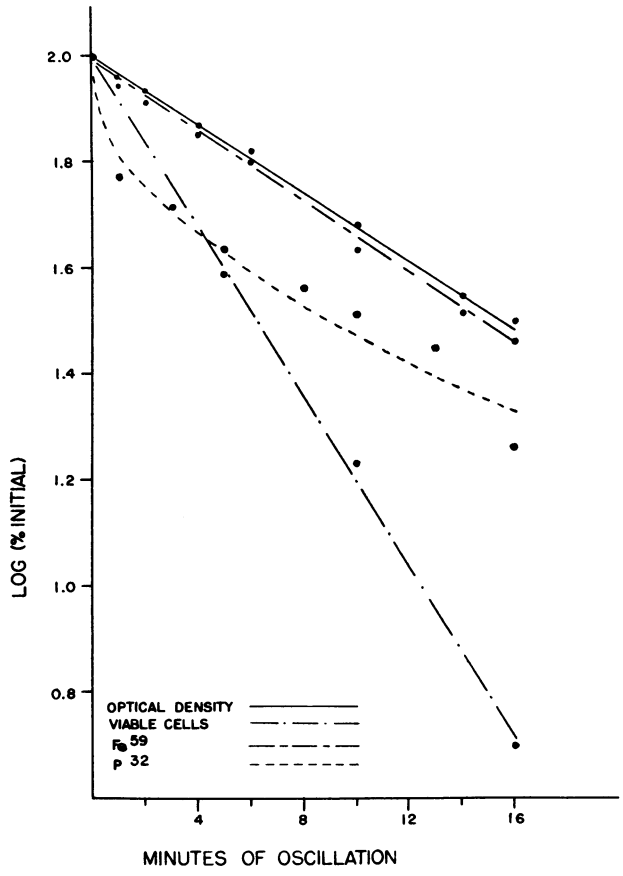

Figure 4. The logarithm of the percentage of the total incorporated P^{32} and Ca^{45} which remained in solution after sedimentation at 10,000 × *g*, and of the percentage remaining of both total viable count and optical density at 660 mμ plotted against the length of time of oscillation of the *Listeria monocytogenes* suspension.

density curve, showing that the iron was present in the wall fraction.

Although data are not available on the proportion of Ca^{45} not sedimented at 10,000 × *g* in 30 min for specific time increments during oscillation, one would expect a kinetic plot of such data to describe an exponential curve which coincided with optical density. Such an assumption seems justified on the basis of the very high specific activity of calcium in the wall fraction and the high proportion of the total calcium of cell which is found in this fraction (table 4).

Monocyte production by fractions. All but one of the 15 mice which received whole cells died within the first 2 postexposure days. Death occurred before postexposure blood films were prepared. At necropsy most mice showed early changes characteristic for listeric septicemia: swollen, yellow-colored livers with or without early focal necrosis and swollen spleen. *L. monocytogenes* was isolated on primary culture from all these animals. The one mouse that survived showed no indication of illness. On the 3rd postexposure day it showed 23 per cent monocytes in the circulating blood. When it was killed the following day there were no gross lesions, but *L. monocytogenes* was isolated from the liver on primary culture.

Of the 15 mice inoculated with wall preparation, one died on the 2nd postexposure day. This was thought to be due to injury during inoculation since there were no gross lesions and *L. monocytogenes* could not be isolated. All surviving mice remained asymptomatic. However, there was a wide variation in the blood picture. Although total leucocyte counts were not made, it

was apparent from the blood films that some mice developed a marked leucocytosis within the first few days with a striking increase in the number of polymorphonuclear cells. During the 10-day observation period some mice showed from 17 to 24 per cent monocytes in the circulating blood, while others showed only slight or no alteration in the monocyte number. These mice were killed at intervals between the 4th and 10th postexposure days. Four showed small necrotic foci on either the liver or spleen. *L. monocytogenes* was isolated on primary culture from all but one of these. After only 2 days refrigeration, the bacterium was also isolated from the latter. Although none of the other mice receiving wall preparation showed gross lesions at necropsy, *L. monocytogenes* was isolated on primary culture from all but two. The viscera of these two animals remained sterile during a 3-month refrigeration period. These findings suggest that all mice in these groups received at least a few viable cells together with the wall material but that the number was too low to initiate symptoms of illness.

None of the 15 mice receiving protoplasmic preparations died. In general, changes in the blood picture were similar to those in mice given wall preparations, but not nearly so striking. The monocytes ranged from 4 to 17 per cent. When these mice were killed only one showed necrotic foci on the liver and *L. monocytogenes* was isolated from it on primary culture. *L. monocytogenes* was isolated from two other mice on primary culture and from one after 2 days refrigeration. It was not isolated during a 3-month period of refrigeration from any of the other mice receiving the protoplasmic preparation.

All control mice showed from 0 to 4 per cent monocytes during the 10-day observation period.

These results indicate that in the amounts given, the terramycin did not prevent multiplication of *L. monocytogenes*. There was no detectable difference between the reactions of the mice receiving the terramycin-treated preparations, those treated with terramycin at the time of exposure, and those which received no antibiotic. The results showed that some viable cells were retained in both wall and protoplasmic preparations, but the number was significantly higher in the wall preparation. However, in neither instance was the number sufficiently high to initiate active infection.

Although somewhat limited, these results suggest that the monocyte-producing fraction of *L. monocytogenes* is contained in either the cell wall or membrane. This is supported by the findings of Stanley (1949, 1950) who demonstrated on the basis of extractability with lipid solvents that the monocyte-producing factor is apparently of lipoidal nature. Although lipids were not determined in the present study, the wall or membrane of many cells contain relatively large amounts of lipid material.

It seems unlikely that the few remaining viable cells were responsible for the monocytosis seen in the mice that were given cell wall material. It is well established that sublethal doses of *L. monocytogenes* seldom elicit a marked monocytosis. The enhanced monocyte production seen in the mice given protoplasmic preparations may have been due to wall material which became comminuted to a degree that it did not sediment with the large wall fragments.

The monocyte-producing ability of *L. monocytogenes* has been something of a laboratory curiosity or a relatively simple means of identifying the bacterium. However, Stanley (1949, 1950) and Girard and Murray (1954) found that in rabbits with a sustained monocytosis, antibody production against several different antigens including *Salmonella typhimurium* (Stanley, 1950), *S. typhosa*, staphylococcus toxoid, and horse serum (Girard and Murray, 1954) was greatly enhanced. They suggested that the monocyte may play an important part in antibody transport and storage. For continuation of these studies a simple method for obtaining the monocyte-producing factor of *L. monocytogenes* would be most desirable.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Jean Martin for her help in culturing the organisms and her help with the mice used for monocyte production. Thanks are also due to Mr. Don Fritts for the drawing and some of the photographic work.

SUMMARY

A method is described for the preparation of cell wall material from *Listeria monocytogenes* by sonic oscillation and differential centrifugation. It is estimated that 30 to 50 per cent of the total cell wall material can be recovered in this way. The chemical composition of the washed cell wall material has been partially determined. It is

found to contain about 20 per cent hexose represented by glucose and galactose which are present in equal concentrations. It also contains about 5 per cent hexosamine. About 50 per cent of the wall material is positive as Kjeldahl protein although only the following five amino acids at the relative concentrations indicated are present: alanine (+++), glutamic acid (++), α,ϵ-diaminopimelic acid (++), aspartic acid (+), and leucine (+). The other 25 per cent of the wall is as yet unaccounted for quantitatively. It is shown, however, that the washed wall material contains at least 5 per cent of the phosphorus, 15 per cent of the iron, and 36 per cent of the calcium content of the entire cell, and may contain as much as twice these values since only 30 to 50 per cent of the wall material is apparently recovered. Kinetic plots of the release of iron and phosphorus during cell breakage show that the former originates in the cell wall fraction, while the latter comes from both cell wall and protoplasm. The results with iron suggest that the cell wall fraction contains adhering cytoplasmic membrane material. Ultraviolet spectra of washed wall preparations reveal no material absorbing at 260 mμ, suggesting the absence of nucleic acid in wall preparations. Preliminary results indicate that the cell wall or membrane may be the cytological location of the monocyte-producing factor. The usefulness of the cell wall fraction in antigen characterization of *L. monocytogenes* is discussed.

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