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Citrate Cycle and Related Metabolism of *Listeria monocytogenes*

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The growth response of *Listeria monocytogenes* strains A4413 and 9037-7 to carbohydrates was determined in a defined medium. Neither pyruvate, acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, nor malate supported growth. Furthermore, inclusion of any of these carbohydrates in the growth medium with glucose did not increase the growth of *Listeria* over that observed on glucose alone. Resting cell suspensions of strain A4413 oxidized pyruvate but not acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, or malate. Cell-free extracts of strain A4413 contained active citrate synthase, aconitate hydratase, isocitrate dehydrogenase, malate dehydrogenase, fumarate hydratase, fumarate reductase, pyruvate dehydrogenase system, and oxidases for reduced nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide phosphate. The α-ketoglutarate oxidation system, succinate dehydrogenase, isocitrate lyase, and malate synthase were not detected. Cytochromes were not detected. The data suggest that strain A4413, under these conditions, utilizes a split noncyclic citrate pathway which has an oxidative portion (citrate synthase, aconitate hydratase, and isocitrate dehydrogenase) and a reductive portion (malate dehydrogenase, fumarate hydratase, and fumarate reductase). This pathway is probably important in biosynthesis but not for a net gain in energy.

Carbohydrate is essential for *Listeria* growth, and glucose is commonly used to meet this demand for carbon and energy (39; Miller and Silverman, Bacteriol, Proc., p. 103, 1959). Under aerobic or anaerobic conditions, the degradation of glucose is essentially homofermentative (Miller and Silverman, Bacteriol. Proc., p. 103, 1959). It has been shown, however, that lactate and pyruvate account for a small fraction of the glucose oxidized by *Listeria* grown on a low (0.2%) glucose concentration (12).

Any role which the citrate cycle and glyoxylate bypass may play in *Listeria* metabolism has received little study. It has been reported that pyruvate (12) and citrate (13) are unacceptable carbon and energy sources. Pyruvate (12) as well as malate, succinate, and α-ketoglutarate are oxidized at low rates by a type 1 *Listeria* (23). In synthetic media, *Listeria* growth does not require glutamate or aspartate (14), amino acids which commonly arise by the transamination of α-ketoglutarate and oxaloacetate of the citrate cycle (31). Since these bacteria contain considerable quantities of glutamate and aspartate (15), they are obviously capable of efficiently synthesizing them. Glutamic oxaloacetic transaminase activity has been detected in many strains of *Listeria* (37). If *Listeria* cells employ the common transaminative pathways to glutamate and aspartate, citrate cycle activity would be expected for the biosynthesis of carbon skeletons, if not for the release of energy.

The purpose of this research was to determine whether *Listeria* possesses the enzymatic facilities for the oxidation of pyruvate, the citrate cycle, and the glyoxylate bypass. Three experimental approaches were employed: growth, oxidation by resting cells, and an enzymatic analysis of cell-free extracts. *Listeria* was tested for its capacity to utilize either pyruvate or citrate cycle intermediates as a source of carbon and energy; the growth response was determined by using these compounds alone or with glucose. The oxidative response of resting cells was then determined manometrically. Finally, cell-free extracts of *Listeria* were assayed for the enzymes which catalyze pyruvate oxidation, the citrate cycle, and the glyoxylate bypass.

[1] This paper is taken from a dissertation presented to the Department of Microbiology and the Graduate Division of the University of Oregon Medical School by Terrence L. Trivett in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Biology, Pacific Union College, Angwin, Calif. 94508.

MATERIALS AND METHODS

Special chemicals. L-Cysteine-hydrochloride; L-leucine; DL-isoleucine; DL-valine; L-arginine-hydrochloride; L-histidine-hydrochloride; DL-methionine; DL-biotin; succinate, sodium; DL-isocitrate, trisodium; L-malic acid; and potassium fumarate were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Nicotinamide adenine dinucleotide (NAD); nicotinamide adenine dinucleotide phosphate (NADP), sodium; reduced nicotinamide adenine dinucleotide (NADH), disodium; reduced nicotinamide adenine dinucleotide phosphate (NADPH), tetrasodium; coenzyme A, trilithium; *S*-acetyl coenzyme A (acetyl-CoA), sodium; diphosphothiamine (DPT); flavine adenine dinucleotide (FAD); glyoxylate, sodium; pyruvate, sodium; α-ketoglutaric acid; crystalline bovine serum albumin; phenazine methosulfate (PMS); 2,6-dichlorophenol indophenol (DCPIP), sodium; tris(hydroxymethyl)aminomethane (Tris); and imidazole were purchased from Sigma Chemical Co., St. Louis, Mo. Reduced glutathione and nitrilotriacetic acid were obtained from Calbiochem, Los Angeles, Calif. Protamine sulfate was purchased from Krishell Laboratories, Portland, Ore. Flavine mononucleotide (FMN) was a gift from Sigma Chemical Co., St. Louis, Mo.

Bacteria. *L. monocytogenes* strains A4413 (smooth, virulent) and 9037-7 (rough, avirulent) were kindly supplied by M. E. Friedman, U.S. Army Biological Laboratories, Fort Detrick, Md. Strain A4413 was employed in all phases of this study, whereas strain 9037-7 was used only in the determination of carbohydrates acceptable for growth. Stock cultures were routinely grown for 16 hr at 37 C on Tryptose Agar (Difco) slants, inoculated to give confluent growth. After quick freezing in an ethanol-dry ice bath, the slants were tightly capped and stored at −20 C for up to 6 months. *Escherichia coli* K-12(λ), graciously furnished by E. L. Oginsky, University of Oregon Medical School, was used in some experiments as a positive control.

Synthetic growth medium. *Listeria* cells were grown on a defined medium, designated D10. It consisted of the following components per liter: K_2HPO_4, 8.50 g; $NaH_2PO_4 \cdot H_2O$, 1.50 g; NH_4Cl, 0.50 g; $MgSO_4 \cdot 7H_2O$, 0.41 g; $FeCl_3 \cdot 6H_2O$, 0.048 g; NaOH, 0.24 g; nitrilotriacetic acid, 0.48 g; L-cysteine-hydrochloride, 100 mg; L-leucine, 100 mg; DL-isoleucine, 200 mg; DL-valine, 200 mg; DL-methionine, 200 mg; L-arginine-hydrochloride, 200 mg; L-histidine-hydrochloride, 200 mg; riboflavine, 1,000 μg; thiamine-hydrochloride, 1,000 μg; D-biotin, 100 μg; α-lipoic acid, 1 μg.

Medium D10 was assembled as follows to prevent the formation of precipitates and to provide for the sterilization of the vitamins by filtration. (i) K_2HPO_4 and NH_4Cl were dissolved in 500 ml of distilled water; (ii) NaOH and then nitrilotriacetic acid were dissolved in 40 ml of distilled water; (iii) $FeCl_3$ was dissolved in 40 ml of distilled water (this $FeCl_3$ solution must be fresh); (iv) the $FeCl_3$ solution was mixed with the sodium nitrilotriacetate solution; (v) $MgSO_4$ was dissolved in 30 ml of distilled water; (vi) the solutions from steps iv and v, the amino acids, and 390 ml of distilled water were added to the solution from step i to give a volume of 1,000 ml which was autoclaved at 15 psi for 15 min. When cool, 10 ml of vitamin solution (100× concentrated) was added. The *p*H of medium D10 was 7.3 to 7.4. The 100× vitamin solution was prepared as follows. A 5-mg amount of α-lipoic acid was dissolved in 200 ml of 70% ethanol. Two milliliters of this solution was combined with 5 mg of biotin, 50 mg of thiamine, and 50 mg of riboflavine in 125 ml of 95% ethanol, after which the volume was brought to 500 ml with distilled water; the solution was filtered through a membrane filter (0.45 μm, Millipore Corp., Bedford, Mass.) because thiamine is heat labile (14). For routine *Listeria* culture, medium D10 was supplemented with glucose; 10 ml of a 20% solution (sterilized by autoclaving) was added to give a final concentration of 0.2%. The vitamin and glucose solutions (concentrated 100×) replaced the fluid lost in autoclaving (about 2%). The amino acids in medium D10 are among those shown to be required or stimulatory for *Listeria* by Friedman and Roessler (14). The vitamins used were as reported by Welshimer (43). Iron is very stimulatory for *Listeria*, as shown by Sword (38), and was included with partially neutralized nitrilotriacetic acid for its chelation (19). Mn^{2+} was excluded because Sword (38) found it to be inhibitory. NH_4^+ was included because Friedman and Roessler (14) found that it could substitute for glutamine in exclusion studies. With reference to its complement of amino acids (seven), medium D10 is thought to be simpler than any medium yet described for the cultivation of *Listeria*.

Ability of various carbohydrates to support Listeria growth. Bacterial growth was measured turbidimetrically at 620 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Optically similar Pyrex tubes (18 by 150 mm) were filled with medium D10, a carbohydrate supplement, and inoculum to a volume of 6 ml. Cultures were incubated at 37 C with or without agitation (Eberbach and Sons, Ann Arbor, Mich.). Carbohydrate supplements included solutions of the sodium salts of pyruvate, acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, or malate which were filtered through membrane filters (0.45 μm) and added to the culture tube to provide a final concentration of 0.2%. Each tube also received a inoculum of about 3×10^8 cells (strain A4413) or about 3×10^7 cells (strain 9037-7). The inoculum was grown on medium D10 + 0.5% glucose for 10 hr at 37 C; the cells were harvested by cold centrifugation at 3,500 × *g* for 15 min in a centrifuge (model RC-2; Ivan Sorvall, Inc., Norwalk, Conn.). All centrifugation in this study was performed in the RC-2. Harvested bacteria were washed once in cold 0.1 M potassium phosphate buffer, *p*H 7.2, and resuspended in this buffer to the turbidity required to provide the desired viable count (*unpublished data*). Carbohydrate-free and glucose controls were included. The effect of iron exclusion and of agitation upon the growth response in medium D10 + glucose was determined by similar methods. In this case, the inoculum consisted of unwashed bacteria grown in the synthetic medium of Welshimer (43) which lacks iron.

Resting cells. Bacteria were grown in 500-ml Erlenmeyer flasks containing 200 ml of medium D10 + 0.2% glucose. Growth was initiated by 0.5 ml of a sus-

pension of cells which were harvested from a freshly thawed stock slant culture by the addition of 0.6 ml of distilled water and the gentle removal and distribution of the slant's growth with a 1-ml pipette. Flask cultures were incubated at 37 C in a gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at medium speed. After 13 hr, each 200 ml culture was harvested in the late-exponential phase of growth by cold centrifugation in a 250-ml centrifuge bottle (Nalge Co., Rochester, N.Y.) at 6,000 × *g* for 15 min. The cell pellet was washed once in cold 0.05 M potassium phosphate buffer (*p*H 7.0) and then resuspended in sufficient buffer to provide the turbidity associated with the level of cellular nitrogen desired (*unpublished data*), as previously determined by a Nesslerization procedure (5).

Cell-free extracts. When destined for disruption, each washed pellet of resting cells was resuspended in 5 ml of cold 0.005 M potassium phosphate buffer (*p*H 7.0). Ordinarily, two resuspended pellets and 10 ml of 0.11-mm glass beads (Bronwill Scientific Co., Inc., Rochester, N.Y.) were placed in a precooled 50-ml stainless-steel flask (Bronwill) with a screw cap. Vaseline was smeared lightly on the threads to prevent leakage. The cells were broken by shaking the flask in a Braun cell homogenizer, model MSK (Bronwill) for 45 sec. The flask was cooled by a stream of CO_2 delivered by a metal capillary (Bronwill) at a micrometer valve (Bronwill) setting of 1. The cell extract debris (≃3 ml) and 10 ml of cold 0.005 M potassium phosphate buffer (*p*H 7.0), used to rinse the flask, were centrifuged cold at 5,000 × *g* for 5 min. The supernatant fluid was centrifuged cold at 5,000 × *g* for 10 min. The supernatant fluid, termed crude extract (CE), was translucent and a faint brownish-yellow color; upon staining with crystal violet, numerous subcellular fragments, but no intact bacteria, were observed microscopically. The *p*H of CE was 7.0. It was found that cell disruption for 90 sec or 180 sec produced little increase in CE protein, although the cooling of the flask was difficult as compared with the 45-sec disruption. The 45-sec disruption time was, therefore, used routinely in these experiments. About 90% of the bacteria were disrupted by this treatment. Approximately 30 mg of CE protein was recovered from each 200 ml of culture. Protein was estimated by a biuret method (28) using bovine serum albumin as standard. To minimize its absorbance in the ultraviolet region, CE was treated in the cold with protamine sulfate (1%, *p*H 6) added dropwise with agitation; 1 mg of protamine sulfate was used for each 3 to 5 mg of CE protein. After standing for 5 min, the mixture was centrifuged cold at 10,000 × *g* for 10 min. This treatment coprecipitated some 30 to 40% of the CE protein. The supernatant fluid was termed protamine-treated extract (PTE); its *p*H was also 7.0.

Manometric methods. Oxygen consumption was measured in an atmosphere of air at 37 C in a conventional Warburg apparatus (Precision Scientific Co., Chicago, Ill.), essentially as described by Umbreit et al. (40). The total volume of all reaction mixtures using cells or extracts was 3.0 ml, including 0.1 ml of 10% KOH in the center well.

Oxidation by resting cells. For determining the oxidation of pyruvate, the reaction mixture contained potassium phosphate buffer (pH 5.0), 200 μmoles; sodium pyruvate, 10 μmoles; coenzyme supplements singly or together in the following amounts (in μmoles), DPT (1), FAD (0.5), CoA (0.5), NAD (0.5), α-lipoate (0.5), $MgCl_2$ (1), and bacteria equivalent to 1.8 mg of nitrogen. For determining the oxidation of acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, and malate, the reaction mixture contained potassium phosphate buffer, *p*H indicated, 200 μmoles; substrate, 10 μmoles; coenzyme supplements (in μmoles), DPT (0.5), CoA (0.1), NAD (1), NADP (1), α-lipoate (0.1), $MgCl_2$ (1), and L-cysteine (1); and bacteria equivalent to 1.8 mg of nitrogen.

Enzyme assays. Absorbancy changes were determined by using 1-cm standard silica cuvettes (Beckman Instruments, Inc., Fullerton, Calif.) in a spectrophotometer (model PMQ II; Carl Zeiss Oberkochen/Württ, West Germany) at room temperature. The following extinction coefficients were used in the calculation of specific activities: cis-aconitate, 3.5 $mM^{-1}cm^{-1}$ (33); fumarate, 2.1 $mM^{-1}cm^{-1}$ (33); NADH and NADPH, 6.2 $mM^{-1}cm^{-1}$ (21); DCPIP, 19.1 $mM^{-1}cm^{-1}$ (4); FMN and FAD, 12.4 $mM^{-1}cm^{-1}$ (18); glyoxylate phenylhydrazone, 16.8 $mM^{-1}cm^{-1}$ (24); acetyl-CoA, 4.5 $mM^{-1}cm^{-1}$ (8). Specific activities are expressed as the number of micromoles of substrate or product transformed or of oxygen consumed per hour per milligram of protein at room temperature unless otherwise indicated. The specific activities are based on the initial rate under optimal conditions as far as these were determined. The reaction mixture given for each method provided approximately optimal conditions for enzyme activity. The effects of alterations in the reaction mixture are noted below. The concentration of substrate required to give half maximal velocity was estimated by the method of Lineweaver and Burk (29). When enzyme activity was not detected in cell-free extracts of *Listeria*, cell-free extracts of *E. coli*, which had been grown, harvested, washed, and disrupted similarly, were used as a positive control for the method.

The pyruvate or α-ketoglutarate oxidation systems were detected manometrically at 37 C. Included in the reaction mixture were CE of strain A4413, 6.6 mg of protein, and (in μmoles), potassium phosphate buffer, *p*H 7.0 (200), sodium pyruvate or sodium α-ketoglutarate (20), NAD (1), DPT (0.5), CoA (0.1), α-lipoate (0.1), L-cysteine (1), and $MgCl_2$ (1).

Citrate synthase (EC 4.1.3.7) was estimated by the chemical determination of citrate, an approach similar to that of Englesberg and Levy (11). The reaction mixture of 2.5 ml in soft rubber-stoppered test tubes (4 inches by ⅝ inch) included PTE of strain A4413, 2.16 mg of protein, and (in μmoles) potassium phosphate buffer, *p*H 7.5, (25) $MgCl_2$ (1), L-cysteine (0.5), acetyl-CoA (1.5), and sodium oxaloacetate (20). Each tube was flushed with nitrogen and incubated at 37 C. At 15 and 30 min, the reactions were stopped by the addition of 2.5 ml of 1.5% trichloroacetic acid, and filtrates were boiled for 15 min to decompose residual oxaloacetate which interferes with the citrate determination (26). Citrate in the filtrates and in standard citrate solutions was estimated by subjecting 1-ml samples to the procedure of Lane and Chen (27) which

combines sensitivity ($\cong 1$ μg) with relative simplicity.

Aconitate hydratase (EC 4.2.1.3) was determined by the method of Racker (33). The increase in absorbancy at 240 nm due to aconitate formation was observed against a reference which lacked substrate. The reaction mixture (3 ml) included PTE of strain A4413, 0.5 mg of protein, and (in μmoles) potassium phosphate buffer, *p*H 7.5 (300), sodium isocitrate (20), or sodium citrate (90).

Isocitrate dehydrogenase (NADP) (EC 1.1.1.42) was measured by a modification of the method of Kornberg (25). The reduction of NADP was followed at 340 nm against a reference lacking substrate. The reaction mixture (3 ml) included PTE of strain A4413, 1.0 mg of protein, and (in μmoles) potassium phosphate buffer, *p*H 7.5 (200), NADP (0.5), sodium isocitrate (30), and $MnCl_2$ (1).

Succinate dehydrogenase (EC 1.3.99.1) was estimated by the Arrigoni and Singer (2) modification of the Ells method (10). The PMS-mediated reduction of DCPIP was monitored at 600 nm against a water reference. The reaction mixture (3 ml) included the following components added in order: potassium phosphate buffer, *p*H 6.5, 100 μmoles; sodium succinate, 60 μmoles; CE of *Listeria* A4413 or *E. coli*, 1.1 mg of protein; KCN adjusted to *p*H 8, 3 μmoles, mixed with the other components by bubbling nitrogen; after allowing the mixture to stand 5 min, the DCPIP, 0.083 μmoles, and PMS, 1 mg, were quickly added, and the final mixture was mixed with bubbling nitrogen.

Fumarate reductase was estimated by the method of Hirsch et al. (18). The fumarate-dependent oxidation of $FMNH_2$ was observed at 450 nm against a water reference. An anaerobic cuvette was required. This was constructed by fusing a 1-cm Beckman standard silica cell to a double sidearm by means of a ground-glass joint. The reaction mixture (2 ml) was added as follows: potassium phosphate buffer (*p*H 7.0), 100 μmoles, and FMN, 0.5 μmole, in the cuvette; sodium fumarate, 20 μmoles, and CE of strain A4413, 0.86 mg of protein, were placed in separate sidearms. The cuvette was twice evacuated (Cenco Hypervac 4, Central Scientific Co., Chicago, Ill.) and flushed with nitrogen; it was then opened, and about 0.3 mg of crystalline sodium hydrosulfite was added and mixed briefly with nitrogen to reduce the FMN. The cuvette was again closed, twice evacuated and flushed with nitrogen, and then evacuated. The fumarate and CE were tipped into the main chamber to initiate the reaction.

Fumarate hydratase (EC 4.2.1.2) was determined by the method of Racker (33). The increase in absorbancy at 240 nm due to fumarate formation was observed against a reference lacking only malate. The reaction mixture (3 ml) contained PTE of strain A4413, 0.5 mg of protein; Tris-hydrochloride buffer (*p*H 8.0), 200 μmoles; and sodium malate, 30 μmoles.

Malate dehydrogenase (EC 1.1.1.37) was detected essentially by the method of Mehler (32). The reduction of NAD was followed at 340 nm against a reference lacking only malate. The reaction mixture (3 ml) included PTE of strain A4413, 1.0 mg of protein, and (in μmoles) glycine-NaOH buffer, *p*H 9.0 (100), sodium malate (60), NAD (0.5), and $MnCl_2$ (1).

Isocitrate lyase (EC 4.1.3.1) was estimated by the method of Kornberg (24). The formation of glyoxylate phenylhydrazone was followed at 324 nm against a reference lacking only isocitrate. Before the actual assay, certain components were premixed to give "R_{im}": 1.0 ml of 0.5 M imidazole buffer (*p*H 6.4, 6.8, or 7.2); 1.0 ml of 0.1 M $MgCl_2$; 0.2 ml of 1 M ethylenediaminetetraacetic acid; 0.8 ml of 0.1 M phenylhydrazine-hydrochloride (preneutralized), and 1.0 ml of distilled water. R_{im} was used fresh or for 2 hr if kept on ice. The reaction mixture (2 ml) contained R_{im}, 0.4 ml; reduced glutathione (preneutralized), 5 μmoles; sodium isocitrate, 2 μmoles; and CE of strain A4413, 1.29 mg of protein, or CE of *E. coli*, 0.75 mg of protein.

Malate synthase (EC 4.1.3.2) was detected by the method of Dixon and Kornberg (8) as modified by Dixon (9) to avoid the use of phosphate. The glyoxylate-dependent cleavage of acetyl-CoA was followed at 232 nm against a reference lacking only glyoxylate and which was set to read 0.50 against water. Cell-free extracts were prepared as previously indicated, except that Tris-hydrochloride buffer supplemented with mM $MgCl_2$ replaced potassium phosphate buffer. The reaction mixture (2 ml) contained PTE of strain A4413, 0.52 mg of protein, or of *E. coli*, 0.58 mg of protein; and (in μmoles), Tris-hydrochloride buffer, *p*H 7.5 (100), $MgCl_2$ (2), acetyl-CoA (0.1), and sodium glyoxylate (1).

The oxidation of NADH or NADPH was measured at 340 nm against a water reference. The reaction mixture (3 ml) contained PTE of strain A4413, 0.25 mg of protein; potassium phosphate buffer, *p*H 7.0 (for NADH) or 6.0 (for NADPH), 200 μmoles; and NADH, 0.5 μmole, or NADPH, 0.375 μmole.

A recording spectrophotometer (model 14; Cary Instruments, Monrovia, Calif.) was used to examine a dense suspension (0.5 mg of nitrogen/ml) and CE (4.2 mg of protein/ml) of strain A4413 for cytochromes by the difference spectra technique (6). Sodium hydrosulfite was added before determining the reduced spectra.

RESULTS

Suitability of synthetic medium D10. Medium D10 + 0.5% glucose supported vigorous growth of *L. monocytogenes* strains A4413 and 9037-7. With respect to strain A4413, population levels of at least 4×10^9 viable bacteria/ml were attained in less than 18 hr at 37 C. The bacteria grew poorly without iron. The extent of growth of strains A4413 and 9037-7 was reduced by two-thirds when iron (as $FeCl_3$) was excluded from medium D10. Doubling the iron content of the medium did not induce more rapid or extensive growth. Shaking increased the rate and extent of growth, but not if iron was excluded.

Inability of pyruvate or citrate cycle intermediates to serve as a source of carbon and energy for strains A4413 and 9037-7. It was of great interest to determine the ability of pyruvate or individual citrate intermediates to support growth. If one or more intermediates supported growth, evidence would be furnished for an active citrate cycle. Furthermore, if an intermediate was acceptable to the bacteria, growth in its presence would be

expected to produce bacteria with higher levels of citrate cycle enzymes than glucose-grown cells, as exemplified in other bacteria (16). Growth was measured turbidimetrically for 48 hr. In two experiments, each conducted in duplicate with and without agitation, strains A4413 and 9037-7 showed no growth on medium D10 containing acetate, pyruvate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, or malate. Controls containing glucose showed excellent growth.

Inability of pyruvate or citrate cycle intermediates to stimulate the growth of strains A4413 and 9037-7 on medium D10 + 0.05% glucose. Because *Listeria* was apparently unable to utilize pyruvate or any citrate cycle intermediate as a sole source of carbon and energy, growth experiments were conducted which used medium D10 containing 0.05% glucose. This glucose content supports definite yet limited growth. Therefore, a growth increase in response to added pyruvate or citrate cycle intermediates would be readily detectable. Such an experimental design was in harmony with the finding that bacteria such as *E. coli* may require an auxiliary carbon source to grow on intermediates such as citrate (41). These experimental results are presented in Table 1. The maximal growth attained by either *Listeria* strain was not significantly different from the controls. These growth experiments indicate that pyruvate, acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, and malate were apparently unacceptable carbon and energy sources for these *Listeria* strains, with or without glucose. Glucose was therefore used routinely to meet the needs of these bacteria for carbohydrate. Since *Listeria* cells grown on high concentrations (0.5 to 1.0%) of glucose show less capacity to oxidize glucose (and possibly other substrates) than those grown on lower (0.2%) concentrations (12), medium D10 was supplemented with 0.2% glucose for the growth of all bacteria to be used in manometric or cell-free experiments.

TABLE 1. *Inability of pyruvate or citrate cycle intermediates to stimulate the crop of Listeria strains A4413 and 9037-7 in medium D10 + 0.05% glucose*[a]

Supplement (0.2%)	Incubation			
	Stationary		Agitation	
	A4413	9037-7	A4413	9037-7
None	0.17	0.16	0.18	0.18
Citrate	0.16	0.16	0.18	0.18
Isocitrate	0.15	0.16	0.16	0.18
α-Ketoglutarate	0.16	0.16	0.17	0.19
Succinate	0.16	0.16	0.17	0.18
Fumarate	0.16	0.19	0.19	0.20
Malate	0.16	0.16	0.17	0.19
Pyruvate	0.16	0.16	0.18	0.19
Acetate	0.16	0.15	0.20	0.18

[a] Each value represents the highest optical density measurement (at 620 nm) observed (after correction for the initial reading at inoculation) throughout the incubation period at 37 C; measurements were made at 0, 8, 16, and 24 hr. Each value is the mean from two experiments, each conducted in duplicate.

Oxidation of pyruvate by resting bacteria. Resting *Listeria* A4413 cells oxidized pyruvate as shown in Fig. 1. The oxidation of pyruvate was not promoted by DPT, FAD, or Mg^{2+}, and only slightly be CoA, NAD, or Mn^{2+}. Oxidation was increased considerably by α-lipoate, however, and even more by the coenzyme mixture. The $Q(O_2)$ N (microliters of oxygen consumed per hour per milligram of nitrogen) was increased from 120 (unsupplemented) to 220 by α-lipoate. The oxidation ratio (moles of oxygen per mole of substrate) was about 0.55, suggesting that pyruvate had been oxidized to acetate. Resting cells oxidized pyruvate more rapidly in potassium phosphate buffer at *p*H 5.0 than at *p*H 5.5, 6.0, 6.5, or in phthalate-NaOH buffer at *p*H 5.0 or 4.5. No oxidation occurred in potassium phosphate buffer at *p*H 7.0 or 8.0, or in phthalate-NaOH buffer at *p*H 4.0.

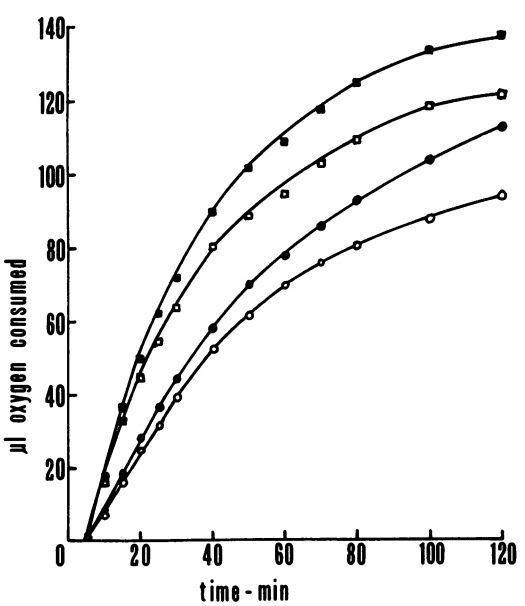

FIG. 1. *Oxidation of pyruvate by resting cells of L. monocytogenes strain A4413 at pH 5 and the effect of coenzyme supplements. Endogenous oxidation was insignificant. Coenzymes were included in the reaction mixture according to the following legend: either DPT, FAD, or Mg^{2+}, or no coenzymes, ○; CoA, NAD, or Mn^{2+}, ●; α-lipoate, □; coenzyme mixture, ■.*

Oxidation of citrate cycle intermediates by resting bacteria. The effect of various citrate cycle intermediates on the oxygen consumption by resting cells of strain A4413 is shown in Fig. 2. With reference to the endogenous oxygen consumption, there was no apparent stimulation by acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, or malate. This nonstimulatory effect was observed at *p*H 6.0, 7.0, and 8.0, with or without the coenzyme mixture.

Enzyme assays with cell-free extracts: the pyruvate oxidation system. Since resting cells oxidized pyruvate, it was not surprising that a cell-free extract was capable of doing so (Fig. 3). Pyruvate was oxidized most rapidly at *p*H 7.0, yielding a specific activity of 2.11. Except for Mg^{2+}, all coenzymes and cysteine were required for maximal oxidation. No oxidation occurred without the coenzymes and cysteine. The exclusion of DPT, CoA, NAD, α-lipoate, or cysteine, in increasing order of effect, significantly decreased the oxidation rate. Without α-lipoate, for example, the rate of oxidation decreased about 70%. The oxidation ratio of 0.47 suggests, as did the oxidation by resting cells, that pyruvate was oxidized to the level of acetate.

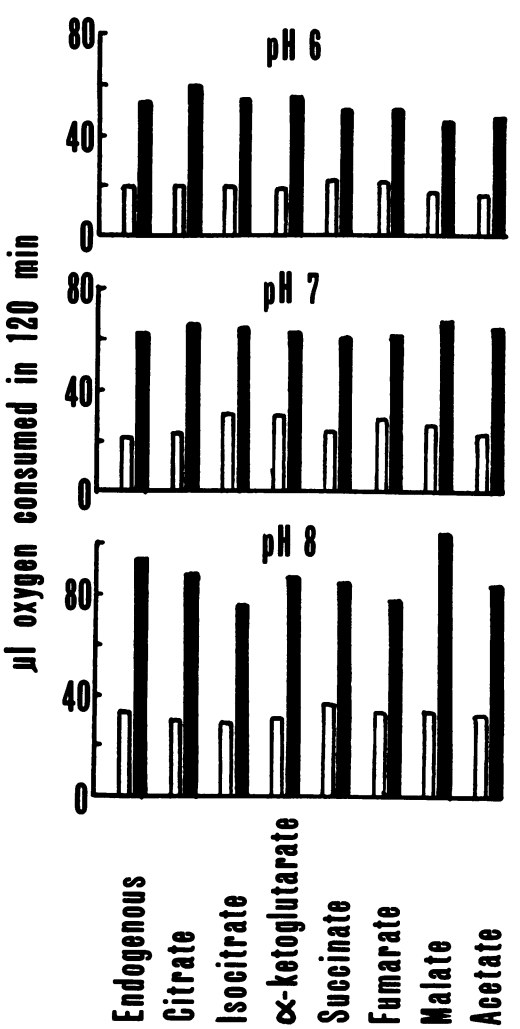

FIG. 2. *Oxidation of citrate cycle intermediates by resting cells of L. monocytogenes strain A4413 at pH 6.0, 7.0, or 8.0. Legend: open columns, without coenzyme mixture; closed columns, with coenzyme mixture.*

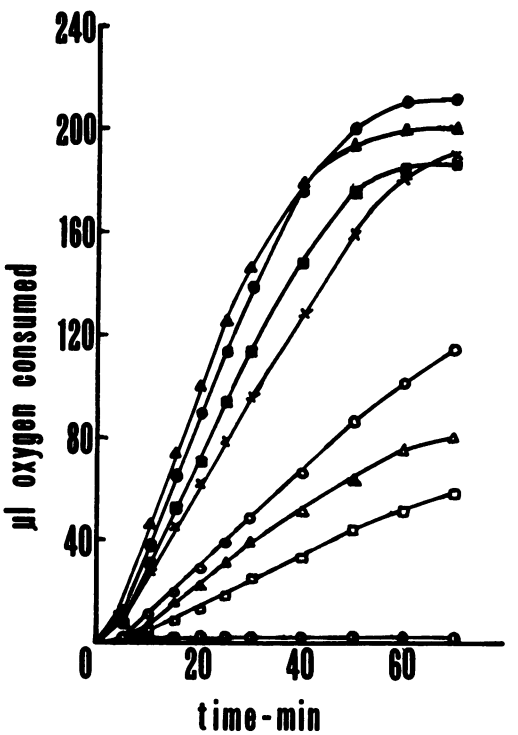

FIG. 3. *Oxidation of pyruvate by a crude extract of L. monocytogenes strain A4413 in potassium phosphate buffer at pH 7.0 and the effect of individual coenzyme deletions. No oxidation occurred without pyruvate. Legend: coenzyme mixture, ●; minus Mg^{2+}, ▲; minus DPT, ■; minus CoA, ×; minus NAD, ○; minus α-lipoate, △; minus cysteine, □; no coenzymes, ◑.*

Enzymes of and related to the citrate cycle. The results of assays for enzymes of the citrate cycle and some enzymes closely related to it are summarized in Table 2. For those enzymes not detected in strain A4413, specific activities of *E. coli* extracts used as positive controls are presented in the right hand column of Table 2. For those enzymes found in strain A4413, specific activities of *E. coli* enzymes from other studies are presented for comparison. Citrate synthase,

TABLE 2. *Enzyme activities in cell-free extracts of L. monocytogenes strain A4413 and some activities of E. coli enzymes for comparison*

Enzyme	*L. monocytogenes*				*E. coli*	
	*p*H[a]	Coenzyme requirement[b]	K_m[c]	Specific activity[d]	Specific activity[d]	Reference
Pyruvate oxidation system	7.0	DPT, CoA, NAD, α-lipoate	ND[e]	2.11	0.44[f]	17
Citrate synthase	ND	ND	ND	0.68	0.63[g], 3.08[h]	16
Aconitate hydratase	7.5	ND	0.80 (citrate) 0.44 (isocitrate)	3.33 6.66	0.97[g], 19.1[h]	16
Isocitrate dehydrogenase	7.5	NADP, Mn^{2+}	0.44 (isocitrate)	0.75	8.32[g], 85.3[h]	16
α-Ketoglutarate oxidation system	(5.0-9.0)	ND	ND	NA[i]	0.40[j]	
Succinate dehydrogenase	(6.0-9.0)	ND	ND	NA	0.97[j]	
Fumarate reductase	7.0	$FMNH_2$ or $FADH_2$	ND	2.82	4.56	18
Fumarate hydratase	8.0	ND	1.01 (malate)	2.24	20.6[g], 50.8[h]	16
Malate dehydrogenase	9.0	NAD, Mn^{2+}	4.0 (malate)	0.87	11.0[g], 225[h]	16
Isocitrate lyase	(6.4-8.0)	ND	ND	NA	1.80[j]	
Malate synthase	(7.0-9.6)	ND	ND	NA	4.02[j]	
NADH oxidase	7.0	(not Mg^{2+} or Mn^{2+})	ND	16.20	5.33[g], 38.2[h]	16
NADPH oxidase	6.0	Mn^{2+}	ND	2.30		

[a] *p*H for optimal activity. Parenthetical *p*H values indicate the range over which activity was sought.

[b] Abbreviations: DPT, diphosphothiamine; CoA, coenzyme A; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; $FMNH_2$, reduced flavine mononucleotide; $FADH_2$, reduced flavine adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; and NADPH, reduced nicotinamide adenine dinucleotide phosphate.

[c] Millimolar substrate concentration giving half maximal velocity.

[d] Expressed as micromoles of substrate transformed or oxygen consumed per hour per milligram of protein.

[e] Not determined.

[f] Reaction mixture lacked coenzyme supplements.

[g] Anaerobically grown in synthetic medium with glucose.

[h] Aerobically grown in synthetic medium with glucose.

[i] Not active.

[j] Positive controls in this research.

aconitate hydratase, isocitrate dehydrogenase, fumarate reductase, fumarate hydratase, malate dehydrogenase, and oxidases for NADH and NADPH, but not the α-ketoglutarate oxidation system or succinate dehydrogenase, were detected. The glyoxylate bypass enzymes, isocitrate lyase and malate synthase, were not found. For each enzyme not detected in cell-free extracts of *Listeria* A4413, activity was readily demonstrated by using a cell-free extract from *E. coli* as a control.

Cytochromes. Difference spectra of strain A4413 cells or cell-free extracts did not reveal the presence of cytochromes.

DISCUSSION

In evaluating the adequacy of medium D10 for growing *Listeria*, the effects of iron exclusion and agitation upon growth were noted. Iron stimulated growth with or without agitation, but agitation improved the growth only in the presence of iron. The stimulation of *Listeria* growth by iron has been pointed out by Sword (38). However, the iron dependence of growth stimulation by agitation has not been reported. The bacteria probably require iron in the synthesis of catalase (12), cytochromes, and other enzymes. Although cytochromes were not detected in this or earlier research (22), these pigments have recently been found in strain A4413 and other strains, provided the bacteria are stored for several days in the presence of a reducing agent (J. H. Jackson, Ph.D. Thesis, University of Kansas, 1969).

The thrust of this study was to determine whether *Listeria* possesses the citrate cycle and related metabolism. Many species of bacteria, including *E. coli* (16) and *Pseudomonas fluorescens* (3), are able to grow with pyruvate or a citrate cycle intermediate as the carbon and energy source. Such is not the case with *L. monocytogenes* strains A4413 or 9037-7, however, at least under these experimental conditions. None of the intermediates tested supported measurable growth. When 0.05% glucose was used as an auxiliary carbohydrate, no intermediate stimu-

lated growth. Citrate (13) and pyruvate (12) have been previously shown to be unsatisfactory carbon and energy sources for *Listeria*.

The finding that resting cells oxidized pyruvate most rapidly at *p*H 5.0, whereas cell-free extracts were most active at *p*H 7.0, suggests that pyruvate enters the cell undissociated (as pyruvic acid). Friedman and Alm (12) found that strain A4413 oxidized pyruvate [$Q(O_2)N$ = 17.7 at *p*H 6.5]. The pyruvate oxidation system in cell-free extracts was most active at *p*H 7.0. The dependence of the pyruvate oxidation system upon DPT, CoA, NAD, and α-lipoate suggests that the enzyme system is similar to or identical with the pyruvate dehydrogenase complex of other organisms (35). The oxidation ratios suggest that pyruvate was apparently degraded to the level of acetate (probably as acetyl-CoA).

Since many of the citrate cycle enzymes were demonstrated in cell-free extracts of strain A4413, the inability of whole cells to oxidize or grow on citrate cycle intermediates suggests that these bacteria lack the requisite permeases for transport across the bacterial membrane (7). The inability of strain A4413 to oxidize these intermediates is in essential agreement with a recent report that resting cells of strain A4413 showed no oxygen uptake on acetate, citrate, or malate, and in the presence of succinate the oxygen consumption was very slight [$Q(O_2)$ N $\leq$ 10.9] (J. H. Jackson, Ph.D. Thesis, University of Kansas, 1969). These results are not in agreement, however, with the report that a type 1 *Listeria* oxidized malate, succinate, and α-ketoglutarate at low rates (23). *Listeria* strains may differ in their oxidative response to these substrates.

Table 2 summarizes the results of enzyme assays using cell-free extracts. For reference and comparison, the specific activities reported by Gray et al. (16) and others for certain enzymes are included in the table. The data of Gray et al. (16) are of interest because they reflect activities from *E. coli* grown aerobically and anaerobically on a synthetic medium with glucose. The specific activities of citrate synthase, isocitrate dehydrogenase, fumarate reductase, fumarate hydratase, and malate dehydrogenase from strain A4413 (which were grown aerobically) were equal to or less than those from anaerobically grown *E. coli*. In contrast, *Listeria* aconitate hydratase and NADH oxidase showed specific activities falling between those of anaerobic *E. coli* and aerobic *E. coli*. Of the citrate cycle enzymes, the α-ketoglutarate oxidation system and succinate dehydrogenase were inactive in strain A4413. The glyoxylate bypass enzymes isocitrate lyase and malate synthase were also inactive.

Aconitate hydratase was twice as active with isocitrate as with citrate. This is in harmony with the characteristics of other aconitate hydratase preparations (33) and with the finding that the equilibrium of this enzyme from many sources favors citrate (30). The K_m of aconitate hydratase (8.0×10^{-4} M, citrate) is also close to that reported by Racker (33) for a mammalian heart enzyme preparation.

Isocitrate dehydrogenase would appear to be NADP specific because no activity was detected when NAD was used as acceptor. This is not certain, however, because at the assay *p*H of 7.5, *Listeria* extract contained NADH oxidase activity which would interfere with the assay. NADP-dependent isocitrate dehydrogenases are common in bacteria, however, whereas those specific for NAD are rare (34). Mn^{2+} was required for full activity.

Malate dehydrogenase is apparently NAD specific, since only traces of activity were observed with NADP. This enzyme is generally NAD dependent (30), although at least one strain of *P. aeruginosa* contains an enzyme which requires NADP (42). Mn^{2+} was required for full activity.

The enzymes of the citrate cycle found in strain A4413 form an enzymatic pathway represented schematically in Fig. 4. Because no oxidation of α-ketoglutarate was demonstrable, the connecting link between α-ketoglutarate and succinate is missing, and a citrate cycle as such

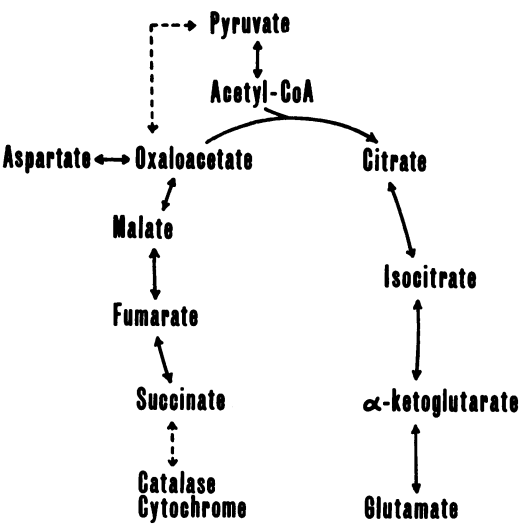

FIG. 4. *Schematic representation of the split citrate pathway apparently present in strain A4413 grown on synthetic medium D10 plus glucose. Solid lines represent enzymatic reactions demonstrated in this or other research; broken lines represent reactions not yet demonstrated.*

cannot function. Instead, a split citrate pathway is likely, having a "right" oxidative aspect and a "left" reductive aspect. For such a pathway to operate, a carbon dioxide fixation reaction with pyruvate or phosphoenolpyruvate would be required to furnish a four-carbon intermediate, because the glyoxylate bypass enzymes are apparently absent. No attempts were made to detect CO_2 fixation in strain A4413.

This split citrate pathway is similar to that found in *E. coli* when grown anaerobically in a synthetic medium plus glucose (1, 16). It is also thought to form a possible metabolic basis for obligate autotrophy (36). Such a pathway would appear to be of small moment in the supply of energy (adenosine triphosphate), which would be furnished by other means, such as glycolysis (16). It would be important, however, for the biosynthesis of amino acids and tetrapyrrole substances. In *E. coli* grown anaerobically on synthetic medium plus glucose, biosynthetic demand is thought to keep the level of α-ketoglutarate so low that the α-ketoglutarate dehydrogenase system is completely repressed (16). Low levels of this enzyme are observed, however, in *E. coli* grown anaerobically on complex medium plus glucose. It is thought that the glutamate supplied by the complex medium relieves the metabolic drain on α-ketoglutarate, thus partially derepressing the synthesis of the dehydrogenase (16). It would be interesting to determine why strain A4413, grown aerobically on defined medium plus glucose, is unable to oxidize α-ketoglutarate. Is a gene deficiency involved, or is the synthesis of the α-ketoglutarate oxidation system repressed by a biosynthetic demand for its substrate?

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LITERATURE CITED

1. Amarsangham, C. R., and B. D. Davis. 1965. Regulation of α-ketoglutarate dehydrogenase formation in *Escherichia coli*. J. Biol. Chem. **240**:3664-3668.
2. Arrigoni, O., and T. P. Singer. 1962. Limitations of the phenazine methosulphate assay for succinic and related dehydrogenases. Nature (London) **193**:1256-1258.
3. Barrett, J. T., and R. E. Kallic. 1953. Terminal respiration in Pseudomonas fluorescens: component enzymes of the tricarboxylic acid cycle. J. Bacteriol. **66**:517-525.
4. Basford, R. E., and F. M. Huennekens. 1955. Studies on thiols. I. Oxidation of thiols groups by 2,6-dichlorophenol indophenol. J. Amer. Chem. Soc. **77**:3873-3877.
5. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1964. Methods in immunology, p. 53-57. W. A. Benjamin, Inc., New York.
6. Chance, B. 1954. Spectrophotometry of intracellular respiratory pigments. Science **120**:767-775.
7. Clarke, P. H., and P. M. Meadow. 1959. Evidence for the occurrence of permeases for tricarboxylic acid cycle intermediates in *Pseudomonas aeruginosa*. J. Gen. Microbiol. **20**:144-155.
8. Dixon, G. H., and H. L. Kornberg. 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**: 3P.
9. Dixon, G. H., H. L. Kornberg, and P. Lund. 1960. Purification and properties of malate synthetase. Biochim. Biophys. Acta **41**:217-233.
10. Ells, H. A. 1959. A colorimetric method for the assay of soluble succinic dehydrogenase and pyridinenucleotide-linked dehydrogenases. Arch. Biochem. Biophys. **85**:561-562.
11. Englesberg, E., and J. B. Levy. 1955. Induced synthesis of tricarboxylic acid cycle enzymes as correlated with the oxidation of acetate and glucose by Pasteurella pestis. J. Bacteriol. **69**:418-431.
12. Friedman, M. E., and W. L. Alm. 1962. Effect of glucose concentration in the growth medium on some metabolic activities of *Listeria monocytogenes*. J. Bacteriol. **84**:375-376.
13. Friedman, M. E., and D. A. Kautter. 1962. Effect of nutrition on the respiratory virulence of *Listeria monocytogenes*. J. Bacteriol. **83**:456-462.
14. Friedman, M. E., and W. G. Roessler. 1961. Growth of *Listeria monocytogenes* in defined media. J. Bacteriol. **82**:528-533.
15. Ghosh, B. K., and K. K. Carroll. 1968. Isolation, composition, and structure of membrane of *Listeria monocytogenes*. J. Bacteriol. **95**:688-699.
16. Gray, C. T., J. W. T. Wimpenny, and M. R. Mossman. 1966. Regulation of metabolism in facultative bacteria. II. Effects of aerobiosis, anaerobiosis and nutrition on the formation of Krebs cycle enzymes in *Escherichia coli*. Biochim. Biophys. Acta **117**:33-41.
17. Halpern, Y. S., A. Even-Shoshan, and M. Artman. 1964. Effect of glucose on the utilization of succinate and the activity of tricarboxylic acid-cycle enzymes in *Escherichia coli*. Biochim. Biophys. Acta **93**:228-236.
18. Hirsch, C. A., M. Raminsky, B. D. Davis, and E. C. C. Lin. 1963. A fumarate reductase in *Escherichia coli* distinct from succinate dehydrogenase. J. Biol. Chem. **238**: 3770-3774.
19. Hodgman, C. D. (ed.) 1959. Handbook of chemistry and physics, 41st ed., p. 1473. Chemical Rubber Publishing Co., Cleveland.
20. Hooper, A. B. 1969. Biochemical basis of obligate autotrophy in *Nitrosomonas europaea*. J. Bacteriol. **97**:776-779.
21. Horecker, B. L., and A. Kornberg. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem. **175**:385-390.
22. Keeler, R. F., and M. L. Gray. 1960. Antigenic and related biochemical properties of *Listeria monocytogenes*. I. Preparation and composition of cell wall material. J. Bacteriol. **80**:683-692.
23. Kolb, E., and H. Seidel. 1960. Ein Beitrag zur Kenntnis des Stoffwechsels von *Listeria monocytogenes* (typ I) unter besonderer Berücksichtigung der Oxydation von Kohlenhydraten und Metaboliten des Tricarbonsaure-Cyclus und deren Beeinflussung durch Hemmstoffe. Zentralbl. Veterinärmed. **7**:509-518.
24. Kornberg, H. L. 1965. Control of biosynthesis from C_2-compounds, p. 193-207. *In* Méchanismes de regulation des activites cellulaires chez les microorganismes. Coloq. Int. Centre Nat. Rech. Sci.
25. Kornberg, A., and W. E. Pricer, Jr. 1951. Di- and triphosphopyridine nucleotide isocitric dehydrogenases in yeast. J. Biol. Chem. **189**:123-136.
26. Kraus, P. 1965. A simple modified micromethod for the

determination of citrate. Clin. Chim. Acta **12**:462–465.
27. Lane, K., and P. S. Chen, Jr. 1960. Rapid determination of citric acid in biological fluids. U.S. At. Energy Comm. Res. Develop. Rep. UR-579, 1–7.
28. Layne, E. 1957. Spectrophotometric methods and turbidimetric methods for measuring proteins, p. 450. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
29. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. J. Amer. Chem. Soc. **56**: 658–666.
30. Lowenstein, J. M. 1967. The tricarboxylic acid cycle, p. 146–270. *In* D. M. Greenberg (ed.), Metabolic pathways, vol. 1, 3rd ed. Academic Press Inc., New York.
31. Mahler, H. R., and E. H. Cordes. 1966. Biological chemistry, p. 667–670. Harper & Row, New York.
32. Mehler, A. H., A. Kornberg, S. Grisolia, and S. Ochoa. 1948. The enzymatic mechanism of oxidation-reductions between malate or isocitrate and pyruvate. J. Biol. Chem. **174**:961–977.
33. Racker, E. 1950. Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim. Biophys. Acta **4**:211–214.
34. Ragland, T. E., T. Kawaski, and J. M. Lowenstein. 1966. Comparative aspects of some bacterial dehydrogenases and transhydrogenases. J. Bacteriol. **91**:236–244.
35. Reed, L. J., M. Koike, and C. R. Willms. 1964. Electron microscopic and biochemical studies of pyruvate dehydrogenase complex of *E. coli*. Science **145**:930–932.
36. Smith, A. J., J. London, and R. Y. Stanier. 1967. Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. J. Bacteriol. **94**:972–983.
37. Stricker, F., J. Fišera, V. Krčméry, and M. Ferenčik. 1963. Comparison of virulence and activity of some enzymes of *Listeria monocytogenes*. Folia Microbiol. **8**:89–92.
38. Sword, C. P. 1966. Mechanisms of pathogenesis in *Listeria monocytogenes* infection. I. Influence of iron. J. Bacteriol. **92**:536–542.
39. Trivett, T. L., and E. A. Meyer. 1967. Effect of erythritol on the in vitro growth and respiration of *Listeria monocytogenes*. J. Bacteriol. **93**:1197–1198.
40. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric techniques, 4th ed. Burgess Publishing Co., Minneapolis.
41. Vaughn, R. H., J. T. Osburn, G. T. Wedding, J. Tabachnick, C. G. Beisel, and T. Braxton. 1950. The utilization of citrate by Escherichia coli. J. Bacteriol. **60**:119–127.
42. Von Tigerstrom, M., and J. J. R. Campbell. 1966. The tricarboxylic acid cycle, the glyoxylate cycle, and the enzymes of glucose oxidation in *Pseudomonas aeruginosa*. Can. J. Microbiol. **12**:1015–1022.
43. Welshimer, H. J. 1963. Vitamin requirements of *Listeria monocytogenes*. J. Bacteriol. **85**:1156–1159.